# Review of Advances in Plant Biotechnology, 1985-88

A. Mujeeb-Kazi and L.A. Sitch, technical editors

International Maize and Wheat Improvement Center
International Rice Research Institute

Cover photo: Regeneration of rice plantlets on a callus.
Courtesy of J. Bouharmont.

2nd International Symposium on Genetic Manipulation in Crops

A. Mujeeb-Kazi and L.A. Sitch, technical editors

**1989**
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**Financial support provided by United Nations Development Programme, the Rockefeller Foundation, the U.S. Agency for International Development, and the Third World Academy of Sciences**

The International Maize and Wheat Improvement Center (CIMMYT) and the International Rice Research Institute (IRRI) are headquartered, respectively, in Mexico and the Philippines. CIMMYT and IRRI are two of 13 nonprofit international agricultural research and training centers supported by the Consultative Group on International Agricultural Research (CGIAR), which is sponsored by the Food and Agriculture Organization (FAO) of the United Nations, the International Bank for Reconstruction and Development (World Bank), and the United Nations Development Programme (UNDP). Donors to the CGIAR system are a combined group of 40 donor countries, international and regional organizations, and private foundations.

CIMMYT receives core support through the CGIAR from a number of sources, including the international aid agencies of Australia, Austria, Brazil, Canada, China, Denmark, the Federal Republic of Germany, Finland, France, India, Ireland, Italy, Japan, Mexico, the Netherlands, Norway, the Philippines, Spain, Switzerland, the United Kingdom, and the USA, and from the European Economic Commission, Ford Foundation, Inter-American Development Bank, OPEC Fund for International Development, UNDP, and World Bank. CIMMYT also receives non-CGIAR extra-core support from Belgium, the International Development Research Centre, the Rockefeller Foundation, and many of the core donors listed above.

IRRI receives support, through the CGIAR, from a number of donors including the Asian Development Bank, the European Economic Community, the Ford Foundation, the International Development Research Centre, the International Fund for Agricultural Development, the OPEC Special Fund, the Rockefeller Foundation, the United Nations Development Programme, the World Bank, and the international aid agencies of the following governments: Australia, Belgium, Canada, China, Denmark, Finland, France, Federal Republic of Germany, India, Italy, Japan, Mexico, The Netherlands, New Zealand, Norway, the Philippines, Saudi Arabia, Spain, Sweden, Switzerland, United Kingdom, and United States.

Responsibility for this publication rests solely with CIMMYT and IRRI.

**Correct Citation**: Mujeeb-Kazi, A., and L.A. Sitch, eds. 1989. Review of Advances in Plant Biotechnology, 1985-88: 2nd International Symposium on Genetic Manipulation in Crops. Mexico, D.F.. Mexico, and Manila, Philippines: CIMMYT and IRRI.

**ISBN** 968-6127-34-8

# Preface

There is great potential for biotechnology as a tool in crop breeding. Despite the risk in such upstream research, many plant breeders are becoming more involved in this work to facilitate the rapid exploitation of new techniques. Genetic manipulation of plants involving tissue culture, restriction fragment length polymorphisms, isozymes, and incorporation of genes from wild species is creating a meteoric revolution for plant breeders.

To keep abreast of the rapid developments in this field, nearly 50 participants from developed and developing countries attended the Second International Symposium on Genetic Manipulation in Crops at the International Maize and Wheat Improvement Center (CIMMYT), El Batán, Mexico, August 29-31, 1988. This meeting was a follow-up to the First International Symposium on Genetic Manipulation in Crops held Oct. 22-26, 1984, in Beijing, China.

The first symposium, which essentially reviewed genetic manipulation work up through 1984, was sponsored by Academia Sinica and the International Rice Research Institute (IRRI). The first meeting was so successful that it prompted CIMMYT to join Academia Sinica and IRRI in co-sponsoring the second symposium.

The second symposium provided the opportunity to analyze research achievements since 1984—hence the title of these proceedings, *Review of Advances in Plant Biotechnology, 1985-88*. The relatively small number of participants (compared to the first symposium) permitted conferees to focus more sharply on research needs and develop the collaborative mechanisms necessary for charting broad pathways for work in this field into the future.

These proceedings start off with a keynote address that looks into the scientific, social, economic, and ethical implication of genetic manipulation in crops. Following this are a selected collection of 27 papers and 6 posters presented during the symposium that update current work in the discipline and review the literature in four specific areas: 1) anther culture and haploid breeding, 2) protoplast culture, somatic hybridization, and transformation systems, 3) distant hybridization; and 4) somatic embryogenesis

and somaclonal variation. A fifth session addressed international collaboration in genetic manipulation of crop plants. Crops covered in these presentations include rice, wheat, maize, barley, triticale, citrus, sugar beet, brassicas, tropical forage legumes, cassava, and cotton.

It is the consensus of plant breeders, geneticists, and other biologists working in crop plant improvement that biotechnology holds the most hope for rapid improvement of crop plants, and for achieving the kinds of advances required for the sustained yield increases demanded in the face of an expanding world population and shrinking land resources. Both CIMMYT and IRRI, currently developing their research strategies towards the year 2000, found the ideas that emerged from this second symposium to be very useful in their program development.

It is now apparent that additional symposia on the subject of Genetic Manipulation in Crops will be held. At this meeting, an organizing committee was formed to start planning for the third symposium, tentatively set for 1991 in Africa.

We take this opportunity to recognize other members of the Second Symposium Organizing Committee, namely: Z.S. Li, H. Hu, and Q.Q. Shao from Academia Sinica; and G.S. Khush from IRRI. We express gratitude to the staff of CIMMYT for being our gracious hosts, and to the United Nations Development Programme, the Rockefeller Foundation, the U.S. Agency for International Development, and the Third World Academy of Sciences for their financial assistance. We thank Gene P. Hettel, science writer/editor for CIMMYT Information Services, for editing these proceedings and coordinating their publication.

A. Mujeeb-Kazi, CIMMYT
L.A. Sitch, IRRI
*Organizing Committee Members*
*and Technical Editors*

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# Genetic manipulation in crops—scientific, social, economic, and ethical implications

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May I first say how happy I am that the discussions we had during the First International Symposium on Genetic Manipulation in Crops in Beijing, China, about the possibility of a series of such symposia taking place once every 3 years, have come true. I am particularly happy to be here at CIMMYT, an institution with which I have had a very long personal association.

Last year when in Beijing, I discussed the overall broad framework for this symposium with representatives of Academia Sinica. It was suggested that, at the beginning of this second symposium, I should discuss some of the key issues involving the broader implications of genetic manipulation both in terms of science and society.

## GREEN REVOLUTION REVISITED

The term "Green Revolution," as some of you might know, was coined in 1968 by the late Dr. William Gaad, then director of USAID. The Green Revolution was really stimulated by the first good wheat harvest in India and Pakistan in April-May 1968. In India, for example, the wheat production jumped from 12 million tons, which was the previous best, to over 17 million tons, a 5 million-ton increase during that year. This was largely caused by the widespread introduction of semidwarf varieties of wheat. The variety largely responsible for that particular jump was Lerma Rojo 64A, which was supplied by Dr. Norman Borlaug, who was working in the pre-CIMMYT program in Mexico. At that time, both India and Pakistan had a fairly large quantum jump in terms of wheat production, which led to Gaad's coining of the term "Green Revolution."

Of course at the same time, IR8, the high-yielding rice variety from the International Rice Research Institute (IRRI), had become available and, so in the next few years, both rice and wheat production started moving forward in many parts of Asia and Latin America and in pockets of Africa as well.

## A 20-YEAR BALANCE SHEET

Now the so-called Green Revolution, even during its early years when great progress was made, created considerable concern. Table 1 is a 20-year balance sheet of benefits and concerns for the 1968-1988 period. Many of the concerns listed here, such as economics, employment, and ecology, have arisen from inadequate interaction with social scientists right from the early planning of such work. Premature expectations were aroused by the term "Green Revolution" and the public was led to believe that all the problems of society would be solved, such as small-scale farmer and large-scale farmer differences, landless laborer problems, and the distinction between scale neutrality and resource neutrality.

## LAND-SAVING TECHNOLOGIES

It is now widely accepted that Green Revolution technologies are really aimed at yield enhancement or land-saving efforts. I call them land-saving because this concept is the most relevant to developing countries, in the sense that all farmers, irrespective of the size of their holdings (scale-neutral), can benefit from the new technologies. But on the other hand, it is not resource-neutral, in as much as inputs are needed for output. The failure to make such distinctions led to a considerable degree of criticism, both in the popular press and the social science journals, that Green Revolution technologies had in-built the seeds of social discrimination.

Of course, many have now tried to sort out the role of technology, the role of the whole set of services, including credit and government policies in input/output pricing, agrarian reform, and rural infrastructure development. But the concerns are broadly in the areas of *economics* (the ability of very poor farmers to adopt such technologies), *equity* (in terms of intragenerational equity and intergenerational equity, particularly the impact on women), *employment* (whether the technologies are labor-displacing or labor-diversifying), *ecology* (such as genetic homogeneity arising from the replacement of a large number of local varieties by one or two high-yielding cultivars over large areas and the problems arising from pesticide residues, excessive fertilizer use, and the emergence of new pests and pathogens), and finally *energy* (in terms of increasing dependence on fossil fuel-based sources).

**Table 1. Green Revolution balance sheet.**

| Benefits | Concerns |
|---|---|
| • Self-confidence | • Economics |
| • Political priority | • Equity |
| • Social prestige | • Employment |
| • Agrarian reform | • Energy |
| • Rural development | • Ecology |
| • Vertical growth | |
| • Disproving Malthusian equation | |
| • Stable or lower food prices | |

But going to the main topic of this symposium, the Green Revolution technologies provide fine examples of genetic manipulation work. For instance, through a complex set of breeding and selection procedures, the Norin dwarfing genes in wheat and the Dee-gee-woo-gen dwarfing gene in rice were introduced into commercially acceptable varieties of wheat and rice, respectively. I think we now have enough experience of planning such work for the future where the potential social impact of these technologies can be anticipated.

Typical results of the Green Revolution technologies can be shown using rice as an example. Figure 1 shows per capita area and per capita production for rice from 1955 through 1986. Of course, there are violent undulations in production because a very large percentage of rice is still rainfed in Asia and the behavior of the southwest monsoon between May and October with its heavy rains and resulting floods, as well as typhoons and periodic droughts, heavily influences production. But the fact remains that per capita production continues to go up and the per capita area continues to go down—very illustrative of the land saving aspect of these technologies.

## HOW MUCH LONGER CAN WE PRODUCE MORE FOOD?

Now the question is how much longer can these trends continue? Can we go on producing more food on less land and continuously increase yield per unit area and per unit of time? For example, Figure 2 shows a projection on world population (Merrick 1986) up to the year 2100. It is well known that population is likely to remain relatively stable in developed countries with the bulk of the population increases taking place in

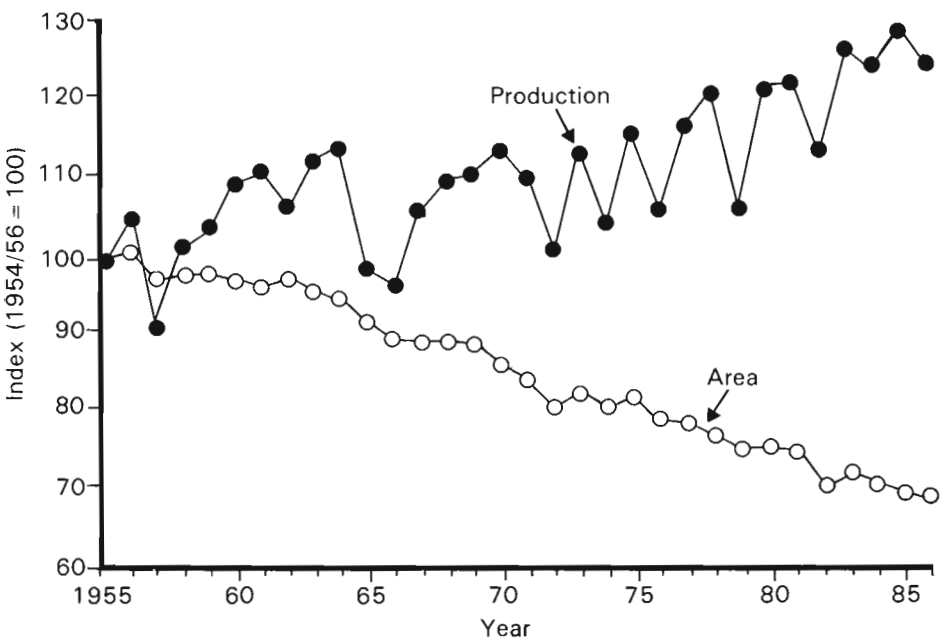


**Figure 1. Trends in per capita paddy production and paddy area, South and Southeast Asia, 1955-86 (Source: IRRI).**

developing countries. Right now, of the 5 billion inhabitants on earth, nearly 4 billion are in developing countries; the developed countries share of the population is not going to increase very much. When world population reaches 8 billion, nearly 6.75 billion will be living in developing countries.

FAO estimates that by the year 2000, 117 developing countries will have exceeded the carrying capacity of their land area and that the population pressure in these countries will be 500 million more than the land can reasonably support. Where will everyone live and get a job? It would seemingly have to be in an industrial zone. However, modern industries are not very labor intensive, so there will be very serious challenges related to producing more food from available land, water, labor and credit resources while, at the same time, making crop production and agriculture, as a whole, much more capable of generating employment in the secondary and tertiary sectors of economic activity, namely the industrial and the services sector.

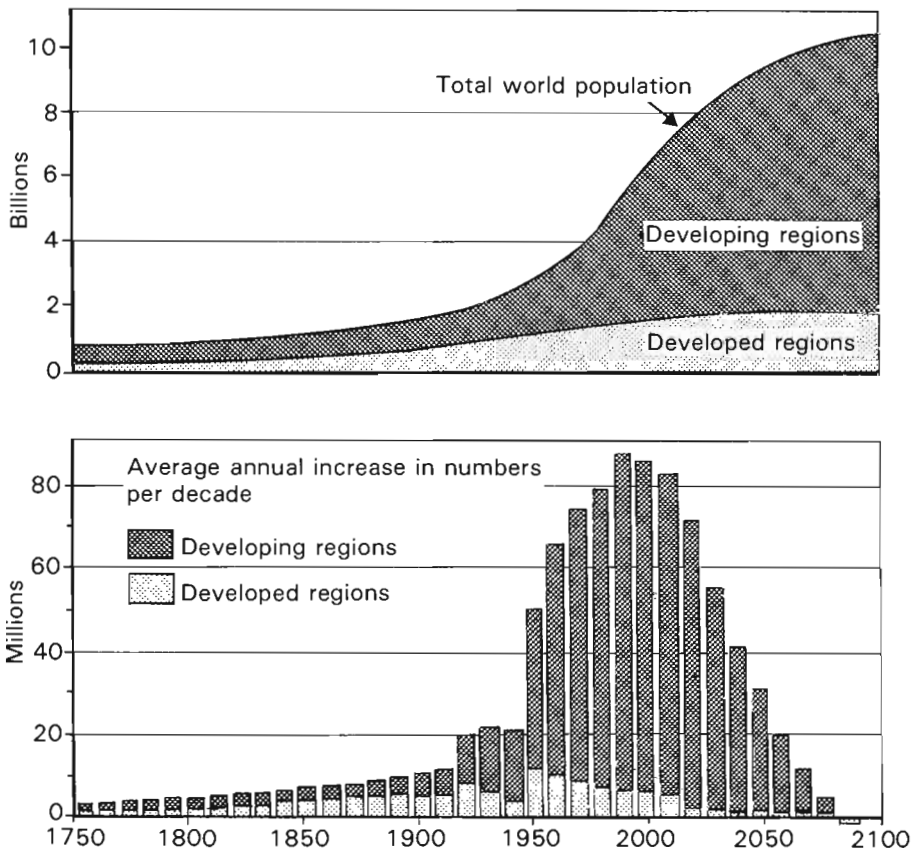


**Figure 2. Population growth of world, developing and developed regions, 1750-2100 (Source: Merrick 1986).**

## SUSTAINABILITY

Now, an important consequence of what I have said so far is the crucial significance of sustainability of the production process. Ecological sustainability involves a time dimension of infinity in terms of production. If we are to integrate considerations of sustainability in productivity, we must look at methodologies of sustainability measurement in terms of production—biological productivity. Production is clear, i.e., crop output produced per year. Productivity, formerly expressed purely in terms of kilograms per hectare, now seems more reasonably expressed as output value/input value, plus changes in environmental capital stocks, particularly in terms of soil. For example, many irrigation projects over the last 30 years have led to salinization and waterlogging and a whole series of associated problems. Therefore, the changes in environmental capital stocks—land and water—become exceedingly important. Sustainability then is stability of productivity as expressed in this way over time, so that today's progress is not at the expense of tomorrow's prospects.

Now, can we do this? Figure 3 illustrates the possibility with wheat. CIMMYT gradually started from the 1950s with Yaqui 50, Pitic 62, and Siete Cerros. Selections from the original parent line of Siete Cerros gave rise to outstanding varieties such as Kalyansona in India and Mexipak in Pakistan. These strains have been used extensively as parental material and have given rise to many important varieties. Then we go on to the Veery lines which now cover more than 4 million hectares all over the world. As you can see from Figure 3, there has been a gradual and steady improvement in yield.

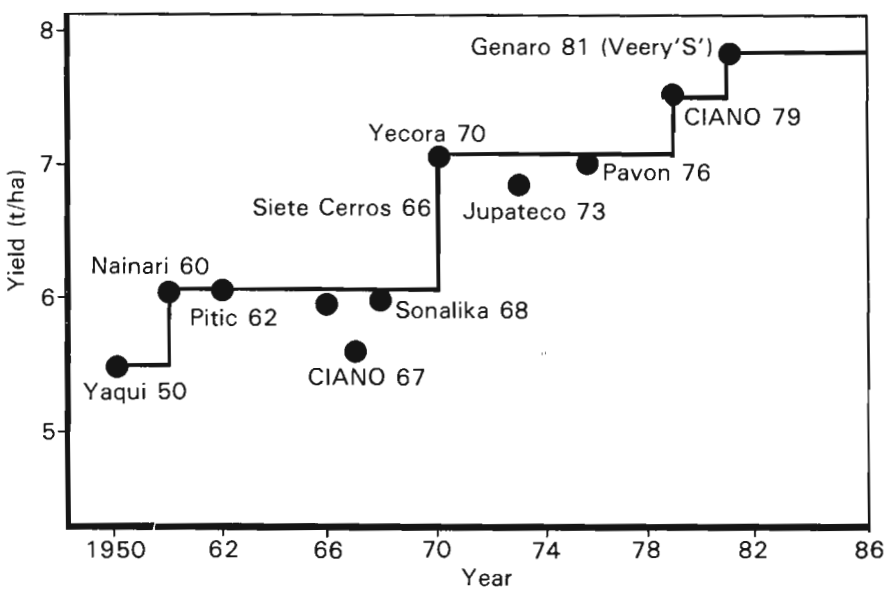


**Figure 3. Pushing back the yield barrier: average yield of Mexican varieties under favorable management and in the absence of disease, yield potential trials at CIANO experiment station, 1982-84 (Source: CIMMYT).**

Now, moving to rice, one of the first things I addressed when I joined IRRI in early 1982, was the clear evidence, both in dry and wet seasons, of a gradual decline in yield (Fig. 4). Calculated over time, this decline comes to about 100 kg/ha per year. The question was: why in such a fine scientific institution as IRRI, with so many experienced scientists, were we not able to achieve the maximum yields achieved in 1967-1968. Data from the All-India Coordinated Research Projects on wheat and rice also confirmed this phenomenon. This decline in yield over a period of time is the exact opposite of the concept of sustainability and we certainly were no longer achieving the goal of producing more and more food from less and less land.

So starting with the 1982 wet season, IRRI initiated a multi-factorial maximum yield experiment to identify the probable factors responsible for this yield decline. What we discovered, I think, is important in terms of genetic manipulation of crops. The first conclusion was that the early varieties such as IR8 had a very stiff straw. Now, over time, quick maturity was emphasized. This meant that, in the breeding plots, per-day productivity was a very important criterion of selection. This resulted in nitrogen harvest indexes and total harvest indexes that were very high in these early duration varieties, otherwise they would not accumulate 90 kg of biomass yield per day.

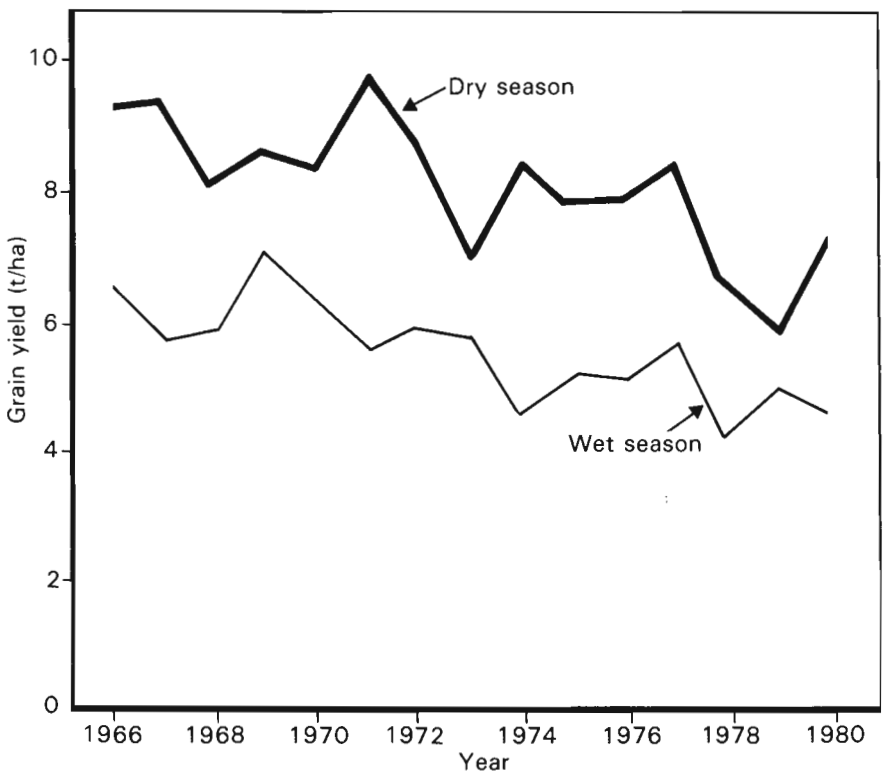


**Figure 4. Highest yield of rice varieties or lines at IRRI during the 1966-80 cropping seasons (Source: IRRI).**

However, we lost in terms of the straw stiffness and as much as 1 t/ha can be lost due to the resulting lodging in the dry season. Varietal selection aimed at increasing per-day productivity and multiple resistance for pests and diseases, i.e., stability of performance, became as important as improvement of yield per se. There were also some new problems—whorl maggot, stem borer, and boron toxicity (primarily an IRRI farm problem) became serious. Finally, cultural practices inappropriate for short-duration rices may have contributed to the yield decline. There was no corresponding agronomic management of very early maturing varieties in terms of seed rate, fertilizer rates and splits, etc. I think it is now clear that we can maintain fairly high yields provided, of course, soil health care and other cultural practices are attended to fairly carefully.

## RAISING THE YIELD CEILING

Now then, how do we go about raising the yield ceiling? Obviously, one approach, apart from the utilization of stiff straw, higher harvest index, etc., is hybrid vigor. Many breeders in China have worked on various aspects of hybrid rice that is based upon the wild abortive (WA) cytoplasm from Hainan Island of China. It is a cytoplasmic genetic restorer system. Now, in all the cases for the various crops shown in Table 2, we had serious problems. For example, Burton's pearl millet material covered very large areas in India at one stage, resulting in downy mildew epidemics associated with this particular genetic makeup. Similarly, you all know the problem with the southern corn leaf blight in the United States. The WA cytoplasm has not yet revealed any serious association with disease problems. However, the concern still arises that if very large areas are planted to hybrid rice of the same genetic makeup, there is a potential for disaster.

As a result, important areas of research to accomplish the diversification of cytoplasm for hybrid breeding include the identification of different cytosterility systems, the use of various chemical gametocides to induce male sterility, functional male sterility, genetic systems, self-incompatibility, and genetic engineering using protoplast fusion techniques.

**Table 2. Cytoplasmic male sterility systems in some crops.**

| Crop | CMS systems |
|---|---|
| Maize | T, C, S |
| Pearl Millet | $S_1$, $S_2$, $S_3$ |
| Sorghum | Milo, $A_2$, Cemunm |
| | Durra, VZM, |
| | M35-1, G1 |
| Rice | BT |
| | WA, Gam |
| | $S_1$, $S_2$, $S_3$, $S_4$ |

Now what are the suggested varietal traits for accomplishing further yield increases? Increased harvest index is very difficult because already it is 0.5 in most of the new varieties of wheat and rice. Obtaining an index above 0.5 may be counterproductive unless the straw is extremely stiff and capable of supporting more grain on top.

Increased biomass production is one trait with which many are working, particularly using distant hybrids. There is evidence some interspecific hybrids produce more biomass and, of course, after having more biomass, attempts must be made to translocate more to the grain. One very extensive study has revealed no clear example of increased biomass production, except in some very distant hybrids. This is a challenge to those who would produce more and more food by increasing total biomass production, in addition, of course, to increasing harvest index.

There is interesting evidence, in the case of rice, for a nitrogen harvest index which enables us to maintain protein content at the same level as the very long-duration varieties, in spite of the higher yield and shorter growth duration. Access to a wide range of germplasm becomes absolutely essential if we are to deal with, not only the existing problems, but also the emerging problems in pest and disease control, soil problems related to toxicities and deficiencies, and above all trying to increase the yield ceiling. Looking at the new varieties, we find in their pedigrees an increasing number of landraces with these special traits.

## GERMPLASM CONSERVATION AND RESOURCES

I am glad that, later during this symposium, we are going to discuss conservation of plant genetic resources. Today, fortunately, we have access to both *in situ* conservation procedures, such as biosphere reserves, national parks, sanctuaries and *ex situ* conservation, both *in vivo* and *in vitro*, the seedbanks, as well as tissue culture, cell culture, and DNA segments. Maintenance of biological diversity in its various forms has become exceedingly important.

Now, the need for additional germplasm is being compounded by the postulated impact of atmospheric changes, particularly in precipitation, temperature, ultraviolet incidence, and ocean warming, which will affect coastal regions. The annual growth rate of fluorocarbons in the atmosphere is still high. In terms of carbon dioxide, the situation is very difficult because many countries depend upon coal, China and India, for example. At the same time, deforestation is still widespread in the tropics and subtropics. The whole area of fossil fuel-based energy is going to be increasingly important, unless there is a major breakthrough in solar energy and biomass utilization.

We can now make computer simulation models on what the likely effects of temperature and precipitation will have on major crops. For example, Tables 3 and 4 are from a recent study of the Indian Agricultural Research Institute which is using the best available prediction models. It has tried to identify for China, India, Indonesia, Bangladesh, and other developing countries what are the likely effects on rice and wheat of doubling atmospheric carbon dioxide and its postulated effect in terms of precipitation and temperature on the productivity of rice and wheat. Altogether I would say this particular study shows global rice area and yield will most likely increase. In fact, if more ocean warming takes place, more deep water rice will be grown because

**Table 3. Effect of doubling of atmospheric $CO_2$ on area and productivity of rice. Total production in 1985: 465 million tons.**

| Country/region | % share of production | Yield (t/ha) | 2 x $CO_2$ climate scenario | | Effects on crop production[a] | |
|---|---|---|---|---|---|---|
| | | | +°C | Soil moisture | Area | Yield |
| China | 37 | 5.3 | 3 | Wetter | + | + |
| India | 20 | 2.2 | 3 | Wetter | + | + |
| Indonesia | 8 | 2.1 | 2 | Drier | - | + |
| Bangladesh | 5 | 4.1 | 3 | Wetter | + | + |
| Developing countries | 94 | 3.1 | 2.4 | Wetter | + | + |

[a] For rice, sterility effects of increasing temperature may neutralize production increase.

Source: S.K. Sinha and M.S. Swaminathan (unpublished).

**Table 4. Effect of doubling of atmospheric $CO_2$ on area and productivity of wheat. Total production in 1985: 510 million tons.**

| Country/region | % share of production | Yield (t/ha) | 2 x $CO_2$ climate scenario | | Effects on crop production | |
|---|---|---|---|---|---|---|
| | | | +°C | Soil moisture | Area | Yield |
| China | 17 | 3.0 | 4 | Some regions wetter | + | - |
| India | 9 | 1.9 | 3 | Wetter | - | - |
| USSR | 16 | 1.6 | 6 | Drier | ++ | - |
| Canada | 5 | 1.7 | 8 | Drier | ++ | - |
| USA | 13 | 2.5 | 5 | Drier | + | - |
| Western Europe | 16 | 4.6 | 6 | Wetter | + | - |
| Australia | 3 | 1.4 | 2 | Wetter | - | - |
| Developed countries | 60 | 2.3 | 5.8 | Drier | ++ | - |
| Developing countries | 40 | 2.1 | 2.4 | Wetter | + | - |

Source: S.K. Sinha and M.S. Swaminathan (unpublished).

its stem elongation gene will allow growth in deeper water. However, in some other areas such as Indonesia, there may be a negative effect. Certainly in India, both wheat area and yield may decrease. So, the very same factors that promote rice production may be detrimental to wheat production.

Now these are just prediction models and what I want to emphasize is the need for a great degree of variability in genetic resources to deal with emerging issues, such as heat tolerance, etc. For example, what are the implications if rainfed rice or other rainfed crops become more important? IRRI conducted a study of what the most optimum combinations might be to improve upland rice. The conclusion was that japonica upland and indica upland crosses would probably provide a complementary set of characters in terms of blast resistance, poor soil adaptation, sheath disease resistance, yield potential, and tillering potential. This is why the tissue culture and anther culture work in the indica/japonica hybrids has become so important in getting a range of material that can be made homozygous fairly fast and tested under a wide variety of conditions.

Summarizing then, we are entering an era where both the efforts to increase the yield ceiling and to stabilize production will be running "head-on" into the emerging requirements of atmospheric changes. As a result, today's and tomorrow's ideal gene pools might be very different as illustrated in Figure 5. This will happen not only because of climatic change, but also because of changing food habits, new processing techniques, and sustainability issues. Sustainability, therefore, has to be viewed as a dynamic concept.

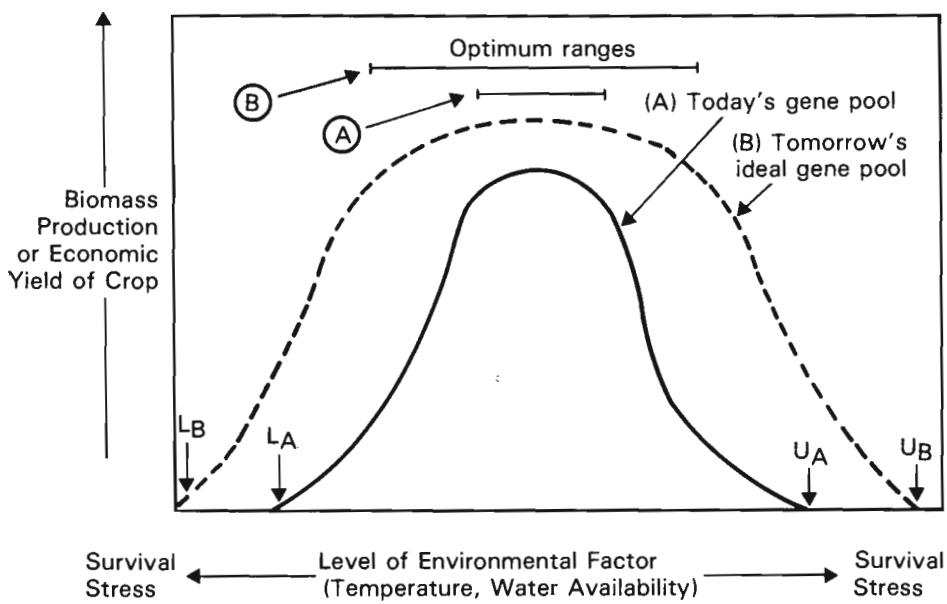


**Figure 5. Efforts to increase the yield ceiling and to stabilize production will be running "head-on" into the emerging requirements of atmospheric changes (Source: U.S. National Academy of Science).**

## PRIVATIZATION

It is in this context that I address the issue of privatization. Now it is a fact of life, in all developed countries, that applied plant breeding and much applied scientific research are being increasingly privatized. This has aroused considerable controversy in international forums. Now on one hand we have a group of people under the umbrella of the International Union for the Protection of New Varieties of Plants, which provides certain kinds of patent rights to plant breeders for private and public sector plant breeding. And on the other hand we have farm families, unknown farm families in most cases, who are responsible for preserving germplasm over a long period of time. What about their rights? What about the role of farm women, who are historically well known in many developing countries to be the seed selectors? For example, a breeder takes a good variety and incorporates one or two genes into it and then immediately patents it. What about those responsible for the source of the original basic stock of material? This has been broadly called farmers' rights, although the name is unfortunate in my view because it looks as though farmers and breeders have contrasting kinds of rights. However, it is clear the contributions of those who contributed the original material need recognition and reward. This, I hope, will be resolved soon.

## ECONOMIC IMPLICATIONS

Turning now to economic implications, I have already mentioned the economic problems associated with the Green Revolution and perceived discrimination against very resource-poor farmers. But the fact remains that consumers have benefitted. Whether for wheat or rice, we can continue extending the downward curve in Figure 6. The exceptions are 1987 and 1988 when drought came to Asia and the United States, respectively—pushing up grain prices. However, by and large, from the 1972-1974 period on, after the escalation of oil and fertilizer prices, grain prices have been, in real terms after adjusting for inflation, coming down.

Now the price of the products that farmers are buying are not coming down and this has created a great deal of difficulties. Developed countries are addressing some of these difficulties with very large subsidies. This year in the United States there will be high subsidies associated with drought relief. During the 1980-86 period, there has been an astounding increase in subsidies in the United States ($2.7 billion to $25.8 billion) and the European Economic Community ($6.2 billion to $21.5 billion). Now the level of subsidies is even higher. The rich countries are able to subsidize their farmers heavily and thereby insulate them from the economic problems. Japan, for example, provides rice farmers a price of almost U.S. $2000/metric ton. In the poor countries, there is no money available for such subsidies. It is in these very poor countries that the challenges are the greatest in terms of increasing productivity on a sustainable basis. While a small farm is ideal for intensive agriculture, a small farm family suffers from many handicaps arising from the cost, risk, and return structure of farming.

Now looking at the costs of production, water and fertilizer can be considered the two most expensive and vital inputs. Seed, itself, is usually not a very high-cost component except in the case of hybrid seeds, which have to be purchased every year. Taking fertilizer as one of the most expensive and effective of the inputs, we find in Figure 7 that, in recent years, the application of fertilizer in Latin America, Africa, and

the Near and Far East is less than 50 percent of the intermediate input level of Europe. In Europe, 180 kg of nitrogen is the average application, while 90 kg is the intermediate level found in some developing countries, of course African countries are far below even 90 kg. Under these conditions, how do we produce genotypes that can respond more to limited fertilizer inputs? How can we reduce the cost of production without reducing yield? This is where there is a new interest in biofertilizers and genetic manipulation and improvement.

## ADVANTAGES OF AZOLLA

Biofertilizers have become an important area of work, particularly with the aquatic plant Azolla, which carries a blue-green algae that is a nitrogen fixer. Azolla has been used extensively in China and Vietnam's Mekong Delta over a period of time. The advantage is two-fold. First, this biofertilizer is the farmer's home-grown input. Second, the leaching losses are very low (Table 5). On many Asian farms during the rainy season (the south west monsoon period), 50 to 70 percent of the urea may be lost,

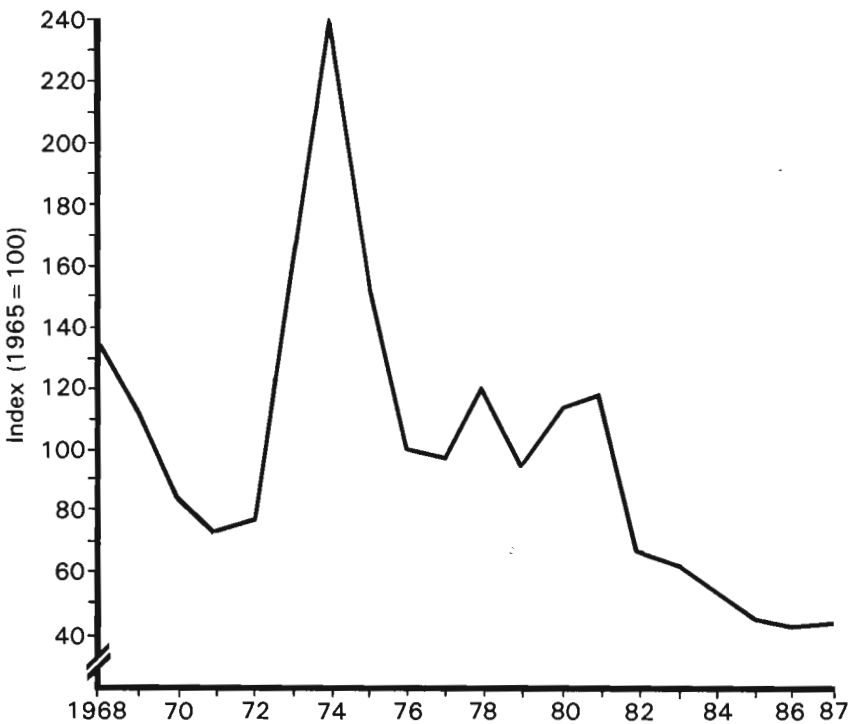


**Figure 6. Trend in real world rice prices, 1968-87 (Sources: 1968-85, World Bank 1986 and 1986-87, FAO 1988).**

through ammonia volatilization, nitrification-denitrification processes, and leaching losses. This is a major reason why biofertilizers are very important for cost reduction.

Great advances have taken place with Azolla in the last few years through joint cooperation of Chinese and IRRI scientists and other scientists. This work has involved interspecific hybridization among Azolla species and exchanging the algal partners to make the nitrogen fixing much more effective.

**Table 5. *Azolla* and urea as N sources for rice.**

| Fertilizer [a] | Grain yield (t/ha) | Plant N uptake (kg/ha) | Fertilizer N loss (%) |
|---|---|---|---|
| *Azolla* | 5.4 | 99 | 6.0 |
| Urea | 5.6 | 92 | 32.6 |
| Control | 3.9 | 63 | |

[a] 60 kg N/ha rate (split application, basal and side-dressed at 42 days after transplanting).

Source: IRRI.

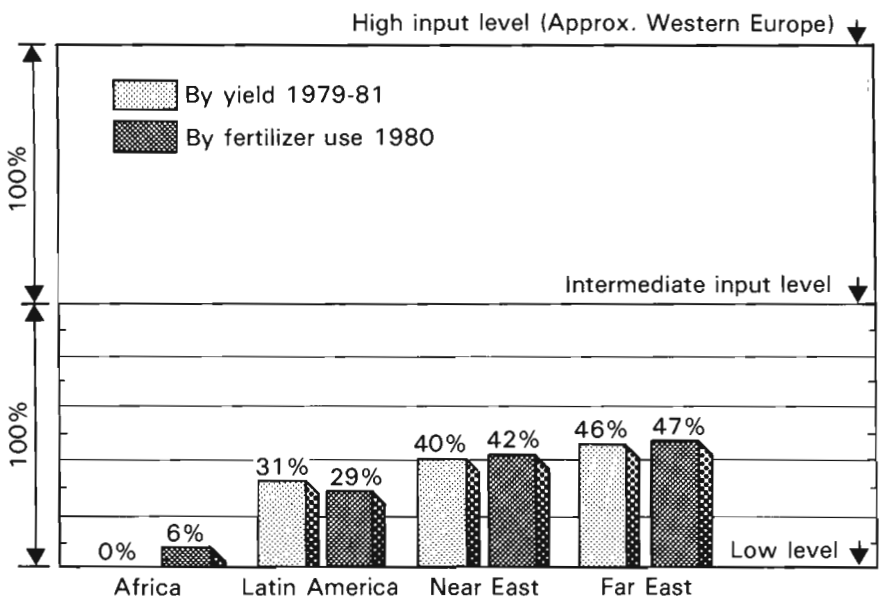


**Figure 7. Present input level by region (Source: FAO).**

## INTEGRATED FARMING SYSTEMS

Research aimed at improving integrated farming systems is another important area of work in the tropics and subtropics. In such systems, several crops and livestock or fish are raised during the year. In these systems, there is a very great need for avoiding the use of chemical pesticides, especially where aquaculture is involved. Data from the International Center for Living Aquatic Resources located in Manila (Fig. 8) show the major rice-fish farming areas of Asia and projected production. There is an enormous amount of increased production for the rice-fish farming system predicted to take place by 1993. Essential elements of the integrated pest management for this rice-fish system are the very high pest and disease-tolerant and resistant varieties of rice and other crops that must be grown along with the fish.

The Rockefeller Foundation-sponsored genetic engineering network is promoting the utilization of the wild rice species with these required traits in interspecific hybridization work. Of course Japan, China, India, and IRRI have, for a very long time, been involved in this very interesting work. For example, the wild rice, *Oryzae nivara*, from the Ganges belt of India provided the donor gene for grassy stunt virus resistance. Examples of other wild rice species with useful traits are given in Table 6. If we were able to incorporate more of these characters into the rice varieties for rice-fish farming, this system could expand without the application of chemicals.

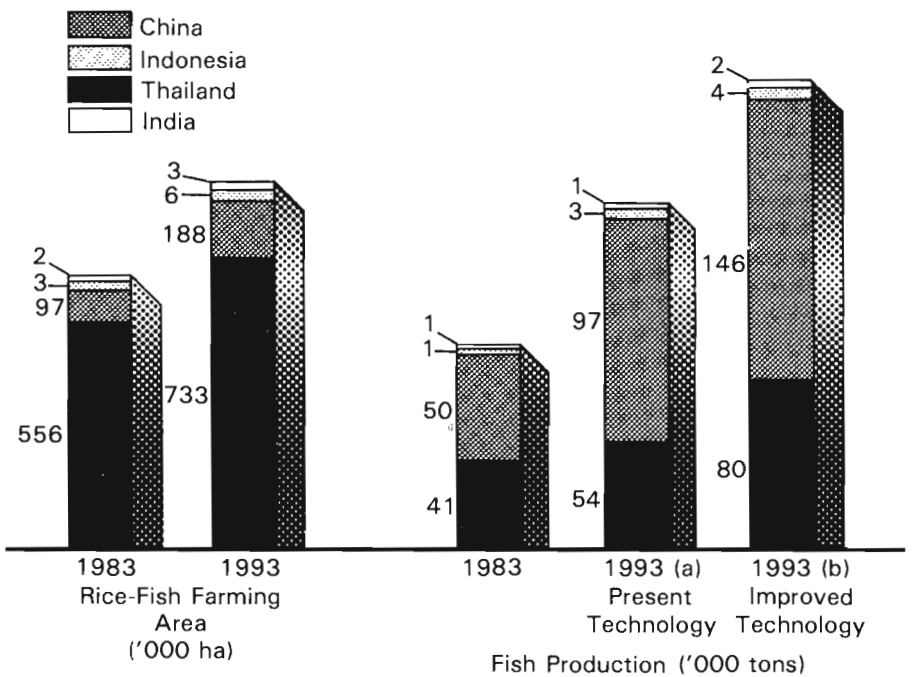


**Figure 8. Major rice-fish farming areas of Asia and projected 1993 production (Source: International Center for Living Aquatic Resources Management).**

While much of the biotechnology work is being done in developed countries, I see the greatest opportunity for its implications in the numerous, very small resource-poor farms of Asia and Africa. Because we can achieve cost reduction without yield loss and we can enhance stability and sustainability of the production process. Also there are opportunities in the postharvest phase of microbiological upgrading of low-grade cellulosic material and in the whole area of bioprocessing.

## BIOSAFETY AND NUTRITIONAL ASPECTS

The biosafety issues of recombinant-DNA work are well known and the development of safety guidelines is well advanced in North America and Europe. But in developing countries, these issues must also be addressed as work in microbiology and plant pathology at the molecular level is becoming more commonplace. Collaboration with international agricultural research institutes and the International Center for Genetic Engineering and Biotechnology located at Trieste, Italy and New Delhi, India could help to improve the capacity of biotechnology research in developing countries.

Another equally important area to study more intensely is the nutritional value of the new genetically engineered materials that are the products of wide crosses. CIMMYT has a wonderful nutritional quality lab that works hand-in-hand with the breeders. This kind of work must also be integrated with new varieties developed from wide crosses. Even though these varieties may be high yielding and disease resistant, what is the quality of their grain? Will the grain carry some kind of toxin harmful to people? So we have biosafety concerns on the one hand and the careful assessment of nutritive value on the other. Calorie deprivation or undernutrition is the mother of malnutrition in cereal-based diets. That is a major problem. And in this situation, it is particularly important that we don't compound the problem.

**Table 6. Wild species of *Oryza* and related genera with useful traits.**

| Wild species | Useful trait |
|---|---|
| *O. perennis* | Tolerance to stagnant flooding and sulfate soils. |
| *O. nivara* | Resistance to grassy stunt virus and blast |
| *O. officinalis* | Resistance to brown planthopper, white-backed planthopper, and green leafhopper |
| *O. australiensis* | Resistance to brown planthopper and drought |
| *O. barthii* | Resistance to bacterial leaf blight |
| *O. longistaminata* | Floral characters for out-pollination |
| *Porteresia coarctata* | Tolerance to salinity |

Source: IRRI.

## CONCLUSION

In closing, I put these questions to you again—how do we raise the yield ceiling? How much higher can the yield ceiling go? Can we go on increasing yield potential, disease resistance, and sustainability? And what are the major reasons for the progress made so far? What made the Green Revolution technologies really work?

I think a very important component is agronomic management, in terms of nutrients and water management. Now, genetic manipulation, unless closely dove-tailed with progress in agronomy and the whole management area, will not accomplish all that much. For example, if water and land are not managed well, then the postulated yield advances may not be realized. I want to emphasize the crucial importance of having genetic manipulation work closely linked with other yield components, particularly land, water, and crop management—and nutrient management.

We must not forget the social scientists in terms of these technologies' potential impact on public policy and welfare. We must make especially sure that these technologies really serve the needs of the poorest farmers in developing countries. If this is to be accomplished, we must be conscious of the importance of proactive relationships with many other disciplines, otherwise there could be a considerable degree of disappointment, criticism, and negative social impact in the long run.

We are happy that we are meeting at an institution, that is headed by an eminent economist and social scientist. CIMMYT, I am sure, will show the way in the coming years of how to do genetic manipulation right. Thank you very much.

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# Doubled haploid breeding: theoretical basis and practical applications

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Doubled haploid systems are now available in a number of crop species. These systems have the unique genetic property of allowing completely homozygous lines to be developed from heterozygous parents in a single generation. In self-pollinating species, this property can be used to increase the efficiency of cultivar production. First, time can be saved in getting selected material ready for commercialization. Second, there is an increase in selection efficiency relative to conventional practices because of an increase in additive genetic variation, an absence of dominance variation and within-family segregation, and a decrease in environmental variation effects through greater replication possibilities. With outcrossing species, doubled haploids can increase selection efficiency in recurrent selection schemes although inserting a generation of haploidization can slow down the cycle time. Nevertheless, overall breeding efficiency should be enhanced. Different schemes that utilize doubled haploids in breeding programs are presented and their relative merits are discussed in relation to the biology of the crop being considered, particularly its breeding system and the type of cultivar required.

In many crop species, particularly the self-pollinating small-grained cereals, very successful systems that produce haploids and double their chromosome number to produce fertile, doubled haploids have been developed (Hu and Yang 1986). These systems now enable doubled haploid lines to be produced in sufficient numbers to contribute directly to breeding programs. Doubled haploids enable breeders to develop completely homozygous genotypes from heterozygous parents in a single generation. No other technique allows this facility and, in essence, doubled haploids are a method of fixing recombinant gametes directly as fertile homozygous lines. This special property has particular genetic consequences that can be exploited to the advantage of

the plant breeder and geneticist. Indeed, in addition to their direct use for varietal production, these systems provide a means of producing unique experimental genotypes that are useful in a range of plant sciences impinging on plant breeding.

This paper considers, in more general terms, the ways in which doubled haploid systems can contribute directly to breeding programs. However, rather than concentrate on the production and utilization in specific crops, attention is focused on the overall genetic consequences of using doubled haploids and in comparing their efficiency relative to conventional breeding and genetic approaches.

## DOUBLED HAPLOIDS AND BREEDING SYSTEMS

The theoretical consequences and practical applications of doubled haploid systems are greatly influenced by the breeding system of the crop in question whether it is predominantly self- or cross-pollinating. This is the primary determinant as to whether it is possible to market a "doubled haploid cultivar" or whether the doubled haploids are used as parental material or even as "transient" genotypes for testing progeny of potential parents.

In self-pollinating species, where a cultivar usually consists of a single homozygous genotype, there is the potential to use these systems to directly generate new cultivars. Thus, recombinant products of a cross are fixed as homozygous lines and then identified and selected according to their agronomic suitability. In addition, however, selected doubled haploids can be used as intermediate parents for further crosses or as parents for $F_1$ hybrids, if a suitable system is available.

In cross-pollinating species, a doubled haploid line is more likely to be used as parental or test cross material than as a cultivar in its own right. Thus, selected lines could provide the parents of single- or double-cross hybrids, or synthetic cultivars where varietal seed arises from random pollinations between several different genotypes. Alternatively, doubled haploids could provide recombinant products of specific genotypes to estimate breeding values or general combining ability, but are not themselves marketable products.

## USE OF DOUBLED HAPLOIDS IN SELF-POLLINATING SPECIES

Doubled haploid systems have the greatest potential use with self-pollinating crops, such as the small-grained cereals. The pedigree system is the traditional method of breeding such crops. $F_1$s are produced by artificial hybridization between selected parents and grown to produce large quantities of $F_2$ seed. $F_2$ individuals are recombinant products of the parental genomes but are, of course, highly heterozygous and successive rounds of selfing are required to stabilize the genotype by the fixation of genes in the homozygous state. While this process is occurring, lines with desired combinations of characters are identified and selected. Generally, sufficient homozygosity is not reached until after five or six generations of selfing, thereby delaying the release of a new cultivar. In addition, selection efficiency on individual plants in early generations is low and response adversely affected by the presence of dominance and by heterozygosity, which reduces the genetic correlations between selected parents in one generation and their progeny performance in the next. Doubled haploid systems can help overcome all of these problems.

### Time-saving advantages

Time saving is the most obvious advantage of a doubled haploid system because yield and other evaluation trials can be done much sooner than with conventional lines, particularly with winter habit material. With winter barley, for example, yield tests are usually commenced at $F_6$, 6 years after the initial cross. If doubled haploid lines are produced from the $F_1$ of the same crosses, yield trials can be done within 4 years after the initial cross, which includes generations of seed multiplication and one cycle of selection (Simpson and Snape 1981). Although single-seed descent can also be used to speed up generation cycles, particularly with spring cereals, the requirement for vernalization to initiate flowering will set an upper limit on the speed of generation turnover with winter crops.

It also takes less time to build up pure stocks of a new cultivar. In the conventional system, stocks are usually derived from a single plant of an advanced generation. Even then, selection and "roguing" are required to achieve statutory levels of uniformity with, subsequently, several generations required to build up sufficient quantities of seed for release. Since doubled haploids are completely homozygous, all stocks are identical and no purification system is required other than isolation to avoid outcrossing.

### Increased selection efficiency

Compared to selection during the early generations of a pedigree program, the "instant" homozygosity obtained from using a doubled haploid system increases the efficiency of selection for both qualitative, major gene characters, and, in particular, for quantitative characters. Thus, it should be easier to identify the superior genotypes in a cross and to produce new cultivars when using a doubled haploid system.

The selection of alleles at major gene loci in a $F_2$ population is most effective if the alleles are dominant. However, if desirable alleles are recessive, then only a proportion $(1/4)^n$, where $n$ is the number of loci segregating, will have the desirable allelic combination. Consequently, the probability of fixation of all desirable alleles is low even if $n$ is relatively small. However, with a doubled haploid population, such genotypes will be at a frequency of $(1/2)^n$. Thus, the frequency of fixation in a $F_1$-derived doubled haploid population is the square root of the probability in a $F_2$ population. Clearly for even small numbers of major genes, the fixation of desirable recessive alleles is greatly facilitated.

Although doubled haploid systems facilitate selection of major gene loci, their greatest advantage is increasing the efficiency of selection of quantitative characters. In the early generations of a pedigree system, efficient selection is hindered by the compounded problems of low additive variance, presence of dominance, within-family segregation, and, particularly at the $F_2$ and $F_3$ generations, interplant competition. Doubled haploids can alleviate all of these difficulties.

The first advantage of using doubled haploid lines is that greater additive genetic variance is expressed between the recombinant products of a cross than between the relative $F_2$s and $F_3$s. Second, dominance variation is absent. These properties are illustrated genetically by comparing the expectations of the variances of $F_1$-derived doubled haploids and the equivalent early conventional generations (Table 1). Here $V_A$

and $V_D$ are the additive and dominance components of variation respectively, defined for an $F_2$ population. Even for traits exhibiting complete dominance, the $F_3$ will not exhibit as much total genetic variation as a doubled haploid population and only half the additive variance. Third, the environmental variance between $F_2$ individuals ($V_{EI}$) is likely to be greater than that between $F_3$ or doubled haploid plots ($V_{EP}$), which are made up of plots of genetically similar individuals. Furthermore, replication can be introduced to reduce $V_{EP}$ so that the individual breeding values of lines can be more accurately assessed. Although $F_3$s can also be replicated, the quantities of seed available are generally less than a doubled haploid line. Further, within $F_3$ plots, dominance variance will obscure true breeding values.

Conventional early generation plots, say $F_3$ or $F_4$, will exhibit genetic differences between individuals within the plots, unlike doubled haploid plots where all individuals are genetically identical (Table 2). This will make visual selection of desirable lines more difficult in early generations compared to doubled haploid plots. This difference is particularly apparent when comparisons are made with the $F_3$ generation, where with complete dominance up to 40% of the genetic variation is segregating within plots and only 60% between plot means, compared to 0 and 100%, respectively, for doubled haploid plots.

Heterozygosity and segregation within pedigree plots will also affect the degree to which selection of individuals in one generation will result in the desired response being expressed in their progenies in the next generation. This can be compared by evaluating the genetic correlations between different generations (Table 3). Doubled

**Table 1. Expectations of phenotypic variances in different generations derived from a cross between inbred parents.**

| Generation | Variance |
|---|---|
| $F_2$ (between individual plants) | $V_A + V_D + V_{EI}$ |
| $F_3$ (between family means) | $V_A + 1/2\ V_D + V_{EP}$ |
| $F_1$ derived DH family means | $2V_A + V_{EP}$ |

**Table 2. Expectations of phenotypic variances within plots of conventional and doubled haploid generations.**

| Generation | Variance |
|---|---|
| Within $F_3$ plots | $1/2\ V_A + 1/2\ V_D + V_{EI}$ |
| Within $F_4$ plots | $1/4\ V_A\ 1/4\ V_D + V_{EI}$ |
| Within doubled haploid plots | $V_{EI}$ |

haploid plants of a line are genetically identical to one another as are their selfed progenies, with a genetic correlation of 1 between generations. However genetic correlations between $F_2$ and $F_3$ and $F_3$ and $F_4$ generations will always be less than 1, except in the extremely unlikely situation that all nonadditive variation is absent.

Overall, the different genetic properties of a doubled haploid population compared to early conventional generations should mean that selection efficiency is increased. This should result in better discrimination between alternative crosses and between genotypes within crosses and, consequently, enhance the probabilities of genetic advance and breeding success.

**Doubled haploid breeding strategies in self-pollinating crops**

The advantages of doubled haploids lines can be obtained very successfully with self-pollinating crops. In such programs different filial generations can be chosen as the parental material for haploidization, although the most common system is to use $F_1$ hybrids, thereby fixing the products of recombination between the two parental genomes at the earliest opportunity. Every doubled haploid produced is a potential cultivar, and field selection is practiced to identify those lines with the desirable combinations of characters.

Successful cultivars of wheat, rice, and barley have been developed using this approach (Hu and Yang 1986, Kasha and Reinbergs 1981). This system allows the greatest time saving compared to a conventional pedigree program. However it also has two disadvantages. One is that a completely random sample of gametes is fixed and, thus, genetic drift will result in a high rejection frequency since only a small proportion of lines will fulfill desired criteria. This can be demonstrated statistically for a quantitative character by considering the expected proportions of lines that transgress

**Table 3. Genetical correlations between generations.**

| Parent generation | Offspring generation | Parent/Offspring Correlation | Maximum Theoretical Value |
|---|---|---|---|
| $F_2$ plants | $F_3$ means | $\frac{(V_A+1/2V_D)}{[(V_A+V_D)(V_A+1/4V_D)]^{1/2}}$ | <1 |
| $F_3$ means | $F_4$ means | $\frac{(1/2V_A + 1/4V_D)}{[(1/2V_A + 1/4V_D)\,(1/2V_A + 1/8V_D)]^{1/2}}$ | <1 |
| $F_1$ DH population | $F_1$ DH selfed population | $\frac{2V_A}{[2V_A \times 2V_A]^{1/2}}$ | 1 |

the best parent for a single character, say yield. Using the theory of Jinks and Pooni (1976), the approximate proportion of a random population of homozygous lines expected to exceed the best parent is given by the value of the one-tailed probability integral corresponding to:

$$\frac{\overline{P}_1 - \overline{x}}{[2V_A]}$$

Where $\overline{P}_1$ is the best parent mean, $\overline{x}$ the doubled haploid population mean, and $2V_A$ the doubled haploid additive genetic variance. Clearly, even with reasonably large values of additive variance, this proportion is likely to be small. If the target value is increased above the best parent, the proportion becomes smaller. Thus, large populations of doubled haploid lines will be required from each cross for a reasonable chance of identifying lines giving genetic advance for even single characters.

This problem is also apparent for major genes. If only a single gene, say for disease resistance, is segregating between the parents, then half of the population is expected to carry the undesirable, susceptible allele. If $n$ major gene loci are segregating, only a proportion of $(1/2)^n$ of the doubled haploids will contain the desired combinations of alleles. Clearly, even for small numbers of loci, this proportion will be small.

The second disadvantage of an $F_1$ system is that only one round of recombination is allowed between the parental genomes before fixation. Thus, the doubled haploid population will be in linkage disequilibrium if linkages between genes are important components of variation. This may result in fewer extreme genotypes for quantitative characters and fewer recombinants for major genes if undesirable coupling or repulsion linkages exist.

The remedy for these disadvantages is to delay haploidization until the $F_2$ or $F_3$ generations and to practice selection prior to haploid production. For example, $F_2$ individuals can be classified for major gene characters and also selected visually for quantitative characters of high heritability, such as flowering time and plant height. Only desirable plants are then used for doubled haploid production. Such a scheme is illustrated in Figure 1 where haploids are produced from the $F_3$ progeny of selected $F_2$ plants. This scheme does not allow much savings in time compared to an $F_1$ system, but does give increased selection efficiency for both major genes and quantitative characters. This should ensure a higher frequency of lines with the desired levels of performance and, consequently, very large population sizes should not be necessary for genetic advance. Indeed, only one or two doubled haploids need to be produced from each selected $F_3$ individual, since most of the genetic differences will be expressed in differences between $F_3$ families rather than within $F_3$-derived families. Since more rounds of recombination are allowed, there will also be less linkage disequilibrium than in an $F_1$-derived population. More advanced generations can also be used in this way, thereby giving more opportunity for selection prior to haploidization. However the benefits of using a doubled haploid system will be eroded as the generations advance.

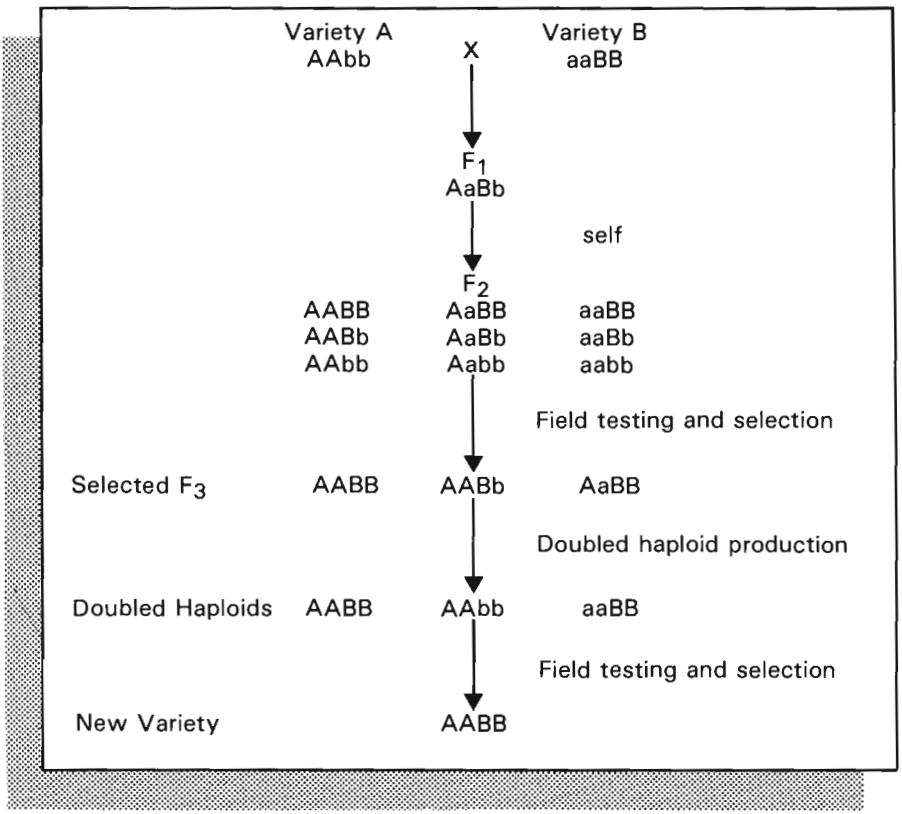


**Figure 1. The selected $F_3$ doubled haploid system for varietal production in self-pollinatng crops.**

In addition to their direct uses as potential varietal material, doubled haploids can also serve other purposes in breeding programs. They can, for example, provide a "test-crossing" system for discrimination between crosses. Under such a scheme a small random sample of lines, say 30, is produced from the $F_1$s of interest and then assessed experimentally. Means and variances for important characters are measured and, using the multivariate prediction equations of Pooni and Jinks (1978), the probabilities of genetic advance over target values are calculated for each cross. This then identifies the crosses worthy of advancement, either using conventional generations or larger populations of doubled haploid lines.

Other more novel schemes of utilizing doubled haploids with self-pollinating crops have been proposed. For example, Choo *et al.* (1979) advocate using doubled haploids in a recurrent selection scheme. Successive cycles of intercrossing, doubled haploid production, and selection are carried out with superior doubled haploids of one cycle providing the parents for hybridization in the next. Cultivars can be extracted at any

generation and gradual improvement of the selected lines is expected. The utility of such different methods has yet to be proven in practice, but they do indicate exciting and novel approaches to breeding problems that doubled haploid systems allow in self-pollinating crops.

## USE OF DOUBLED HAPLOIDS IN CROSS-POLLINATING CROPS

In outcrossing species, new cultivars are generally either improved random mating populations, synthetic populations produced from a small sample of inbreds or selected parents, or single- or double-cross hybrids. Doubled haploid systems can contribute to increasing the efficiency of varietal production with all of these breeding methods.

### Recurrent selection schemes

With random mating populations, selection of superior genotypes in one generation provides the parents for intercrossing which produce the population for the next assessment generation. Cultivar seed can be extracted at any cycle and a gradual improvement of population performance with time can be expected. If techniques are available, a generation of doubled haploid production can be inserted into each cycle. In the simplest scheme, doubled haploids are produced from a random sample of parents and used as the assessment generation (Fig. 2). Thus, selection is practiced to identify the superior parents through the superior doubled haploids, which then provide the seed for the next generation.

The advantage of such a procedure in improving selection efficiency for quantitative characters can again be demonstrated by considering the genetic variances of conventional and doubled haploid generations (Table 4), where $V_{AR}$ and $V_{DR}$ are the additive and dominance components of a random mating population, respectively, and $V_A$ the additive component of a derived doubled haploid population. When increasing and decreasing alleles are at equal frequency at all loci, then $V_{AR}=V_A$ and the derived doubled haploid population has twice the additive genetic variance of the random mating population. Even when gene frequencies are not equal, the doubled haploid population will always exhibit more additive variance. Also as before, no dominance variance is present in the doubled haploid population. The consequence of these

**Table 4. Expectations of phenotypic variances of random mating and derived doubled haploid populations.**

| Population | Variance |
|---|---|
| Random mating | $V_{AR} + V_{DR} + V_{EI}$ |
| Doubled haploid | $2V_A + V_{EP}$ |

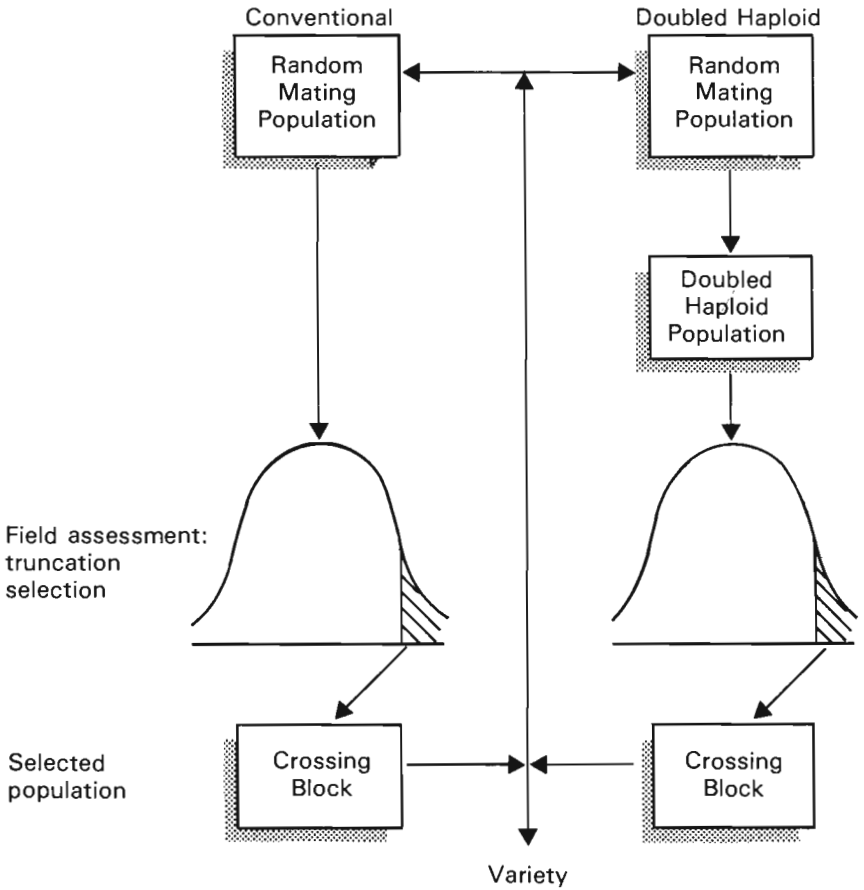


**Figure 2. Conventional and doubled haploid recurrent selection schemes for population improvement breeding programs.**

changes in variance components is to increase the response to selection in the doubled haploid population relative to the conventional population. Griffing (1975) showed that the incremental changes in population mean, in terms of the variance components, would be greater by using a doubled haploid generation. For example, Table 5 shows the changes for two common methods of genotypes assessment—individual and general combining ability (GCA) selection. For different genetic models considered by Griffing, these equations show that doubled haploid selection is always more efficient compared to conventional methods. This advantage is maintained even when population sizes are restricted, an important criterion in many breeding programs.

A disadvantage of such schemes, however, is that a generation of haploidization and chromosome doubling slows down each breeding cycle. The increase in selection efficiency may compensate for this, and efficiency comparisons with conventional schemes should consider the rate of response per year as well as the magnitude of response per cycle.

## Inbred line production

Many cross-pollinating species are dioecious or have self-incompatibility mechanisms that prevent the easy development of inbred lines. Hence, this restricts the production of $F_1$ hybrid cultivars in such crops. Even where forced selfing or inbreeding by sib-mating is possible, it is not always commercially viable. However, doubled haploids can provide a quick and novel solution to this problem and can be used to produce homozygous lines directly from selected parents. Such lines can be evaluated for specific combining abilities, and hence form hybrid or synthetic cultivar seed that provide a uniform and more acceptable product.

In some species, doubled haploids are the only means of producing suitable homozygous lines. For example, to produce $F_1$ hybrid lines of asparagus it is necessary, because of the XY sex system, to have YY and XX lines. This can be done by producing haploids from XY males and then doubling to produce YY supermales and XX females (Bassett 1970). In potatoes, it is very difficult to produce true homozygous lines because the species is autotetraploid and hence has, potentially, four alleles at each locus. However, by producing dihaploids and then monoploids, and then doubling back to the dihaploid and quadrupled haploid, true breeding lines can be developed. These open up the possibilities for $F_1$ hybrid potatoes and uniform "true seed" potatoes.

The value of these novel schemes has not yet been realized in practice in crop plants, except in a few isolated cases. Indeed, questions still remain concerning the practicalities of producing completely homozygous lines from highly heterozygous plants. In particular, it has not yet been established whether or to what extent inbreeding depression will express itself in the derived doubled haploids from normally outcrossing populations. If this is extreme, then doubled haploids may be sterile or of too low vigor for proper field evaluation, and this will restrict their usefulness. However, if this is not a major problem, then doubled haploids may even open the avenue to homozygous cultivars that perform equal to the best hybrids or populations of outbreeding species.

**Table 5. Changes in population mean for individual and general combining ability (GCA) selection.**

| Selection method | Conventional genotypes | Doubled haploids |
|---|---|---|
| Individual | $\dfrac{i \times V_{AR}}{V_{AR} + V_{DR} + V_{EI}^{1/2}}$ | $\dfrac{i \times 2V_A}{2_{VA} + V_{EP}^{1/2}}$ |
| GCA | $\dfrac{i \times 1/2V_{AR}}{\frac{(n+3)}{4n} V_{AR} + \frac{V_{DR}}{n} + \frac{V_{EP}^{1/2}}{n}}$ | $\dfrac{i \times V_A}{\frac{(n+1)}{2n} V_{AR} + \frac{V_{DR}}{n} + \frac{V_{EP}^{1/2}}{n}}$ |

i = selection intensity.
n = replication.

## CONCLUSIONS

Doubled haploid systems offer a novel method of increasing the efficiency of varietal production in crop species regardless of the breeding system. The benefits are likely to be greatest with self-pollinating species, although the ability to produce completely homozygous lines in cross-pollinating species opens up the avenue for new breeding methods. However, their general application requires very efficient production systems and, to date, this has only been achieved in a few species, notably the small-grained cereals.

Generally, doubled haploids will be more expensive and more resource dependent to produce than conventional material. Consequently, in most programs only elite genotypes, those with the greatest chance of genetic advance, will be subject to such systems rather than all breeding material. The proportions of the resources devoted to doubled haploid production will thus vary with the requirements of the breeding program and the relative efficiencies of production. Because of this, doubled haploid systems are likely to be complementary rather than an alternative approach to conventional breeding methods. Nevertheless, the successes already achieved in using them, and their theoretical advantages, more than justify the research efforts being devoted to developing and increasing the efficiencies of doubled haploid production in a wide range of crop species.

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**RESUMEN**

En diversas especies de cultivos existen hoy día sistemas haploides dobles, que presentan la característica genética única de permitir la creación de líneas enteramente homocigotas a partir de progenitores heterocigotas en una sola generación. En las especies autógamas, esta propiedad puede utilizarse para incrementar la eficacia de la producción de variedades, ya que, en primer lugar, se puede ahorrar tiempo en la preparación de material seleccionado para la comercialización y, en segundo, se produce un aumento en la eficacia de la selección en comparación con las prácticas convencionales a causa del incremento de la variación genética acumulativa, la ausencia de variación en las características dominantes y de segregación dentro de una misma familia y la reducción de los efectos causados por la variación ambiental gracias a las mayores posibilidades de repetición. Los haploides dobles pueden, sin necesidad de cruzar especies, incrementar la eficacia de la selección recurrente, a pesar de que la inserción de una generación para la obtención de haploides puede reducir un poco el ciclo. De cualquier manera, aumenta la eficacia del mejoramiento en su conjunto. Se presentan diferentes esquemas que hacen uso de los haploides dobles en los programas de mejoramiento y se analizan las ventajas que ofrece cada uno en relación con la biología del cultivo en cuestión, en especial en cuanto se refiere a su sistema de mejoramiento y al tipo de variedad que se requiere.

# The practicability of anther culture breeding in rice

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Beginning in 1972, the Crop Breeding and Cultivation Institute, Shanghai Academy of Agricultural Sciences, has conducted studies on rice anther culture breeding and its theory to find new rice breeding methods. After 15 years of systematic research and breeding practice, the procedures of rice anther culture breeding have been established and the research on systematization and standardization of the culture techniques have been completed, and can now be put into practice. The main progress includes the following:

- Experiments have revealed that the age of the callus can affect callus differentiation. The differentiation ability decreases with increasing callus age and more albino plants occur. Different rice genotypes have different responses to 2,4-Dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), and Kinetin (KT). Generally speaking, rice cultivars that are difficult to anther culture give better results by using a mixture of several hormones. The physiological status of donor plants can directly influence the culture results. If the meiotic divisions proceed under high temperature (above 35° C), more albino plants can be formed in the process of anther culture. Higher nitrogen content in the donor plant can increase the rate of green plantlet regeneration.
- The culture ability is controlled by the genotype. Regression analysis has shown that there is relatively higher overdominance for culture ability. Therefore, making the cross combinations among the parents with different culture abilities can overcome the culture difficulties.
- Identification and analysis of various generations of the same pollen plant (second through fifth generations) show that there is no degeneration of vigor among the generations. The offspring of pollen plants are genetically stable. By multiple crosses and several anther cultures, good characters from several parents can be accumulated together. With this method, the rice cultivar 'Hua Han Zhao' was

developed, which confirmed that accumulating breeding by anther culture is a feasible breeding method.

- Stable and fully fertile pollen plants can be obtained from japonica/indica hybrids. So, anther culture of japonica/indica hybrids for breeding can be done on a large scale.
- Two rice cultivars, developed by anther culture breeding, have been put into commercial use in large areas. 'Xin Xun' was the first in 1976; 'Hua Han Zhao' with the traits of early maturity, cold tolerance, and good quality was developed in 1981.

In the mid-1930s, haploid plants were discovered in maize, tobacco, and rice that led to the idea of their utilization for crop breeding. Through doubling the chromosome number of haploid plants derived from hybrids, we can obtain homozygous diploid plants, which can stabilize hybrid characters and expedite the breeding progress. In 1966 pollen plantlets of *Datura innoxia* were first obtained by anther culture. Subsequent successful culture of tobacco and rice (Niizeki and Oono 1968) anthers brought attention to the exploration and research of haploid breeding.

Beginning in 1972, the Shanghai Academy of Agricultural Sciences began exploring rice anther culture breeding and conducting research on the practicability and systematization of this technology for application to agriculture and for finding a new method of rice breeding. If anther culture breeding is to become a feasible breeding method, the following essential problems must be studied and solved:

- Finding a method to obtain enough pollen plants for breeding selection, evaluation, and utilization.
- Confirmation of genetic stability of the progeny of the pollen plants.
- Character expression of pollen plants, selective effects in the process of anther culture, and their practical problems in rice breeding.
- Standardization of breeding procedures and systematization of the anther culture technique.
- Because of limitations to gene recombination in $F_1$ anther culture, it is necessary to identify an efficient complementary method for anther culture breeding.
- Development of new cultivars to confirm the practical use of anther culture breeding.
- The variability of homozygous pollen plants and their utilization in rice breeding.

Since 1972 the Shanghai Academy of Agricultural Sciences has systematically studied the above problems. This paper discusses the achievements and progress to date.

## COMPLETING SYSTEMATIZATION AND STANDARDIZATION TESTS OF THE ANTHER CULTURE TECHNIQUE

In anther culture breeding, it is essential to obtain enough pollen plants for breeding selection, evaluation, and utilization. Inducing a microspore to develop into a regenerated plant has involved basic biological research. In order to make this research applicable to agriculture, it will be imperative to develop methods that allow large numbers of pollen plants to be obtained simply and efficiently. At present, the anther

culture ability of japonica rice has reached 8% (an average of eight pollen plants can be formed from 100 inoculated anthers). Upon planting, seedling survival rate is over 93%. Furthermore, many analyses and comparisons have been made on the culture techniques and methods for improving the green plantlet formation rate. The general results follow.

**Selection of media and supplemental components**

After analyzing material from about 1000 hybrid combinations, it was found that calluses induced on N6 medium with 2 mg/L of 2,4-Dichlorophenoxy acetic acid (2,4-D), 1-2 mg/L of naphthalene acetic acid (NAA), and 1-2 mg/L of Kinetin (KT) have high differentiation rates. It was also found that the age of the callus can influence differentiation; the differentiation rate decreased with increasing age of the callus. At the same time the albino plantlet rate increased with callus age (Table 1).

Different genotypic combinations give different responses to 2,4-D and KT. Genotypes from which it is difficult to induce callus give better results if several auxins are mixed. However, the total concentration of auxins should not be more than 5 mg/L

**Table 1. The effect of callus age on the callus differentiation rate in material from approximately 1000 hybrid combinations produced at Shanghai Academy of Agricultural Sciences.**

| | | | | Root | | Bud | | Albino | | Green plants | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Medium | No. of materials tested | Culture days from inoculation | No. of tubes | No. of tubes | % | No. of tubes | % | No. of tubes | % | No. of tubes | % |
| Miller | 55 | 30-40 | 42 | 2 | 4.76 | 2 | 4.76 | 7 | 16.67 | 1 | 2.38 |
| + | | 41-50 | 78 | 11 | 14.14 | 5 | 6.41 | 13 | 16.56 | 5 | 6.41 |
| 2,4-D | | 51-60 | 118 | 25 | 21.19 | 6 | 5.08 | 34 | 28.81 | 20 | 16.97 |
| (2 mg/L) | | 61-70 | 79 | 20 | 25.32 | 6 | 7.59 | 17 | 21.52 | 7 | 8.86 |
| CM 15% | | 71-80 | 51 | 8 | 15.19 | 1 | 1.96 | 7 | 13.73 | 4 | 7.84 |
| | | 81-90 | 50 | 12 | 24.00 | 5 | 10.00 | 7 | 14.00 | 3 | 6.00 |
| | | 91-100 | 21 | 1 | 4.76 | 2 | 9.52 | 5 | 23.81 | 2 | 9.52 |
| | | 101-110 | 8 | 1 | 12.50 | 0 | - | 2 | 25.00 | 0 | - |
| | | 111-120 | 27 | 0 | - | 0 | - | 3 | 11.11 | 1 | 3.70 |
| Miller | 44 | 31-40 | 62 | 7 | 11.29 | 8 | 12.9 | 9 | 14.5 | 0 | - |
| + | | 41-50 | 66 | 10 | 15.15 | 3 | 4.55 | 6 | 9.09 | 2 | 3.03 |
| 2,4-D | | 51-60 | 87 | 9 | 10.34 | 4 | 4.59 | 11 | 12.64 | 2 | 3.30 |
| (2 mg/L) | | 61-70 | 56 | 4 | 7.14 | 0 | - | 3 | 5.36 | 2 | 3.57 |
| CM 15% | | 71-80 | 21 | 2 | 9.52 | 2 | 9.52 | 3 | 14.29 | 3 | 14.29 |
| | | 81-90 | 30 | 6 | 20.00 | 1 | 33.33 | 6 | 20.00 | 0 | - |
| | | 91-100 | 21 | 3 | 14.29 | 1 | 4.76 | 5 | 23.81 | 0 | - |
| | | 101-110 | 17 | 0 | - | 0 | - | 0 | - | 0 | - |
| | | 111-120 | 34 | 4 | 11.77 | 0 | - | 2 | 5.88 | 0 | - |

(Table 2). Adding plant extract to the medium facilitates green plantlet formation and subsequent strong seedlings. Experiments show that adding 15% (V/V) coconut milk can increase the green plantlet development.

### Physiological condition of donor plants and anther culture ability

Rice exposed to high temperatures at meiosis easily forms albino plants. High nitrogen content in the donor plant can enhance callus formation and green plantlet regeneration (Tables 3 and 4). Experiments show that microspores formed in the glasshouse between March and April have the highest rates of callus induction and differentiation in the Shanghai area.

### Uninuclear stage—a critical point where rice microspores can be redifferentiated

Anthers of donor plants different in their suitable collection time in different seasons. In the greenhouse (20-28° C), the ideal collecting time is from 0800 to 0900 hrs during March and April. In the field (25-30° C), the proper time is from 0500 to 0600 hrs during

**Table 2. Differences in genotype pollen responses to 2,4-D and NAA using Miller's medium.**

| Material | Component added[a] (mg/L) | Time culture (days) | Anthers inoculated (no.) | Calluses gaincd | |
|---|---|---|---|---|---|
| | | | | no. | % |
| Mei Zao/Mi Nian $F_2$ | 2,4-D: 2 | 72 | 1524 | 38 | 2.5 |
| single line | NAA: 2 | 73 | 1388 | 50 | 3.6 |
| Nong Hu 6/7623 $F_1$ | 2,4-D: 2 | 63 | 1620 | 191 | 11.8 |
| | NAA: 2 | 61 | 819 | 42 | 5.1 |
| | NAA: 4 | 76 | 485 | 73 | 15.0 |
| Guang Zuan 78/ | 2,4-D: 2 | 51 | 1385 | 182 | 13.1 |
| Nong Hu 6//Tai | NAA: 2 | 48 | 687 | 57 | 8.3 |
| Nan 13 $F_1$ | NAA: 4 | 51 | 723 | 69 | 9.5 |
| 302/A10-4 $F_1$ | NAA: 2 | 55 | 271 | 51 | 18.8 |
| | NAA: 4 | 54 | 108 | 38 | 35.2 |
| Tai Zhong Yu//Ken | 2,4-D: 2 | 55 | 585 | 22 | 3.8 |
| Gui/Ai 55 $F_1$ | NAA: 2 | 55 | 232 | 6 | 2.4 |
| Ai Huang Zhong/ | 2,4-D: 2 | 60 | 1330 | 89 | 5.7 |
| Ai Pi Nuo $F_1$ | NAA: 4 | 60 | 313 | 48 | 15.4 |
| 302/Gong Qin 21 $F_1$ | 2,4-D: 2 | 70 | 1229 | 76 | 6.2 |
| | NAA: 2 | 68 | 199 | 1 | 0.4 |

[a] 2 mg/L = 6% saccharose concentration, 4 mg/L = 3% saccharose concentration.

June and July. During August and September, the proper time is from 1000 to 1100 hrs. In summary, the key points for obtaining large amounts of green plantlets by anther culture are:

- Correct selection and accurate preparation of the medium.
- Control of physiological conditions for the donor plants.
- Identification of the correct developmental phase of microspores (pollen cells at uninuclear stage).
- Timely detection of the callus and differentiation culture.

**Table 3. Nitrogen content of donor plants of two rice cultivars.**

| Cultivar | Treatment | Total N g/plant | Protein-N g/plant |
|---|---|---|---|
| | Low fertilizer | 1.63 | 10.19 |
| Hua Han Zhao | Intermediate fertilizer | 2.03 | 12.69 |
| | High fertilizer | 1.90 | 11.88 |
| | Low fertilizer | 1.19 | 7.44 |
| Ti Gui Fong | Intermediate fertilizer | 1.52 | 9.50 |
| | High fertilizer | 1.60 | 10.00 |

**Table 4. The effect of N content in the main stem and fertilizer treatment on rates of callus induction and green plant regeneration in two rice cultivars.**

| Variety | N content | No. anthers inoculated | Rate of callus induction (%) | Green plants (%) |
|---|---|---|---|---|
| | **Main stem** | | | |
| Hua Han Zhao | low | 1671 | 4.6 | 1.21 |
| | intermediate | 2010 | 5.5 | 1.84 |
| | high | 1756 | 4.3 | 2.05 |
| | **Fertilizer** | | | |
| | low | 1098 | 3.6 | 0.27 |
| | intermediate | 978 | 13.8 | 2.56 |
| | high | 1018 | 11.1 | 2.85 |
| | **Main stem** | | | |
| Ti Gui Fong | low | 747 | 2.8 | 0.07 |
| | intermediate | 763 | 2.1 | 0.39 |
| | high | 1085 | 6.1 | 0.83 |
| | **Fertilizer** | | | |
| | low | 1855 | 8.7 | 4.30 |
| | intermediate | 1970 | 11.0 | 3.45 |
| | high | 1565 | 9.4 | 4.86 |

- Skillful operation.
- Cultivation of pollen plantlets.

Based on these standard techniques, the mean anther culture ability of japonica rice can reach 8%. The callus-inducing rate can reach 15-20%. At present, the Academy obtains from 8,000 to 11,000 clumps of green plantlets every year (Table 5).

## STUDIES ON ANTHER CULTURE ABILITY

In anther culture, different genotypic combinations differ in their callus induction and differentiation rates. At the early stages of this work, the calculation method did not fully agree with the concept of culture ability. We suggest that:

*rice anther culture ability = callus induction rate (%) /callus differentiation rate (%)*

So, from this equation, the effects of the callus induction rate and the callus differentiation rate on the anther culture ability can clearly be seen. The effect of genotype on the two processes (induction and differentiation) can also be distinguished. Experiments reveal that culture ability is controlled by the genotypes of both parents.

**Table 5. Total number of green plantlets obtained annually using rice anther culture at Shanghai Academy of Agricultural Sciences, 1979-87.**

| Year | Material | Total No. green plants (clumps) |
|---|---|---|
| 1979-1980 | japonica/japonica<br>japonica/indica | 7,221 |
| 1980-1981 | japonica/japonica<br>japonica/indica | 6,929 |
| 1981-1982 | japonica/japonica | 10,164 |
| 1982-1983 | japonica/japonica | 11,135 |
| 1983-1984 | japonica/japonica<br>japonica/wild rice | 11,210 |
| 1984-1985 | japonica/japonica<br>japonica/indica | 7,921 |
| 1985-1986 | japonica/japonica<br>japonica/indica | 9,846 |
| 1986-1987 | japonica/japonica<br>japonica/indica<br>japonica/wild rice | 11,092 |

Regression analysis indicates that there is relatively high overdominance for culture ability (Zhang 1985). Therefore, transferring the gene for high culture ability to parents or hybrids is the critical step in overcoming difficulty in the culture of some parents and improving the efficiency of anther culture breeding (Gu 1986) (Tables 6 and 7).

**Table 6. Differences of anther culture ability among hybridized combinations from different parents.**

| Parent | Hybridization combinations | Total No. anthers | No. anther-forming calluses | Callus induction rate (%) |
|---|---|---|---|---|
| Female parent | Yuan 11570 | 788 | 91 | 11.5 |
| Male parents | x Dong Nong 418 | 745 | 49 | 6.6 |
| | x 50334 | 623 | 72 | 11.5 |
| | x 72-597 | 397 | 81 | 20.4 |
| | x 2010 | 540 | 49 | 9.1 |
| | x Shong Qian | 540 | 72 | 13.3 |
| | x $m_9$ | 241 | 29 | 12.0 |
| Mean callus ind. rate (%) | | | | 12.2 |
| Female parent | Dong Nong 418 | 962 | 32 | 3.3 |
| Male parents | x Yuan 11570 | 514 | 27 | 5.2 |
| | x 50334 | 677 | 39 | 5.7 |
| | x 72-597 | 663 | 65 | 9.8 |
| | x 2010 | 414 | 35 | 8.5 |
| | x Shong Qian | 394 | 50 | 12.7 |
| | x $m_9$ | 515 | 33 | 6.4 |
| Mean callus ind. rate (%) | | | | 8.4 |
| Female parent | $m_9$ | 706 | 143 | 20.2 |
| Male parents | x Yuan 11570 | 322 | 59 | 18.3 |
| | x Dong Nong 418 | 363 | 65 | 17.3 |
| | x 50334 | 527 | 89 | 16.9 |
| | x 72-597 | 521 | 98 | 18.8 |
| | x 2010 | 426 | 54 | 12.7 |
| | x Shong Qian | 448 | 79 | 17.6 |
| Mean callus ind. rate (%) | | | | 16.9 |

## STUDIES ON THE GENETIC STABILITY OF CHARACTERS OF THE POLLEN PLANT AND ITS PROGENY

Can the characters of homozygous diploid offspring be stable? Is the vigor of homozygous diploid pollen plants and their offspring normal or reduced? In conventional breeding, the vigor of a hybrid decreases in advanced generations and some good characters are lost, resulting in the failure of breeding selection. If such phenomena exist in pollen plant offspring, anther culture breeding can not be successful. Different generations (second through the fifth) of one pollen plant were tested and analyzed under the same conditions. The results show that the vigor had not progressively decreased in advanced generations; the characters were quite stable. This suggests that selection can be made during the early generations (Table 8).

## STUDIES ON MULTIPLE CROSS AND ACCUMULATION BREEDING BY ANTHER CULTURE

With anther culture on the $F_1$ hybrid, characters can be stabilized quickly. However, imposing homozygosity in the early generations reduces the opportunity of recombination for various characters. Whereas, by doing multiple crosses and several phases of anther culture, the characters of several parents can be accumulated. Pollen line 175 was obtained following anther culture of a hybrid of Jia Nong 485/Labalat//Tainan 13. It was then crossed with 'Ke C 1669' and a second anther culture cycle was made to obtain the cultivar 'Hua Han Zhao' with early maturity, cold tolerance, and good quality.

**Table 7. WrVr analysis of three characters in 5 x 5 diallel cross. Based on 1/2p(p + 1) data.**

| Characters | Array | Wr | Vr | $\hat{Wr}$ | Wr' | Wr-Vr | Wr+Vr | Yr |
|---|---|---|---|---|---|---|---|---|
| Callus induction rate | 1 | 8.530 | 5.306 | 3.694 | -0.075 | 3.224 | 13.836 | 2.7 |
| | 2 | -3.588 | 11.652 | 5.472 | 7.921 | -15.240 | 8.064 | 2.9 |
| | 3 | 10.252 | 11.523 | 5.442 | 7.758 | -1.271 | 21.775 | 2.1 |
| | 4 | 29.366 | 25.601 | 8.111 | 25.496 | 3.765 | 54.967 | 17.8 |
| | 5 | 4.249 | 11.623 | 5.465 | 7.884 | -7.374 | 15.872 | 6.6 |
| Green plantlet rate | | | | | | | | |
| | 1 | 0.456 | 0.156 | 0.615 | 0.106 | 0.300 | 0.612 | 1.6 |
| | 2 | -0.320 | 0.661 | 1.267 | 0.534 | -0.981 | 0.341 | 1.0 |
| | 3 | 1.018 | 1.073 | 1.614 | 0.882 | -0.055 | 2.091 | 0.6 |
| | 4 | 1.389 | 1.491 | 1.903 | 1.236 | -0.102 | 2.880 | 4.5 |
| | 5 | 0.727 | 0.634 | 1.241 | 0.511 | 0.093 | 1.361 | 2.6 |
| Differentiation rate | | | | | | | | |
| | 1 | 6.948 | 4.403 | 11.378 | 2.069 | 2.545 | 11.351 | 1.9 |
| | 2 | -0.866 | 8.409 | 15.724 | 6.584 | -9.275 | 7.543 | 1.6 |
| | 3 | 8.638 | 7.378 | 14.729 | 5.422 | 1.260 | 16.016 | 1.1 |
| | 4 | 18.082 | 16.033 | 21.712 | 15.176 | 2.049 | 34.115 | 14.0 |
| | 5 | 2.812 | 8.207 | 15.534 | 6.356 | -5.215 | 11.019 | 5.6 |

By using anther culture breeding, pollen from hybrids can be cultured to produce pollen plants (homozygous diploid), with uniform genotype and phenotype. Selecting pollen plants derived from various hybrid combinations, crossing them together, and repeatedly culturing $F_1$ anthers to obtain pollen plants can overcome the difficulty of gene recombination and avoid the interference of dominance which results from the zygote formation.

## ANTHER CULTURE OF JAPONICA-INDICA HYBRIDS

Distant hybridization has generally been used as a special breeding method to create cultivars with pest resistance and good grain quality and to create new plant types. But it is difficult to utilize distant hybrids in large-scale breeding programs because their seed sets are abnormal and their characters are not easily stabilized. With pollen plants, some difficulties in distant hybridization can be overcome. Although distant hybrids are often sterile or semi-sterile, their anthers can be used as material for culturing pollen plants because their pollen sterility occurs after the uninucleate stage of pollen development. So, a number of green plantlets can be obtained by culturing the anthers at the early-uninucleate stage. It was found that some japonica-indica combinations can approach or surpass the japonica-japonica combinations in anther culture ability. Experiments have proved that:

- Most of the pollen plants and their offspring derived from anther culture of japonica-indica hybrids can set seed normally. Some of them (about 10%) were semi-sterile and only a few (1-2%) were multiploids or aneuploids.

**Table 8. The comparison of characters of the generation of (Ken Gui Ke Qin 3) pollen plants (Line 302).**

| Material | Plant height (cm) | Panicle length (mm) | Flag leaf length (mm) | No. of panicles per plant | 1000-grain weight (g) |
|---|---|---|---|---|---|
| $H_2$ 1973 | 81.3+3.0<br>3.6 | 15.1+1.2<br>7.7 | 16.8+2.4<br>2.4 | - | - |
| $H_2$ 1974 | 84.7+3.5<br>4.2 | 13.8+0.7<br>5.4 | 13.7+2.4<br>17.2 | 6.9 | 27.0 |
| $H_3$ | 81.6+2.2<br>2.7 | 13.6+0.6<br>4.3 | 14.8+2.4<br>16.1 | 7.2 | 27.1 |
| $H_4$ | 84.3+3.2<br>3.8 | 14.1+0.7<br>5.3 | 15.5+2.7<br>17.5 | 6.9 | 28.0 |
| $H_5$ | 82.0+3.0<br>3.6 | 14.2+0.7<br>4.5 | 15.6+2.5<br>15.8 | 6.8 | 27.0 |
| $H_5$ | 99.9+4.6<br>4.6 | 15.3+1.1<br>7.3 | 21.0+4.6<br>22.1 | 6.2 | 27.0 |

- Of all the pollen plant offspring from japonica-indica hybrids, most of them were of the japonica type or tended towards the japonica type. Few were of indica or intermediate types.
- The characteristics of pollen plant offspring from japonica-indica hybrids remained stable. For example, offspring of semi-sterile pollen plants remained semi-sterile.

## FEASIBILITY OF ANTHER CULTURE BREEDING

Can anther culture breeding be applied to crop breeding? Is anther culture breeding a more efficient breeding method? Since 1972, we have conducted research on rice anther culture breeding. From anther culture of the $F_1$ (Ken Gui/Ke Qin3), we obtained strain '302'. Further identification and evaluation was performed in 1973. In 1974, a yield test on this line showed promising results; multilocation testing was done in 1975. This line was named 'Xin Xun' in 1976 and officially became one of the first cultivars developed by the anther culture technique (Crop Breeding and Cultivation Institute 1976). Therefore, it seems feasible that the anther culture technique is an alternative breeding method for rice. Through the accumulation breeding method, by anther culture, the characters from several cultivars can be accumulated. The rice cultivar 'Hua Han Zhao' was developed by this method, which combined cold tolerance, early maturity, and good quality (Zhang 1983).

## VARIABILITY OF POLLEN CELLS AND SELECTION OF VARIANTS

By using variation of pollen clones and somaclones, variants with special agronomic characteristics, such as dwarf plant type, large plump grains, and disease resistance can be selected. The young panicles from haploid pollen rice plants were used as the initial explant to study somaclonal variation in rice. Experiments show that, in the series of subcultures, variation in the rate of callus differentiation and plantlet regeneration exists among the calluses derived from same original material. The ploidy level was increased by subculture. Cytological observation reveals that aneuploidy can be attributed to one source of trait variation. Analysis of variance of traits in SS1 and SS2 indicates that great variation occurs within and/or between somaclone lines, with significant F values. The possible mechanism of the variation may involve aneuploidy, chromosomal aberrations, gene mutation, as well as somatic recombination and segregation (Chu and Zhang 1985).

Studies on aneuploids derived from pollen plants have been done for 4 years. We found that anther culture can induce a high frequency of aneuploidy, such as trisomics (6.7%), tetrasomics, monosomics, and nullisomics (Chu *et al.* 1985).

The culture filtrates from *P. oryzae* were used as a medium supplement to screen for mutants resistant to the culture filtrate. Sixteen plantlets were obtained from 29,945 inoculated anthers. Laboratory and field identification indicates that the resistance of two pollen plants was actually genetic (Zheng *et al.* 1985 a,b). All this research indicates that obtaining resistant variants by anther culture is an important new approach in rice breeding.

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## RESUMEN

En 1972, el Instituto de Cultivo y Mejoramiento de Cultivos de la Academia de Ciencias Agrícolas de Shanghai, comenzó a realizar estudios sobre el mejoramiento de arroz mediante el cultivo de anteras y sobre la teoría que lo sustenta con el fin de encontrar nuevos métodos de mejoramiento de arroz. Tras 15 años de investigación y práctica sistemáticas del mejoramiento, se han establecido procedimientos para el mejoramiento de arroz mediante el cultivo de anteras, y se ha dado por terminada la investigación sobre la sistematización y normalización de las técnicas de cultivo, de tal manera que ya se pueden poner en práctica. Entre los adelantos más importantes cabe mencionar:

- Los experimentos revelaron que la edad del callo puede afectar su diferenciación, ya que la capacidad de diferenciación del callo disminuye al aumentar la edad de éste y se producen más plantas albinas. Los distintos genotipos del arroz presentan diferentes respuestas a ácido 2,4-D-diclorofenoxiacético (2,4-D), ácido naftalén acético (ANA) y quinetina (KT). En términos generales, las variedades de arroz que presentan dificultades en el cultivo de anteras dan mejores resultados si se emplea una mezcla de diferentes hormonas. El estado fisiológico de las plantas donadoras puede afectar en forma directa los resultados del cultivo. Si las divisiones meióticas tienen lugar a temperaturas elevadas (más de 35 °C), es posible que se formen más plantas albinas en el proceso del cultivo de anteras. Un elevado contenido de nitrógeno en la planta donadora puede incrementar la velocidad de regeneración de plántulas verdes.
- El genotipo controla la capacidad de cultivo. El análisis de regresión ha demostrado que existe un predominio relativamente mayor en cuanto a la capacidad del cultivo; por tanto, es posible superar las dificultades encontradas en el cultivo haciendo combinaciones de cruzamientos entre progenitores que presenten diferente capacidad de cultivo.

• La identificación y análisis de diversas generaciones del mismo progenitor masculino (de la segunda a la quinta generación) demuestran que el vigor no degenera entre las generaciones. La descendencia de los progenitores masculinos es genéticamente estable. Mediante cruzamientos múltiples y varios cultivos de anteras, es posible acumular las características positivas de diversos progenitores; con este método se creó la variedad de arroz 'Hua Han Zhao', la cual confirmó que el mejoramiento acumulativo mediante el cultivo de anteras constituye un método factible de mejoramiento.
• Es posible obtener progenitores masculinos estables y completamente fértiles de híbridos japónica/índica; por tanto, se podría emprender el cultivo en gran escala de anteras de híbridos japónica/índica con fines de mejoramiento.
• En regiones extensas se han introducido comercialmente dos variedades de arroz creadas gracias al mejoramiento mediante el cultivo de anteras. La primera fue la 'Xin Xun' en 1976; en 1981 se creó la 'Hua Han Zhao' que presenta características de madurez precoz, tolerancia al frío y buena calidad.

# Use of isozyme markers to monitor recombination and assess gametic selection among anther culture derivatives of remote crosses of rice (*Oryza sativa* L.)

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Among sexual and anther culture derivatives of rice distant hybrids, the specific advantages in studying the segregations and recombinations of natural heterozygous isozyme markers are described in this paper. Aside from their wide expression within microspore-derived calluses, they permit an assessment of the germinal origin, the original feature and the representativeness towards the gametic population of the anther culture derivatives. Moreover, their mostly scattered and known locations on chromosomes give an insight into comparative recombinant recoveries occurring through the selfing and androgenetic processes in rice distant hybrids.

During the last decade, the anther culture (AC) technique in rice has been increasingly used as an alternative to the conventional pedigree breeding method. Its main advantage is the rapid production of homozygous lines, mainly from $F_1$ crosses, in international research institutions and national programs (Zapata *et al.* 1983, Pulver

and Jennings 1986, Chen 1986, Guiderdoni *et al.* 1986, and Chung 1987). Although this methodology has already resulted in the release of several cultivars, mainly in China, it is of primary importance for its wide integration into breeding schemes and genetic studies to know whether AC derivatives represent a random array of the hybrid gametic population. Experiments that test the representativeness of the embryogenic microspore pool have been carried out on several crops to compare the segregations of heterozygous markers within the AC derivatives with those observed within the $F_2$ population. These studies have made contradictory conclusions about the existence of gametic selection (Nakata and Kurihara 1972, Ha and Pernes 1982, Kao *et al.* 1983, Foroughi-Wehr and Friedt 1984, Orton and Browers 1985, Powell *et al.* 1986) or its absence (Raquin 1982; Chen *et al.* 1982, Chen *et al.* 1983, and Snape *et al.* 1986). In distant crosses of rice (e.g., japonica/indica), the pedigree method is frequently hampered by the partial sterility of the progenies, the slow fixation of the derived lines (sometimes up to the $F_9$ or $F_{10}$ generations) and the low recovery of useful recombinants, and a corresponding high frequency of parental types. The exploitation of such crosses through doubled haploid breeding appears more promising. Aside from these applied aspects, genetic analysis of AC derivatives of distant hybrids, whose $F_2$ progeny are commonly prone to segregation distortions and recombination restrictions, permits the concurrent study of mechanisms controlling gametic selection during both microsporogenesis and androgenesis.

This paper presents the theoretical and practical advantages of using isozyme markers as an analytical tool in distant hybrid anther culture in rice.

## NATURAL ALLELIC VARIATION OF RICE ISOZYME MARKERS

In recent years, isozymes have been widely used to analyze the genetic structure of the *Oryza* genus (Second 1982) and to set up a classification of *O. sativa* L., the cultivated rice of Asian origin (Glaszmann 1985, 1987). A total of 36 polymorphic isozyme loci can be surveyed during the growth of the rice plant. The natural allelic variation at these loci provides convenient heterozygous markers in the hybrids. Their potential use in rice breeding has been reviewed recently (Glaszmann *et al.* 1988). The number of isozyme markers available in a given hybrid increases with genetic distance between the parental cultivars: from 6 to 16, 0 to 14, and 0 to 5 in the young shoots of japonica/indica, indica/indica, and japonica/japonica hybrids, respectively (Glaszmann *et al.* 1988). Eighteen of these markers have been assigned to 8 of the 12 rice chromosomes. Linkage status among several of these loci is known (see Wu *et al.* 1988 for an updated review) and their scattered location provides an insight into genomic recombinations. This property has been used to monitor recombinations within interspecific (Jena, pers. comm.) and intersubspecific progenies (Pham, pers. comm.). In the latter, the high number of isozyme markers available and the location of several of them on the chromosomes, known to bear hybrid sterility such as 1, 3, 4, and 12, allow assessment of the impact of such genes on segregation and recombination.

Segregation and recombination of isozyme markers at the polymorphic isozyme loci can also be monitored in AC-derived plants, irrespective of their ploidy level. Moreover, isozyme loci are widely and reliably expressed within the microspore-derived calluses from their emergence 4 to 8 weeks after anther plating until 3 weeks

after their subculture onto regeneration medium (Guiderdoni *et al.* 1988a) (Table 1). This permits an analysis of segregation among a larger number of AC derivatives and, therefore, a larger number of crosses before plant regeneration.

## USE OF ISOZYME MARKERS IN RICE DOUBLED HAPLOID BREEDING

The methods used in producing AC derivatives from rice hybrids and in analyzing isozyme patterns of microspore-derived calluses and plants have been described (Guiderdoni *et al.* 1988b,c). Second and Trouslot (1980) and Glaszmann (1985) described the electrophoresis techniques used. The isozyme phenotypes of 444 microspore-derived calluses and 90 AC-derived lines stemming from the japonica/indica hybrid (IRAT177/Apura) were surveyed at 9 and 13 loci, respectively. Segregation and recombination data at these loci were compared with those observed among 178 $F_2$ plants at 12 loci. The experimental procedure used is summarized in Figure 1. Isozyme analyses of microspore calluses derived from two japonica/indica crosses and three hybrids from the cross between indica IR lines—belonging to the "indica" isozyme group of Glaszmann (1987)—and indica traditional cultivars from Bangladesh—known for their high quality grain, falling under the "Basmati" isozyme group—were also carried out (Table 2). These preliminary data are being compared with the data on $F_2$ population patterns.

### Legitimate AC derivatives

Heterozygous isozyme markers, aside from checking the hybrid nature of the donor plants, also make an unambiguous distinction between the somatic and the germinal origin of the AC derivatives. An isozymic survey of more than 1500 microspore calluses derived from the above mentioned hybrids has shown that almost all of the

**Table 1. Isozyme loci expressed in the microspore-derived callus of rice (Guiderdoni *et al.* 1988a).**

| | |
|---|---|
| *Adh-1* | *Amp-1* |
| *Sdh-1* | *Amp-2* |
| *Icd-1* | *Amp-3* |
| *Cat-1* | *Amp-4* |
| *Pgi-1* | *Acp-1* |
| *Pgi-2* | *Acp-2* |
| *Pgd-1* | *Acp-3* |
| *Pgd-2* | *Acp-4* |
| *Got-1* | *Est-1* |
| *Got-2* | *Est-2* |
| *Mal-1* | *Est-9* |

*Pox-2* and *Est-7* never appeared and *Est-5* showed an altered expression in our experiments.
Data recorded on the following cultivars and crosses: Taipei 309, Aus 454, Fujiminori, Tetep, Dinorado/BR319, Up1Ri5/CNA4121, and IRAT177/Apura.

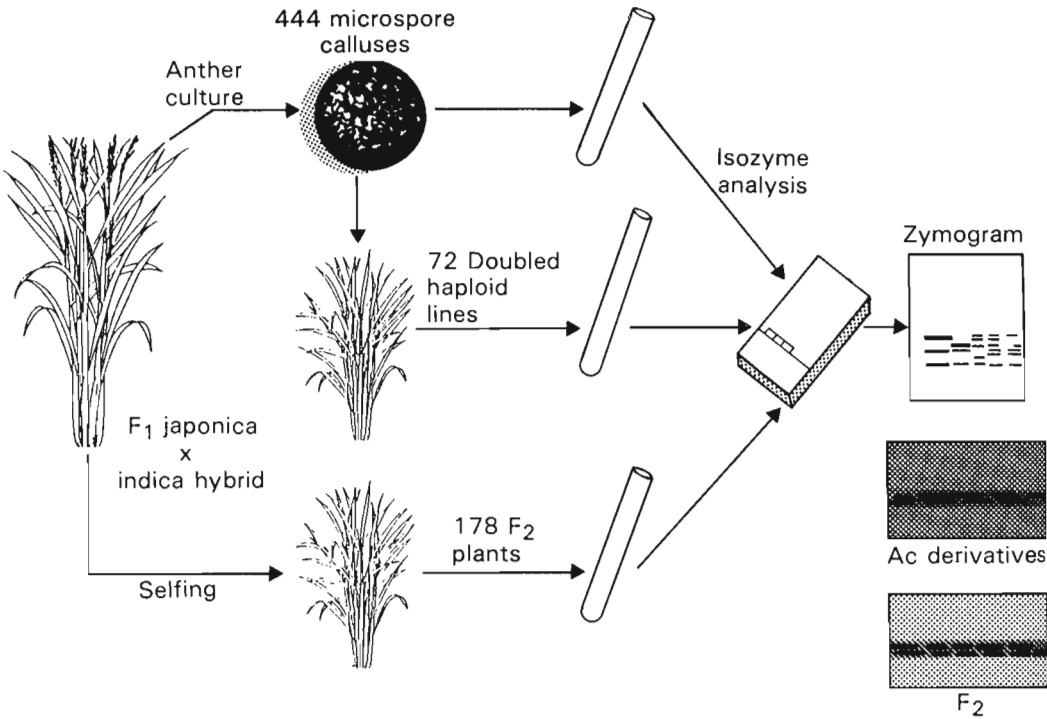


**Figure 1. Experimental protocol followed in the analysis of selfing and anther culture derivatives of the cross IRAT177/Apura.**

**Table 2. Number of microspore calluses and isozyme loci surveyed during the isozyme analysis of AC derivatives of several japonica/indica and indica/indica crosses.**

| | Isozyme loci surveyed | Number of microspore calluses analyzed |
|---|---|---|
| **Japonica/indica crosses** | | |
| IRAT216/IRAM31/5/6 | 11 | 407 |
| IRAT216/Eloni | 10 | 436 |
| **Indica/indica crosses** | | |
| IR43518-62-2-2-1-3/Randhanipagal | 10 | 175 |
| IR42312-17-1-1-4-2/Tulsimanjari | 10 | 172 |
| IR21567-9-2-2-3-1-3/Ramjaewen | 10 | 315 |

samples displayed nonheterozygous phenotypes. The observation that anther wall cells very rarely give rise to callus in rice is consistent with previous reports of Kinoshita (1982) and Chen *et al.* (1982, 1983) who used morphological markers. This also indicates very low incidence of nonreduction of the microspore mother cell in the crosses studied. This has, however, been reported with a 1% frequency in rice (Chen and Li 1978). For the IRAT177/Apura cross, 86 of the 90 AC-derived lines displayed nonheterozygous products of recombination of the parental phenotypes. Nevertheless, 4 of the 90 AC lines analyzed segregated at several loci (Table 3), presumably due to intermating of partially sterile plants. Heterozygous isozyme markers have also been used in AC of wild rice (Wu and Kiang 1979), male sterile tomato (Zamir *et al.* 1981), broccoli (Orton and Browers 1985), *Digitalis* (Corduan 1975), and *Arabidopsis* (Keathley and Scholl 1983) to demonstrate the microspore or sporophytic origin of the AC derivatives.

When the number of isozyme markers surveyed is high (i.e., more than 10), the probability of obtaining two identical isozyme phenotypes from two different meiosis products is low. For instance, among the 86 doubled haploid (DH) lines of the cross IRAT177/Apura, 13 lines displayed identical isozyme phenotypes with at least one of the other lines. This presumably resulted from early fragmentation of the microspore callus, followed by unwitting independent transfers of the fragments onto the regeneration medium. This phenomenon, already cytologically reported by Mercy and Zapata (1987) and confirmed by subsequent field evaluation of the duplicated lines, is being quantified in the other crosses cited. It may affect genetic studies dealing with AC of species whose androgenetic pathway involves mainly a callus phase (e.g., rice and barley in cereals). The large number of heterozygous markers handled in isozyme analysis of distant hybrids permits these duplicates, undesirable from geneticists' and breeders' points of view, to be discarded.

As indirect organogenesis or embryogenesis are the main morphogenetic pathways described in rice AC, several plants may sometimes be independently regenerated from different sectors of the same microspore callus. Besides field evaluation of the derived lines, isozyme analysis provides a means to test the identicalness of these plants. In the four cases reported in Table 3, the AC-derived lines that originated from the same callus displayed identical isozyme phenotypes and phenol reaction. This confirmed their identical field performances noted for 15 traits. Field evaluation generally confirms the identicalness of such series of plants in 98% of the cases (Courtois, unpublished results). On the other hand, both diploid and haploid plantlets, like plantlets presenting several pigmentation types, can be regenerated from a single callus (Guiderdoni *et al.* 1986). These results are consistent with those reported by Kinoshita (1982) in rice, using six morphological markers. Therefore, to consider the several plants regenerated from one single callus as original genotypes in yield estimation studies is meaningless. Moreover, it entails unnecessary, additional field work.

**Table 3. Isozyme phenotype and phenol reaction of the 90 anther culture (AC)-derived lines of the cross IRAT177/Apura.**

| Locus | pH | Sdh 1 | Pgi-1 | Amp 2 | Est-9 | Est-1 | Icd-1 | Acp-4 | Acp-1 | Acp-2 | Pgd-1 | Pox-2 | Est-7 | Mal-1 | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IRAT 177 | – | F | F | S | S | A | F | S | F | A | F | A | P | S | |
| Apura | + | S | S | F | F | P | S | F | S | P | S | P | A | F | |
| AC line 1 | + | S | S | F | F | P | S | S | F | A | S | A | A | F | |
| 2 | – | S | F | F | S | A | S | F | S | P | S | P | A | S | |
| 3 | – | F | F | F | F | A | S | F | F | A | S | A | A | F | |
| 4 | + | F | F | S | F | P | S | S | S | P | S | A | A | F | |
| 5 | – | F | F | F | F | A | S | F | F | A | S | A | A | F | id 3 |
| 6 | – | F | F | F | F | A | S | F | F | A | S | A | A | F | id 3 |
| 7 | – | F | F | F | F | A | S | F | F | A | S | A | A | F | id 3 |
| 8 | + | S | F | S | S | P | F | S | S | P | S | P | P | F | |
| 9 | – | F | F | S | F | A | S | S | F | A | S | A | P | S | |
| 10 | + | S | F | S | S | A | F | S | S | P | S | P | P | F | |
| 11 | + | S | F | S | F | P | F | S | S | P | F | P | P | F | |
| 12 | + | S | F | S | S | A | F | S | S | P | S | P | P | F | id 10 |
| 13 | + | F | F | F | S | P | S | S | S | P | S | A | A | F | |
| 14 | – | S | F | S | F | A | S | S | S | A | S | P | P | S | |
| 15 | + | F | F | F | F | A | S | F | S | P | S | A | P | S | |
| 16 | – | S | S | F | S | A | S | F | F | A | F | P | A | F | |
| 17 | – | S | F | S | F | A | S | S | S | P | F | P | P | S | |
| 18 | – | F | F | F | F | A | S | F | F | A | S | A | A | F | id 3 |
| 19 | – | F | F | F | F | A | S | F | F | A | S | A | A | F | id 3 |
| 20 | + | F | F | F | F | P | F | S | F | A | F | A | P | F | |
| 21 | + | F | F | S | S | P | F | F | F | A | S | A | P | F | |
| 22 | + | F | F | F | S | A | F | S | F | A | S | P | P | S | |
| 23 | + | F | F | S | S | P | F | S | F | A | S | A | P | S | |
| 24 | + | S | F | F | F | P | F | S | F | A | F | A | P | S | |
| 25 | – | S | S | F | S | A | F | S | F | A | S | P | P | S | |
| 26 | – | F | F | F | F | A | F | F | F | A | F | A | A | S | |
| 27 | – | F | F | F | F | A | S | F | S | A | S | A | P | S | |
| 28 | + | F | S | S | S | P | F | S | F | A | F | A | P | S | |
| 29 | – | F | S | F | S | P | S | S | F | A | F | A | P | S | |
| 30 | + | F | F | F | F | P | F | F | F | A | S | A | A | F | |
| 31A | + | S | F | F | F | A | F | S | F | A | S | P | P | S | |
| 31B | + | S | F | F | F | A | F | S | F | A | S | P | P | S | id 31A |
| 32 | + | S | F | F | F | A | F | F | F | A | S | P | P | S | |
| 33 | + | S | S | F | F | P | F | S | F | A | S | P | P | F | |
| 34 | – | F | F | F | F | A | F | F | F | A | F | A | A | S | |
| 35 | – | F | F | S | S | A | S | S | S | P | F | A | A | S | |
| 36 | + | S | F | S | F | P | S | S | S | P | S | P | P | S | |
| 37 | – | S | F | S | F | A | S | S | S | P | F | P | P | S | |
| 38 | – | S | F | F | S | A | S | F | S | P | S | P | A | S | |
| 39A | – | F | S | F | F | A | S | S | S | P | S | A | P | S | |
| 39B | – | F | S | F | F | A | S | S | S | P | S | A | P | S | id 39A |
| 40 | – | S | S | F | F | A | S | S | S | P | F | P | A | S | |
| 41 | + | S | S | S | F | A | S | S | S | P | S | P | A | S | |
| 42 | – | S | F | F | S | A | S | S | S | P | S | P | P | S | |
| 43 | – | S | S | S | F | A | F | S | S | P | S | A | A | S | |
| 44A | – | S | F | S | F | P | F | S | S | P | S | P | P | F | |
| 44B | – | S | F | S | F | P | F | S | S | P | S | P | P | F | id 44A |
| 44C | – | S | F | S | F | P | F | S | S | P | S | P | P | F | id 44A |
| 45 | – | S | S | S | S | P | S | F | F | A | S | P | A | F | |

| Locus | pH | Sdh-1 | Pgi-1 | Amp-2 | Est-9 | Est-1 | Icd-1 | Acp-4 | Acp-1 | Acp-2 | Pgd-1 | Pox-2 | Est-7 | Mal-1 | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IRAT 177 | – | F | F | S | S | A | F | S | F | A | F | A | P | S | |
| Apura | + | S | S | F | F | P | S | F | S | P | S | P | A | F | |
| 46 | – | F | S | S | F | A | S | S | F | A | S | A | P | F | |
| 47 | – | F | S | F | S | P | S | S | F | A | F | A | P | F | id 29 |
| 48 | + | F | S | S | S | P | S | S | F | A | S | A | P | S | |
| 49A | + | S | F | F | S | P | S | S | F | A | F | P | P | F | |
| 49B | + | S | F | F | S | P | S | S | F | A | F | P | P | F | id 49A |
| 50 | +/– | – | – | – | – | – | – | – | – | – | – | – | – | – | segregating |
| 51 | +/– | – | – | – | – | – | – | – | – | – | – | – | – | – | segregating |
| 52 | +/– | – | – | – | – | – | – | – | – | – | – | – | – | – | segregating |
| 53 | – | F | F | S | S | P | F | S | S | P | F | A | P | F | |
| 54 | +/– | – | – | – | – | – | – | – | – | – | – | – | – | – | segregating |
| 55 | – | F | F | S | S | P | F | S | F | A | F | A | P | S | |
| 56 | – | F | F | S | S | P | F | S | F | A | F | A | P | S | id. 55 |
| 57 | – | F | F | S | S | P | F | S | F | A | F | A | P | S | id. 55 |
| 58 | + | S | F | F | S | A | F | S | S | P | S | P | A | S | |
| 59 | – | S | S | F | S | A | S | S | S | P | S | P | P | F | |
| 60 | – | F | F | S | S | A | F | S | S | P | S | P | P | S | |
| 61 | – | F | F | S | F | A | F | S | S | P | F | A | P | S | |
| 62 | – | F | F | S | S | P | F | S | S | A | F | P | A | S | |
| 63 | + | S | F | F | S | A | F | S | F | A | F | P | P | S | |
| 64 | + | F | F | F | F | A | F | F | F | A | S | A | A | S | |
| 65 | – | S | S | S | F | P | S | S | S | P | S | P | P | F | |
| 66 | – | S | F | F | S | P | S | S | F | P | S | P | A | F | |
| 67 | + | S | S | S | F | P | S | F | S | P | S | P | P | S | |
| 68 | – | S | F | S | S | P | S | S | S | P | S | P | P | F | |
| 69 | – | S | F | S | S | P | S | S | S | P | S | P | P | F | id 68 |
| 70 | – | S | F | S | S | P | S | S | S | P | S | P | P | F | id 68 |
| 71 | – | F | F | F | S | P | F | S | F | A | S | A | P | S | |
| 72 | + | S | S | S | F | P | S | F | S | P | S | P | A | S | |
| 73 | + | S | S | S | F | P | S | F | S | P | S | P | A | S | id. 72 |
| 74 | + | S | S | S | F | P | S | F | S | P | S | P | A | S | id 72 |
| 75 | + | S | S | S | F | P | S | F | F | P | S | A | P | S | |
| 76 | – | F | F | F | F | A | F | S | S | P | S | A | P | S | |
| 77 | – | F | S | F | S | P | S | F | S | P | S | P | P | F | |
| 78 | – | S | F | F | S | A | S | S | F | A | S | P | A | S | |
| 79 | + | S | F | F | F | A | S | S | S | P | S | P | P | S | |
| 80 | + | F | S | F | S | P | F | S | S | P | S | A | A | F | |
| 81 | – | F | F | S | S | A | F | S | F | A | S | A | P | S | |
| 82 | + | F | S | S | F | P | F | S | S | P | F | A | P | F | |
| 83 | – | F | F | S | S | P | F | S | F | A | S | A | P | F | |
| 84 | + | S | S | S | S | P | F | S | S | P | S | A | P | F | |
| 85 | – | S | S | F | S | P | S | F | F | A | S | P | P | S | |
| 86 | – | S | S | S | F | P | F | S | S | P | F | A | P | F | |
| 88 | + | S | S | F | S | P | F | S | S | P | F | P | P | S | |
| 89 | + | F | F | S | F | P | S | S | S | P | S | A | P | S | |
| 90 | – | F | F | S | F | A | F | S | F | A | F | A | P | S | |

The AC plants are numbered according to the date of subculture onto the regeneration medium of the corresponding callus (Id = identical to; F = fast; S = slow; A = absence; P = presence).
The letters following the same AC line number correspond to plants regenerated from the same microspore-derived callus.

### Assessing gametic selection

In simultaneously monitoring the segregations at several loci, isozyme analysis of the AC derivatives of an $F_1$ hybrid, when compared to $F_2$ or backcross data, gives insights into the extent of gametic selection occurring among the cultured microspore population during the androgenetic pathway. Moreover, the survey of both microspore calluses and AC-derived plant populations reveals whether selection occurred during the regeneration process.

The results of segregation of heterozygous isozyme markers among selfing and AC derivatives of the IRAT177/Apura hybrid are shown in Table 4. Significant departures from the expected 1:1 isozyme phenotype segregation were observed at two loci (i.e, *Pgd-1* and *Acp-4*) among both the AC derivatives (calluses and lines) and among the $F_2$ plant population. Such deviations are probably due to hybrid sterility breakdown, which has been tentatively explained in rice by several genetic mechanisms (Oka 1974, Nakagahara 1986, and Ikehashi and Araki 1986). De Buyser *et al.* (1988) reported that the gametic sample produced from a wheat monosomic is biased *in vivo*, and produced consistent deviations among the *in vitro* AC derivatives. This indicates that the gametic selection occurring during the selfing of rice distant hybrids may also interfere with the androgenetic process, and hence, with the nature of AC derivatives.

**Table 4. Comparison of segregation of heterozygous isozymes markers among microspore calluses, doubled haploid lines, and $F_2$ plants derived from the cross IRAT177/Apura.**

| | L O C U S | | | | | |
|---|---|---|---|---|---|---|
| | Ph R | Sdh-1 | Pgi-1 | Amp-2 | Est-9 | Est-1 |
| IRAT 177[a] | - | F | F | S | S | A |
| APURA | + | S | S | F | F | P |
| MICROSPORE[b] CALLI | | 216:226 | 182.264 | 186:258 | 214:186 | 135:115 |
| DOUBLED HAPLOID LINES | 32:40 | 38:34 | 25:47 | 36:36 | 35:37 | 35:37 |
| $F_2$ PROGENY | | 43:102:44 | 54:95:40 | 50:74:64 | 57:87:43 | 41:148 |

(a) The allele designations adopted are F (Fast) vs S (Slow) when the parents differed in the migration speed of the allozymes and A (Absence) vs P (Presence) when the parents differed in the presence of a band—.

(b) Segregation of isozyme phenotypes following the order SS:SF:FF or AA:AP+PP for the $F_2$ progeny and SS:FF or AA:PP for the AC-derived calluses or lines; +/-: for the phenol reaction locus.

Moreover, significant deviations at two additional loci (i.e., *Pgi-1*, *Est-7*) were noticed only among the AC derivatives, indicating that such derivatives do not represent a fully random gametic array. A significant departure from the expected 1:1 ratio was also observed among the AC calluses and lines at locus *Mal-1*, but the weak expression of malic enzyme among screenhouse-grown $F_2$ plants did not permit the conclusion that a specific allelic selection took place during the androgenetic pathway. Therefore, the AC process imposes significant deviations at a proportion of the loci studied.

Segregation data (not shown in this paper) in at least 10 loci among the microspore calluses derived from the five crosses (listed in Table 2) have shown deviations at several loci for both japonica/indica and indica/indica distant hybrids. However these data still have to be compared to $F_2$ data for variable comments on gametic selection. This contrasts with the random assortment patterns previously reported by Chen *et al.* (1982) and Chen *et al.* (1983) on several hundred AC plants derived from three hybrids of rice, each bearing two heterozygous morphological markers. This discrepancy could be explained by their use of japonica/japonica crosses, which are not prone to the hybrid breakdown phenomenon, and of only two markers. Electrophoresis of microspore-derived calluses facilitates the study of a critical number of markers and samples, thereby allowing segregation significant analysis even when crosses between indica cultivars—of which lower anther culturability is known—are used.

| | | | | L O C U S | | | |
|---|---|---|---|---|---|---|---|
| Icd-1 | Acp-4 | Acp-1 | Acp-2 | Pgd-1 | Pox-2 | Est-7 | Mal-1 |
| F | S | F | A | F | A | P | S |
| S | F | S | P | S | P | A | F |
| 234:210 | (c) | 92:90 | (c) | 293.197 | (d) | (d) | 131:59 |
| 36:36 | 54:18 | 39:33 | 34:38 | 51:21 | 36:36 | 22:50 | 45:27 |
| 54:95:40 | 37:31:24 | 49:93:43 | 44:144 | 63:71:44 | 47:142 | 30:86 | (c) |

(c) Number of samples successfully analyzed too small.
(d) Isozymes not detectable in microspore callus analysis.
*Chi Square Tests for Homogeneity:*
$F_2$: fit to a 1:2:1 or a 1:3 segregation; AC-derived calli or lines: fit to a 1:1 segregation ▨ : significant at the 5% level; □ : significant at the 1% level; ■ : not significant at the 5% level.

### Monitoring recombinations among AC derivatives

Comparison of linkages between isozyme loci estimated from DH and $F_2$ data indicates whether or not AC influences the recovery of meiotic products bearing recombinant allelic associations. When it does not, genetic analysis of AC derivatives would provide a simplified way to evaluate recombination rates between markers and traits. Linkage estimates between isozyme loci, borne by chromosome 6, evaluated from the doubled haploid lines derived from the cross IRAT177/Apura appeared consistent with those evaluated from the $F_2$ data and data previously recorded on several japonica/indica crosses (Fig. 2). F2 and DH data permitted detection of a linkage between the *Acp-4* and *Est-7* loci, although they remain unassigned to a rice chromosome. Moreover, phenol reaction and *Est-1* and *Mal-1* loci appeared loosely linked within the DH data. This may be due to pseudolinkages between independent genes, which are commonly observed among progenies of rice distant hybrids. On the other hand, it may indicate that these three markers are located on chromosome 12 to which the *Ph* locus has already been assigned. If this linkage is physically confirmed by further studies, AC would have considerably simplified the genetic analysis, since the exact genotype of an $F_2$ plant for phenol reaction and *Est-1* genes can only be known through analysis of the seeds and leaves of several $F_3$ plants.

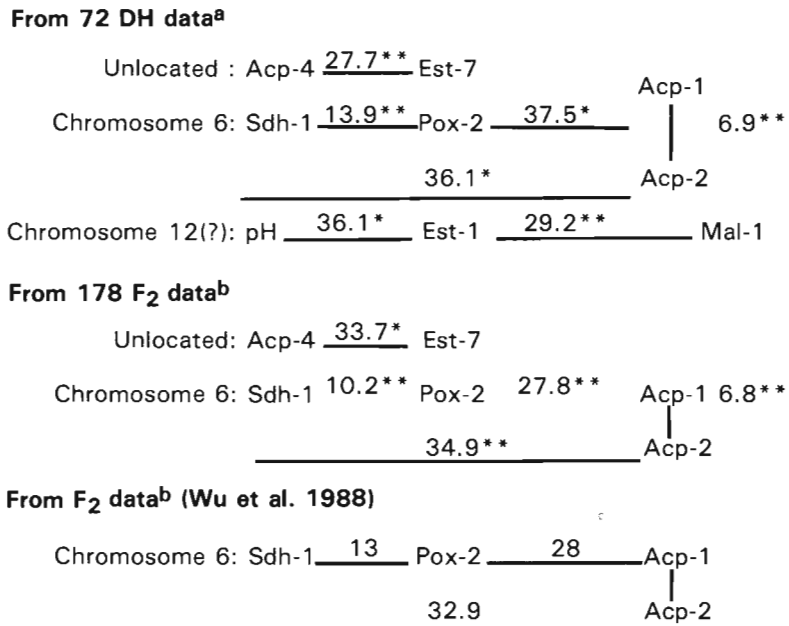


**Figure 2. Representation of linkages between isozyme loci estimated from $F_2$ and DH data of the cross IRAT177/Apura.**

[a] Rough estimates obtained directly from DH data in calculating the percentage of recombinant associations.

[b] Recombination rates evaluated through the maximum likelihood method.

*;**: chi square independent test significant at the 5% and 1% level, respectively.

## CONCLUSIONS

The main advantages in using isozyme markers in rice DH breeding are to assess the germinal origin of the AC derivatives and, when distant hybrids are exploited, to unambiguously identify duplicates resulting from early fragmentation of the microspore-derived calluses. Isozyme analysis of AC derivatives also offers a sensitive test to study their similarity with the gametic population and to study linkage relationships. It could also be used to detect new evidence of co-segregation of useful traits with isozyme genes among doubled haploid lines. This preliminary study is therefore being broadened to include numerous rice crosses exhibiting a wide range of hybrid sterility, to come up with general rules about the comparative impacts of gametic selection and distant hybrid sterility factors on recombinant recovery during selfing and AC processes. Such materials could also appropriately test the controversial theory of pollen dimorphism. Moreover, DH lines from hybrids between distantly related cultivars—which usually display contrasting agronomic traits, isozyme phenotypes, and DNA patterns—also form, once they reach a significant number, a precious set of homozygous materials which can be analyzed to study the cosegration of useful agronomic traits with isozyme and restriction fragment length polymorphism (RFLP) markers now available in rice (McCouch *et al.* 1988).

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## RESUMEN

En este artículo se analizan las ventajas específicas de estudiar las segregaciones y recombinaciones de marcadores heterocigotas naturales de izoenzimas en los derivados sexuales y por cultivo de anteras de híbridos distantes de arroz. Aparte de su amplia expresión dentro de los callos derivados de microsporas, hacen posible la evaluación del origen germinal, la característica original y la representatividad en la población gamética de los derivados por cultivo de anteras. Por otra parte, su ubicación dispersa y conocida en los cromosomas arroja nuevas luces sobre las recuperaciones recombinantes comparativas que se producen a través de los procesos androgenéticos y de autofecundación de los híbridos distantes del arroz.

# Chromosome engineering by anther culture

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Anther culture was carried out on the pure lines of wheat varieties. About 90% of the derivatives were haploids and homozygous diploids and 10% aneuploids, including nullisomics, monosomics, trisomics, tetrasomics, and different telocentrics. These aneuploids are the basic materials of chromosome engineering. The hybrids of secondary distant hybrids crossed with common wheat were used as materials for anther culture. Anther culture allowed various gametic genotypes with different chromosome compositions to be fully expressed and provided new forms of pollen-plants that have 46 chromosomes that are difficult to obtain by conventional methods. Besides these materials, alien substitution lines, addition lines, and translocation lines were directly produced via anther culture. The success frequency using alien addition lines by anther culture was 5.36%, which coincided with the theoretical expectation and was 18 times higher than from the conventional method. Among the products of chromosome engineering by anther culture, there were desirable new strains that possessed good agronomic characters such as disease resistance, early maturity, and reduced plant height. This new technique of chromosome engineering by anther culture appears to be easier and more efficient than the conventional method.

At present, there is a serious lack of germplasm for crop improvement. This problem can be solved by chromosome engineering. The aim of classical chromosome engineering is transferring desirable nonhomologous or alien genes into cultivars as a means of changing and improving their genetic characteristics. Thereby, genetic resources or new cultivars can be provided for research and breeding purposes. Feldman and Sears (1981) proposed and summarized efficient chromosome engineering methods, including distant hybridization, to produce the basic materials of chromo-

some engineering, such as haploids, nullisomics, and monosomics. Through direct crosses, backcrosses, self-pollination, and X-ray treatment, the desirable nonhomologous or alien substitution lines and translocation lines might be obtained.

During the last decade, much progress had been made in the use of anther culture for crop improvement and genetic studies. Our research work indicates a number of advantages with anther culture: 1) homozygosity is achieved rapidly; 2) recessive characters are expressed easily, thereby enhancing selection efficiency; 3) various types of recombinant gametes can be fully expressed at the homozygous state; and 4) through gametoclonal variation, recombinants, variants, and novel genotypes may be created that are usually difficult to obtain by conventional methods (Hu and Huang 1987). This paper reports the application of this new method of chromosome engineering for breeding practices and genetic research.

## STABILITY AND VARIABILITY

For many years, we have been working on Karyotypic analysis of somatic cells derived from pollen. Through the investigation of the chromosome configuration of pollen mother cells (PMCs) and genetic analysis of anther culture (AC)-derived wheat plants, we found that both genetic (chromosomal) stability and variability occurred in the anther culture procedure. Results obtained over a period of several years from unselected populations of AC plants in wheat (*Triticum aestivum*) (Hu *et al.* 1978, 1980), rice (*Oryza sativa*), maize (*Zea mays*), and tobacco (*Nicotiana tabacum*) indicate that about 90% of the AC-derived plants are haploids or homozygous diploids (doubled haploid), whereas 10% are aneuploids. This is an important genetic characteristic of AC plants.

## GAMETOCLONAL VARIATION

Variability of the chromosome number and in the structure of plant cells regenerated *in vitro* is a common phenomenon (D'Amato 1978). The same phenomenon has also been observed in AC-derived plants. The technology of introducing genetic variation by using cell culture has been termed somaclonal and gametoclonal variation (Sharp *et al.* 1984).

To further verify the production of aneuploids directly from anther culture, experiments were carried out from 1981 through 1983, using the inbred spring wheat cultivar 'Orofen'. The root tip somatic cells of 472 AC-derived plants and PMCs of some plant lines were examined cytologically. According to the basic chromosome number, these AC-derived plants were classified into four types: euploids, aneuploids, heteroploids, and mixoploids. Among 472 AC-derived plants, 85.8% were euploids, 9.3% were aneuploids (including nullisomics, monosomics, etc.), 1.7% were heteroploids, and 3.2% were mixoploids. It is worth noting that of the 472 plants, 18 aneuploid plants (3.8%) showed aberrations in chromosome structure. From the AC-derived plants of 'Orofen,' plant line Q31 was monotelosomic with a chromosome configuration of 2n=41+t'. Its spike form was similar to that of speltoid, and therefore significantly different from the parent 'Orofen'. These results indicate that AC gives rise to variation in chromosome number and structure. Another line, D19, was derived from AC of the winter wheat, 'Kedong 58'. This line is ditelosomic, showing a 2n=41+2t' chromo-

some configuration. The progenies of D19 were examined cytologically. Among 10 plants ($H_2$), five were disomic (2n=42), one was mixoploid, three were ditelosomic (2n=41+2t'), and one was double ditelosomic for chromosome 1B with a chromosome configuration of 2n=40+4t'. Sears and Sears (1978) noted that the principal uses of telosomics are as follows: 1) identifying aneuploids and guarding against univalent shift; 2) locating and mapping genes; 3) determining chromosome morphology; 4) transferring genes from alien chromosomes; and 5) determining degrees of meiotic pairing and somatic association. Therefore, these AC-derived telosomics and other aneuploids are very useful resources for studying cytogenetics and chromosome engineering.

## GAMETE ANALYSIS

Since pollen grains from $F_1$ hybrids are heterozygous, different gene combinations of both parents of a cross occur in each $F_2$ progeny. If pollen grains of $F_1$ hybrids are induced to develop plants by anther culture, the plants ($H_1$) show various phenotypes of both parents and their recombinants. Figure 1 shows that the most common AC system is to use $F_1$ hybrids as the parental material for haploid production, thereby fixing the new products of recombination between the parental genotypes at the earliest possible opportunity.

This principle, in general, is difficult to test by conventional methods, but there is significant evidence when distant hybrids are used for anther culture. We have conducted a series of investigations on this topic. Figure 2 illustrates two systems that produce hybrid gametes at the homozygous plant level.

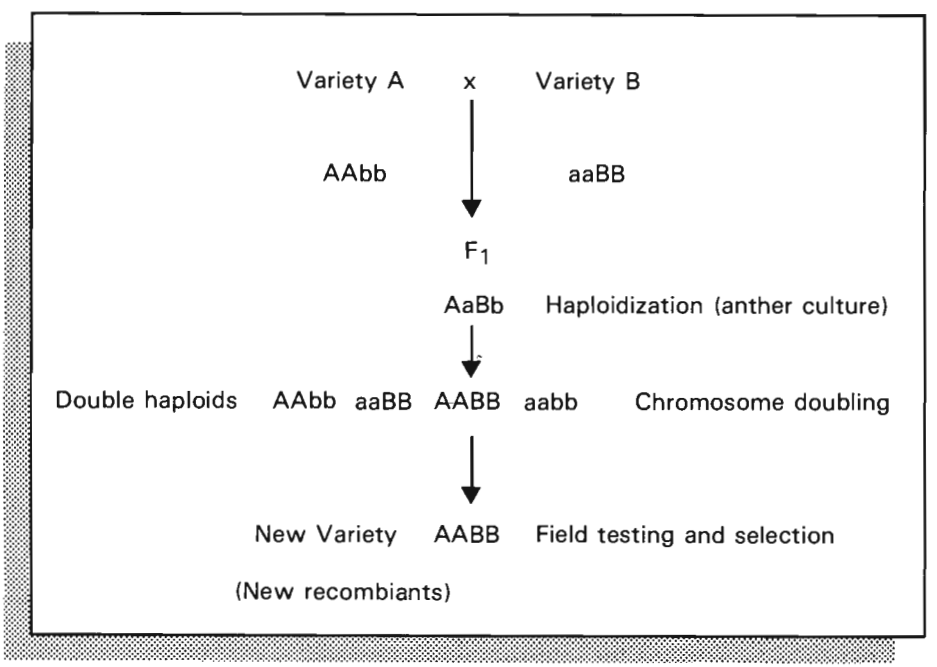


**Figure 1. $F_1$ doubled haploid system.**

**Gamete genotypes fully expressed at plant level**

In conventional breeding, crosses between hexaploid triticale (AABBRR) and hexaploid bread wheat (AABBDD) yield hybrids with the chromosome constitution AABBDR, in which the D and R chromosomes are present as two sets of univalents. At meiosis, the two sets of univalents are distributed into daughter cells with frequencies ranging from 0 to 7; therefore, the pollen grains have diverse chromosome constitutions (Fig. 2, system A). Fifteen kinds of gamete genotypes may be formed with chromosome numbers ranging from 14 to 28. However, Müntzing (1979) indicated that, due to natural selection, in practice it is difficult to combine the same gamete types into one zygote. So, the various gamete genotypes of hybrids are difficult to fully express in sexual hybridization.

From a similar cross between 'Rosner' (6x) triticale and 'Kedong 58' (6x) bread wheat, 10 recombinant types of AC plants were obtained, whereas only four types of $F_2$ plants were produced by conventional methods. The frequencies of chromosome composition types of $H_1$ AC-derived plants and $F_2$ plants produced by conventional methods were 37.04% and 11.76%, respectively (Wang 1984). The difference between these values was significant at the 2% level, which indicates that a high number of recombinant types may be obtained in a shorter time and from a smaller population by anther culture than by conventional methods (Wang and Hu 1985).

Table 1 shows our results using the A system. Repeated experimental data indicate that from 681 AC plants, there were 16 kinds of gamete genotypes with different chromosome constitutions, including 11 with chromosome numbers ranging from 17 to 27 (the corresponding diploids after spontaneous chromosome doubling), two aneuploids (one monosomic and one trisomic), and other groups comprising heteroploids,

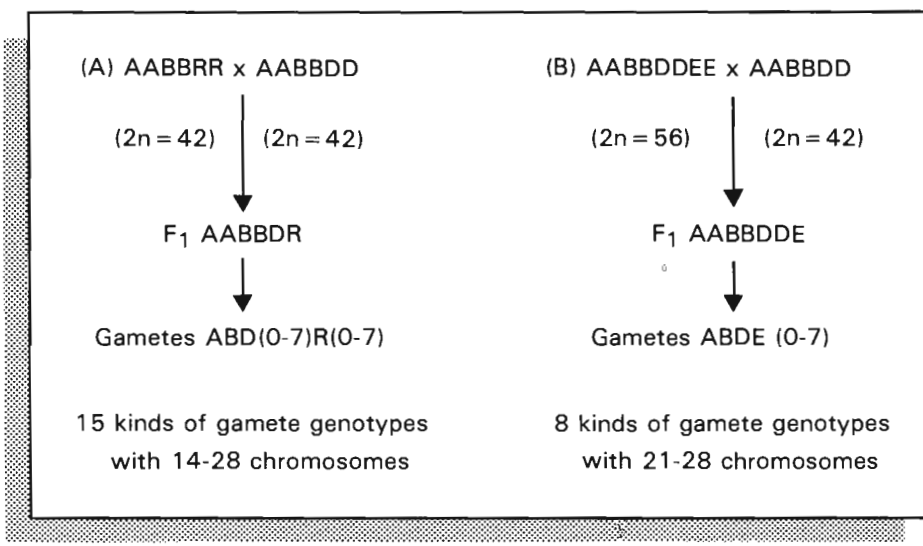


**Figure 2. Two systems that express hybrid gametes at the homozygous plant level.**

**Table 1. Number of chromosomes in AC plants derived from different hybrids between triticale(6x) and bread wheat (6x).**

| Material | Chromosome Numbers | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $F_1$ | 17 | 18 | 19 | 20 | | 21 | | 22 | 23 | 24 | 25 | 26 | 27 | | | | |
| | 34 | 36 | 38 | 40 | 41 | 42 | 43 | 44 | 46 | 48 | 50 | 52 | 54 | I[a] | II[a] | III[a] | Total |
| Rosner/Kedong 58 | | | | | | 1 | | 1 | 7 | 3 | 4 | 1 | 1 | | 9 | | 27 |
| Beagle/Kedong 58 | 1 | 1 | 2 | 6 | | 40 | | 40 | 113 | 88 | 32 | 18 | 6 | 5 | 91 | 23 | 466 |
| Beagle/Jinghua 1 | | | 2 | 1 | | 3 | | 11 | 17 | 16 | 10 | 4 | 2 | 2 | 22 | 5 | 95 |
| Beagle/Orofen | | | 1 | 2 | 1 | 6 | 1 | 17 | 18 | 18 | 13 | 3 | | | 13 | | 93 |
| Total numbers of plants | 1 | 1 | 5 | 9 | 1 | 50 | 1 | 69 | 155 | 125 | 59 | 26 | 9 | 7 | 135 | 28 | 681 |

[a] I = Heteroploids, II = Mixoploids, III = Chromosome structure variants.

The data of Rosner/Kedong 58, Beagle/Kedong 58, and Beagle/Jinghua 1 derived from X.Z. Wang and H. Hu (1985).

mixoploids, and chromosome structure variants. This means that many diverse AC plants with different chromosome constitutions were produced.

Based on the same principle, in the B system (Fig. 2), anther culture of $F_1$ hybrids of the octoploid *Triticum-Agropyron* and bread wheat (Intermediate type No. 3 x 'Orofen') was carried out (Miao *et al.* 1988). We obtained 112 green AC-derived plants. Table 2 shows that by using the $X^2$ test, the distribution of various types having different numbers of chromosomes coincided with that expected by theoretical calculation on the basis of binomial distribution of each univalent at metaphase I of meiosis. Table 2 also shows that we obtained 10 kinds of AC plants with different chromosome constitutions, eight kinds of AC plants with various chromosome constitutions, one trisomic, and 20 mixoploids.

These results indicate that the possible recombinant types of $F_1$ hybrid pollen, i.e. recombined gametes, might be fully expressed at the plant level by means of anther culture.

**Creating new forms**

Anther culture might be an approach to create new forms. From Table 3, we can see that W66-2 is a substitution-addition line with 46 chromosomes including three pairs of rye chromosomes; 1R, 4R, and 6R. This line is also resistant to powdery mildew (*Erysiphe graminis*). Its chromosome constitution has been maintained for seven generations (from $H_2$ to $H_8$), as it has maintained its disease resistance. Meanwhile the AC line m27 derived from 'Beagle' crossed with the bread wheat 'Jinghua 1', has been shown, by the C-banding technique, to be a multi-addition line with 46 chromosomes that possesses two pairs of rye chromosomes, 1R and 6R (Table 5). It sets seed normally and is morphologically uniform. It is also resistant to powdery mildew. Based on the apparent chromosome stability over three to seven generations and the stability of characteristics such as powdery mildew resistance, it is believed that the two 46-chromosome lines are relatively stable. These are new genotypes difficult to acquire either naturally or with conventional crosses. In these examples, the gametes with 23 and 24 chromosomes were shown to be the predominant portion of the whole regenerated AC plants.

Such data indicate that through AC each gamete type might be fully expressed at the haploid plant level, and after chromosome doubling, is again expressed in the regenerated plants with 46 and 48 chromosomes. By contrast, through self-pollination and fertilization, especially under distant hybridization conditions, gamete selection occurs. Those gametes with 23 and 24 chromosomes are unable to compete, compared to the normal gametes with 21 chromosomes. Therefore, this results in the loss of such 23- and 24-chromosome gamete types. Meanwhile, the probability of fusion between a female and a male with the same chromosome composition is very low. Anther culture might be useful for recovering and creating new forms that are difficult to obtain through conventional methods.

**Table 2. Number of chromosomes of AC plants derived from $F_1$ between 8x *Triticum-Agropyron* and 6x bread wheat (Intermediate type No. 3 x 'Orofen').**

| Item | Chromosome Number | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 21 | | 22 | 23 | 24 | 25 | 26 | 27 | 28 | Mixoploids | Total |
| | 42 | 43 | 44 | 46 | 48 | 50 | 52 | 54 | 56 | | |
| No. plants examined | 1 | 1 | 6 | 21 | 22 | 23 | 10 | 6 | 2 | 20 | 112 |
| Percentage | 0.9 | 0.9 | 5.36 | 18.75 | 19.64 | 20.53 | 8.9 | 5.36 | 1.8 | 17.86 | 100 |

Data derived from Miao *et al.* 1988.

**Table 3. Pedigrees of AC plants (2n=46).**

| Rosner/Kedong 58 | | | Beagle/Kedong 58 | | |
|---|---|---|---|---|---|
| Generation (year) | Strain | Chromosome constitution (2n) | Generation (year) | Strain | Chromosome constitution (2n) |
| $H_1$ 1980-81 | W66 | Mixoploid 48,49 | $H_1$ 1983-84 | m17 | 48 |
| $H_2$ 1981-82 | 66-2 | 46[a] | $H_2$ 1984-85 | 17-1 | — |
| $H_3$ 1982-83 | 66-2-4 | 46[a] | $H_3$ 1985-86 | 17-1-7 | 46[a] |
| $H_4$ 1983-84 | 66-2-4-10 | 46[a] | $H_4$ 1986-87 | 17-1-7-3 | 46[a] (1R"6R")[a,c] |
| $H_5$ 1984-85 | 66-2-4-10 -7 | 46[a] (1R"4R"6R")[a,b] | $H_5$ 1987-88 | 17-1-7-3 | 46 (1R"6R")[a,c] |
| $H_6$ 1985-86 | 66-2-4-10 -7-3 | 46[a] (1R"4R"6R")[a,b] | | | |
| $H_7$ 1986-87 | 66-2-4-10 -7-3-10 | 46[a] (1R"4R"6R)[a,b] | | | |
| $H_8$ 1987-88 | 66-2-4-10 -7-3-10-4 | 46[a] (1R"4R"6R)[a,b] | | | |

[a] Plant with powdery mildew resistance.
[b] Substitution-addition line.
[c] Multi-addition line.

**Table 4. The theoretical frequency of alien addition lines.**

| Methods | Addition lines (2n=44) frequency of 7A7B7D+1E |
|---|---|
| a. Anther culture | 1/18.3=5.46% |
| b. Conventional (Selfing) | 1/333.3=0.3% |
| a/b | 18.2 |

### Desirable new forms

Theoretically, the selection efficiency of a desired gamete genotype is much higher through anther culture than through conventional self-pollination. Table 4 shows the theoretical frequency of alien addition lines from $F_1$ hybrids of octoploid *Triticum-Agropyron* crossed with the bread wheat, 'Orofen'. Table 2 shows that the experimental data of alien addition lines from anther culture is 6/112=5.36%, which is similar to the theoretical value of 5.46% shown in Table 4. With AC and subsequent chromosome doubling, the alien addition lines derived from distant hybrids might be obtained directly and more efficiently. The frequency is 18 times higher through AC of the $F_1$ than through the conventional method.

Up to now, different kinds of substitution lines and addition lines from rye with desirable agronomic traits have been obtained in our laboratory. Table 5 shows different desirable forms that possess the characteristics of disease resistance (i.e., powdery mildew and scab), early maturity, and reduced plant height. These strains with alien (rye) chromosomes, having desirable agronomic characteristics, might be used directly in crop improvement programs.

**Table 5. Different substitution and addition lines from hybrids of hexaploid triticale and hexaploid wheat.**

| | | | |
|---|---|---|---|
| **Substitution lines 2n=42** | | | |
| | m08 | with one pair 1R | short stature |
| | m17 | with one pair 1R | early maturity |
| | m24 | with one pair 6R replaced 6B by 6R | resistant to powdery mildew |
| **Translocation line 2n=42** | | | |
| | m05 | translocated $5R^L$(?) | hairy neck ($5R^L$Hp), susceptible to powdery mildew, big grains |
| **Minotelosomic 2n=42+t** | | | |
| | m15 | with $5R^s$ monotelos | |
| **Addition lines 2n=44** | | | |
| | m25 | with one pair 1R | early maturity |
| | m09 | with one pair 6R | resistant to powdery mildew |
| **Multi-addition line 2n=46** | | | |
| | m27 | with one pair 1R and one pair 6R | resistant to powdery mildew |

m27 from Beagle/Jinghua 1; m05, m08, m09, m15, m17, m24, and m25 from Beagle/Kedong 58.

## CONCLUSION

Anther culture is an excellent system for investigation of genetic recombination through gamete analysis in hybrids and gametoclonal variation in pure lines.

Our research work indicates that many diversified gamete genotypes, including recombinants and variants, might be fully expressed at the plant level, while using the pure lines of varieties in wheat as materials where a number of aneuploids—including nullisomics, monosomics, and different telosomics—are obtained directly from AC. These are the basic materials of chromosome engineering. Using distant hybrids as material for AC, the alien chromosomes (genes) might be transferred into commercial cultivars. With this, alien substitution lines, addition lines, translocation lines, and even substitution-addition and multi-addition lines can be acquired directly. In addition to the desirable agronomic traits of these alien addition lines, their frequency by AC is also higher than that obtained from conventional methods. Based on these results, chromosome engineering via AC may be a more efficient and simple method than conventional methods.

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**RESUMEN**

Se realizaron cultivos de anteras de líneas puras de variedades de trigo. Alrededor del 90% de los derivados fueron haploides y homocigotas diploides y el 10% fueron aneuploides, incluyendo nulisómicos, monosómicos, trisómicos, tetrasómicos y diversos telocéntricos. Los aneuploides constituyen los materiales básicos de la ingeniería de cromosomas. Se utilizaron los híbridos de híbridos secundarios distantes cruzados con trigo común como materiales para el cultivo de anteras, que hizo posible la plena expresión de varios genotipos gaméticos con diferentes composiciones cromosómicas. Se generaron algunas formas nuevas de progenitores masculinos que resultan difíciles de obtener con métodos convencionales. Además de estos materiales, se empleó el cultivo de anteras para producir líneas de sustitución extrañas, líneas de acumulación y líneas de translocación. La frecuencia de éxito cuando se usaron líneas de sustitución extrañas en el cultivo de anteras fue de 5.36%, porcentaje que coincidió con las expectativas teóricas y que fue 18 veces mayor que el obtenido con el método convencional.

# Studies on the *in vitro* culture of unpollinated sugarbeet (*Beta vulgaris* L.) ovules and plant regeneration

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The most important method for haploid production in crops is anther culture. However, haploid production in sugarbeet (*Beta vulgaris* L.) through anther culture has been disappointing. Since the technique of unfertilized ovary and ovule culture was first established, many scientists have reported experimental results of sugarbeet ovule culture. In this paper, experimental results of sugarbeet ovule culture and plant regeneration during the 1985-1987 period are reported. We were able to obtain high frequencies and efficiencies when female sugarbeet gametophytes were induced to form haploid plantlets.

At present, the most important method for haploid production in crops is anther culture. However, haploid production in sugarbeet (*Beta vulgaris* L.) through anther culture (AC) has been disappointing (Li 1987). Since Hosmans and Bossoutrot (1983) first established the technique of unfertilized ovary and ovule culture, many scientists have reported experimental results of sugarbeet ovule culture (Bornman 1985, Gibson 1987, Goska 1985, Keimer and D'Halluin 1985, Li *et al.* 1988, Luo *et al.* 1987, Shao *et al.* 1988, Wang *et al.* 1987). In this paper, we report our experimental results of sugarbeet ovule culture and plant regeneration during the 1985-1987 period.

## MATERIALS AND METHODS

Materials used in these experiments included: polygerm diploid lines of Sh 21 system materials, polygerm tetraploid lines of Sh 4 system materials, monogerm diploid lines of seven materials collected from China and other countries, and a monogerm tetraploid Sh 1 line.

### Preparation of explants

We took a section of immature inflorescence and cut off the top 3 cm. Ovules were removed from the ovary and placed on MS (Murashige and Skoog 1962) or modified

MS medium containing Kinetin (KT), boric acid + 0.05-0.2 mg/L of 2,4-Dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA), 0.5-1.5 mg/L of indole-3-butyric acid (IBA), and 3-8% sucrose at a pH between 5.8 and 6.2.

### Clonal propagation and plant regeneration

Haploid plantlets were propagated in MS + boric acid (1.2-2.5 mg/L), KT (0.5-1.5 mg/L), and gibberellic acid (GA) (0.05-0.1 mg/L). Rooting was stimulated by half-strength MS + IBA or NAA (1.2-3.0 mg/L). The culture temperature and relative humidity were 28-30 °C and 70-80%, respectively.

### Chromosome counting

Chromosome counts were made using the Ferric Ammonium Haematoxylin preparation method on plant material fixed in Carnoy's solution.

## RESULTS

### Morphological observations of ovule-derived plantlets in sugarbeet

After 20 days in culture, embryoids were formed from the ovules. Embryoids differed in morphology and color, e.g. white, light yellow, light green, dark green, and pink. After an additional 5-10 days in culture, the plumule, the plumule axis, and radicle had formed. These plantlets generally grew slowly. The rate of plantlet growth and the thickness of the plumule axis varied with the genotype and culture conditions.

### Factors affecting embryoid induction frequency

The frequency of embryoid induction from sugarbeet ovule culture depended on the genotypes, culture medium, and culture environment. The experiments showed that the different genotypes gave different induction frequencies while growing in the same medium and the same genotype gave different induction frequencies in different media or combinations of media (Tables 1 and 2). Table 1 shows the embryoid induction obtained from diploid and tetraploid sugarbeet plants grown in different media. The embryoid induction frequency of the diploid-derived ovules varied from 1.0 to 5.0% and that of the tetraploid-derived ovules varied from 1.4 to 5.0%. Table 2 shows the embryoid induction frequency of different genotypes and suggests that differences in the frequency of haploid embryoid induction are under genetic control. In addition, our experiments showed that suitable culture conditions appear to be between 70-80% relative humidity and 28-30 °C.

### *In vitro* plant propagation and plant regeneration

Embryoids or callus were moved to MS medium supplemented with boric acid (0.5-2.5 mg/L), KT (0.5-1.4 mg/L), and GA (0.05-0.1 mg/L). After 20-25 days in culture, these embryoids developed into young buds, subsequently forming many adventitious buds. The efficiency of clonal propagation varied depending on the type and concentration of growth hormone used. The results are shown in Table 3. Boric acid (2.0 mg/L) gave the highest efficiency of clonal propagation. To obtain good efficiency of clonal propagation, it is important to frequently change the hormone dosage in the medium during long-term continuous culture.

Plantlets derived from continuous culture were rooted in half-strength MS + IBA (1.2-3.0 mg/L). The frequency of rooting was higher. However, the ability of root differentiation and regeneration was reduced with a greater time in the culture.

**Genetic stability of regenerants and observations of ploidy level**

From Table 4 we see that, of the 85 plantlets derived from diploid genotypes, 66 plantlets were haploid (2n=x=9). Of the 15 plantlets derived from tetraploid genotypes, 14 plantlets were amphihaploid (2n=2X=18). The chromosomes of haploid plantlets doubled spontaneously with a certain frequency during continuous culture. The chromosome number of amphihaploids was stable during continuous culture. In addition, we found a few mixoploid plantlets.

**Table 1. Embryoid induction frequency from ovules of sugarbeet (*Beta vulgaris* L.) in different media (1987).**

| Materials | Medium No. | No. ovules inoculated | No. embryoids regenerated | Frequency (%) |
|---|---|---|---|---|
| **Diploid** | 1 | 110 | 2 | 1.8 |
| | 2 | 310 | 13 | 4.2 |
| | 3 | 270 | 11 | 4.1 |
| | 4 | 130 | 2 | 1.5 |
| | 5 | 290 | 6 | 2.1 |
| | 6 | 350 | 7 | 2.0 |
| | 7 | 270 | 3 | 1.1 |
| | 8 | 440 | 5 | 1.1 |
| | 9 | 100 | 2 | 2.0 |
| | 10 | 100 | 3 | 3.0 |
| | 11 | 70 | 1 | 1.1 |
| | 12 | 100 | 1 | 1.0 |
| | 13 | 100 | 5 | 5.0 |
| | 14 | 100 | 4 | 4.0 |
| | 15 | 90 | 4 | 4.4 |
| | 16 | 90 | 2 | 2.2 |
| **Tetraploid** | 17 | 70 | 1 | 1.4 |
| | 18 | 20 | 1 | 5.0 |
| | 19 | 140 | 4 | 2.8 |
| | 20 | 40 | 1 | 2.5 |

**Table 2. Embryoid induction frequency from ovules of different sugarbeet (*Beta vulgaris* L.) materials (1986).**

| Medium No. | Materials | No. ovules inoculated | No. embryoids regenerated | Frequency (%) |
|---|---|---|---|---|
| 1 | Sh 8 | 70 | 4 | 5.7 |
| 2 | Sh 8 | 130 | 4 | 3.1 |
| 3 | Sh 8 | 50 | 6 | 12.0 |
| 4 | Sh 8 | 10 | 1 | 10.0 |
| 5 | Sh 8 | 10 | 2 | 20.0 |
| 6 | Sh 8 | 30 | 2 | 6.7 |
| 7 | Sh 8 | 20 | 2 | 10.0 |
| 8 | Sh 8 | 20 | 0 | 0.0 |
| 9 | Sh 8 | 30 | 2 | 6.7 |
| 10 | Sh 8 | 10 | 3 | 30.0 |
| 11 | Sh 6 | 90 | 7 | 7.7 |
| 12 | Sh 6 | 40 | 2 | 5.0 |
| 13 | Sh 6 | 70 | 1 | 1.4 |
| 14 | Sh 6 | 70 | 1 | 1.4 |
| 15 | Sh 6 | 50 | 0 | 0.0 |
| 16 | 86P1 | 40 | 1 | 3.3 |
| 17 | 86P6 | 30 | 1 | 2.5 |
| 18 | 86P6 | 10 | 1 | 10.0 |
| 19 | 86P6 | 40 | 0 | 0.0 |
| 20 | Sh 8/Sh 6 | 70 | 1 | 1.4 |
| 21 | Sh 8/Sh 6 | 50 | 2 | 2.5 |
| 22 | Sh 8/Sh 6 | 80 | 1 | 2.0 |
| 23 | Sh 8/Sh 6 | 10 | 2 | 20.0 |
| 24 | Sh 8/Sh 6 | 10 | 1 | 10.0 |
| 25 | Sh 8/Sh 6 | 40 | 3 | 7.5 |
| 26 | Sh 8/Sh 6 | 30 | 1 | 3.3 |
| 27 | Sh 8/Sh 6 | 40 | 4 | 10.0 |
| 28 | Sh 8/Sh 6 | 20 | 1 | 5.0 |
| 29 | Sh 8/Sh 6 | 100 | 9 | 9.0 |
| 30 | Sh 411-12 | 70 | 1 | 1.4 |
| 31 | Sh 413 | 20 | 1 | 5.0 |
| 32 | Sh 413 | 40 | 1 | 2.5 |
| 33 | Sh 413 | 40 | 1 | 0.0 |
| 34 | Sh 413 | 10 | 0 | 0.0 |
| 35 | Sh 413 | 110 | 0 | 0.0 |
| 36 | Sh 413 | 20 | 1 | 5.0 |
| 37 | Sh 413 | 40 | 1 | 2.5 |
| 38 | Sh 413 | 40 | 0 | 0.0 |
| 39 | Sh 413 | 140 | 4 | 2.8 |
| 40 | Sh 413 | 40 | 2 | 5.0 |

## DISCUSSION

From our experimental results, high frequencies and efficiencies were obtained when female sugarbeet gametophytes were induced to form haploid plantlets. Our research group has studied sugarbeet anther culture for 12 years (1973 to 1985). A summary of the results is shown in Table 5. Anther culture produced a very low frequency of haploid induction and in some cases it was impossible to achieve. Many scientists have reported the differences in the response of sugarbeet to ovule and anther culture (Wang

**Table 3. Efficiency of haploid plant propagation in sugarbeet (*Beta vulgaris* L.) using two growth hormones (1987).**

| Hormone | Concentration (mg/L) | No. explants inoculated | Increase no. | Efficiency (%) |
|---|---|---|---|---|
| Boric Acid | 0.5 | 3 | 4.5 | 150 |
| | 1.0 | 3 | 4.9 | 163 |
| | 1.5 | 3 | 5.6 | 187 |
| | 2.0 | 3 | 7.1 | 237 |
| | 2.5 | 3 | 6.0 | 200 |
| Kinetin | 0.5 | 3 | 3.5 | 117 |
| | 1.0 | 3 | 4.2 | 140 |
| | 1.5 | 3 | 5.0 | 167 |

**Table 4. Ploidy level of ovule-derived sugarbeet (*Beta vulgaris* L.) plantlets (1986).**

| Materials | No. plants observed | Haploid no. | Amphihaploid no. | Frequency (%) |
|---|---|---|---|---|
| Sh 8 | 38 | 36 | | 95 |
| Sh 8-1 | 2 | 2 | | 100 |
| Sh 8-2 | 2 | 2 | | 100 |
| Sh 6 | 1 | 1 | | 100 |
| Sh 6-1 | 11 | 5 | | 45 |
| Sh 8/Sh 6 | 13 | 6 | | 46 |
| Sh 4 | 2 | 2 | | 100 |
| Sh 6-84 | 2 | 1 | | 50 |
| 87P6 | 4 | 3 | | 75 |
| Sh 6/Sh 5 | 1 | 1 | | 100 |
| Sh 5/Sh 6 | 2 | 2 | | 100 |
| 7208 | 2 | 1 | | 50 |
| 7208/Sh 1 | 1 | 1 | | 100 |
| Sh 410 | 6 | | 6 | 100 |
| Sh 413 | 5 | | 5 | 100 |
| Sh 415 | 3 | | 2 | 66 |
| Sh 403 | 1 | | 1 | 100 |

*et al.* 1983, Li 1987, Goska 1985). We think that such differences may be under genetic control. It was found that frequency of sex cell-derived plants is a quantitative character under polygenic control (Hu 1985). Whether the difficulties experienced in inducing sugarbeet anther-derived plants are due to experimental techniques or not needs to be investigated further.

The frequency of diploid ovule-derived plantlets in our experiment was lower than that obtained from others (Hosmans and Bossoutrot 1985). This may be due to the time at which the samples were taken. We can use male fertile lines as the method for taking samples. It was not clear whether the diploid ovule-derived plants are derived from the fertilized ovule, a somatic cell, or from the fusion of the synergids. Our research group is currently studying the problem.

**Table 5. Sugarbeet (*Beta vulgaris* L.) anther cultures made during 1973-1985.**

| Medium | Materials | No. plants regenerated | Year |
|---|---|---|---|
| 7301-04 | Sh 403 | 4 | 1973 |
| 7504 | Sh 8 | 2 | 1975 |
| 7716 | Sh 403 | 1 | 1977 |
| 7703-2 | Sh 403 | 1 | 1977 |
| 8007-1 | Sh 403 | 1 | 1980 |
| 8007 | Sh 403 | 1 | 1980 |
| 8020 | Sh 403 | 1 | 1980 |
| 8007-2 | Sh 403 | 1 | 1980 |
| 8021 | Sh 403 | 1 | 1980 |
| 8213 | Sh 5 | 1 | 1982 |
| N6 | Sh 5 | 1 | 1983 |
| N6 | Sh 406 | 1 | 1983 |
| 8401 | Sh 8 | 1 | 1984 |
| 8517 | Sh 6 | 1 | 1985 |

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## RESUMEN

En la actualidad, el método más importante para la producción de haploides en los cultivos es el cultivo de anteras; no obstante, la producción de haploides en la remolacha azucarera (*Beta vulgaris* L.) mediante el cultivo de anteras no ha tenido éxito. Desde que se estableció por primera vez la técnica del cultivo de ovarios y óvulos no fecundados, muchos científicos han presentado resultados experimentales del cultivo de óvulos de remolacha azucarera. En este artículo se presentan los resultados experimentales del cultivo de óvulos y la regeneración de plantas de remolacha azucarera en el período 1985-1987. Fue posible obtener alta frecuencia y eficiencia cuando los gametofitos femeninos de la remolacha azucarera fueron inducidos a formar plántulas haploides.

# Protoplast culture in crops: techniques, status, and potential

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Protoplasts are required for many aspects of plant genetic manipulation. The efficient regeneration of plants from protoplasts is required for the application of this protoplast technology in agriculture. In cereals, regeneration of plants is possible from maize and rice protoplasts. By direct interaction of protoplasts with plasmids, transgenic maize and rice plants can now be readily attained. Legume protoplasts are also responsive, leading to the production of somatic hybrids and transgenic plants. In both tomato and the legumes, protoplast fusion, coupled with adequate division and plant regeneration, is being utilized for the introgression of desired genes from wild into cultivated species.

There is continuing interest in the extent to which protoplast technology will contribute to crop improvement. Protoplasts are used for a range of genetic manipulations; protoplast fusion has produced a range of somatic hybrid and cybrid plants and direct interaction of protoplasts with plasmids has produced a range of transgenic plants. Two major difficulties face those wishing to apply protoplast technology to agriculture. The first difficulty is that, generally, agriculture has been very successful in utilizing sexual crossing and mutation breeding for crop improvement. The other difficulty is that, ideally, one would like to be able to transfer specific genes for the improvement of a desired agronomic trait. Quite apart from the fact that often many genes are usually involved, making transformation of protoplasts very difficult, only very rarely have the genes actually been identified. Confounding the whole approach is the on-going lack of much basic knowledge of plant cell and developmental biology and of plant molecular genetics.

For genetic manipulation techniques, such as somatic hybridization and direct gene transfer into protoplasts, to be applied to crop improvement, efficient procedures must be available for plant regeneration from protoplasts of the species in question.

Also for any utilization of protoclonal variation for crop improvement, efficient procedures for plant regeneration from protoplasts are also required.

## CEREAL PROTOPLASTS

Recently there have been several reports of the regeneration of plants and plantlets from rice protoplasts (Coulibaly and Demarly 1986, Fujimura *et al.* 1985, Yamada *et al.* 1986). However, either low frequencies of plant regeneration were obtained in these studies or adequate details were lacking of the culture methods employed. At Nottingham a system has been developed for the efficient, reproducible regeneration of fertile rice plants from protoplasts (Abdullah *et al.* 1986). The procedures involve the culture of cell suspension-derived protoplasts of embryo, leaf, root, and anther origin in an agarose-solidified medium following heat shock treatment. The resulting colonies, when plated directly into a hormone-free medium, produce green plants rapidly, principally through somatic embryogenesis. Regeneration can be obtained as quickly as 7 weeks from the time of protoplast isolation from the japonica rice cultivars Taipei 309 and Fujisaka 5. Recently, regeneration has been achieved from protoplasts of Nipponbare, another japonica rice cultivar, utilizing cell suspension cultures derived from the scutellum of mature seeds. Key considerations involve the establishment of the suspension cultures from embryogenic callus, and the maintenance of the suspension cultures (Finch *et al.* 1989). Nurse cultures may be beneficial for the culture of the isolated rice protoplasts but, if used, it is essential that their origin be fully described if the protocols recommended are to be reproduced in other laboratories.

Kyozuka *et al.* (1987) found that nurse cultures were essential for sustained division of their rice protoplasts. It is now clear that the combination of a heat shock (Thompson *et al.* 1987), protoplast culture in agarose (Thompson *et al.* 1986), and direct rapid plant regeneration from protoplast-derived callus through somatic embryogenesis (Abdullah *et al.* 1986) provides a generally applicable system for efficient plant regeneration from japonica rice protoplasts isolated from cell suspensions of a wide range of explant origins. Efficient plant regeneration from indica rice protoplasts has not, as yet, been achieved. Studies on the response in culture of indica/japonica hybrids may be useful in resolving this difficulty; many commercially important rice cultivars are indica/japonica hybrids (Glaszmann 1987).

A somewhat similar approach, involving the use of protoplast isolated from cell suspension cultures, has also been successfully utilized for the regeneration of plants from maize protoplasts (Cai *et al.* 1987, Rhodes *et al.* 1988). However, wheat and barley still present an ongoing challenge in this respect. While it is possible to obtain cell suspension cultures suitable for protoplast isolation, by the time such cell suspension cultures have been produced, the plant regeneration capability of their protoplasts has been lost. A recent detailed investigation of the control and maintenance of plant regeneration in sugarcane callus cultures may provide additional insight. Techniques for controlling and maintaining long-term regeneration from sugarcane callus cultures, including the effect of electric current treatment on plant regeneration, have been described (Chen *et al.* 1988a). It is of interest that electric current utilizing field pulses ranging from 250 to 2000 V of 10 to 50 μsec duration promoted division and enhanced plating efficiency of protoplasts of *Glycine*, *Pyrus*,

and *Solanum* spp. (Rech *et al.* 1987). Recently an assessment of the factors affecting the division and plant regeneration of sugarcane protoplasts has highlighted the importance of source material for the initiation of cell suspension and callus cultures (Chen *et al.* 1988b).

In the case of rice, the ability to obtain seed progeny from plants regenerated from japonica rice protoplasts has enabled potentially useful variation to be detected in plants derived from seed produced by protoplast regenerated rice plants (Abdullah *et al.* 1986).

The use of protoplast fusion is of particular interest for attempted gene flow between sexually incompatible cereal species, e.g. attempted somatic hybridization of highly salt tolerant *Porteresia coarctata* with *Oryza sativa*. Protoplasts can be readily isolated from the basal regions of the leaves of *P. coarctata* and fused with these from cell suspension cultures of *Oryza sativa*, using the improved polyethylene glycol fusion procedure (Chand *et al.* 1988a). Selection of heterokaryons is currently being undertaken using fluorescent activated cell sorting (Cocking 1986). Alternatively, or in combination with cell sorting, selection can be undertaken by inactivation of rice protoplasts by iodoacetamide, coupled with the inability of the *P. coarctata* protoplasts to divide in culture, paralleling the selection procedure used in the production of rice and barnyard grass somatic hybrids (Terada *et al.* 1987). Apart from the use of protoplast fusion in relation to the transfer of cytoplasmic-based male sterility and herbicide resistance, the opportunity now arising in the cereals to produce unique nuclear-cytoplasmic combinations has additional implications (Kumar and Cocking 1987).

The current failure to achieve *Agrobacterium*-induced transformation in cereals has led to increased interest in assessing other transformation procedures for the production of transgenic plants, especially direct DNA uptake into cereal protoplasts (Cocking and Davey 1987). There has also been renewed interest in microinjection into microspore-derived embryoids, paralleling the approach recently developed for the microinjection of developing microspores of *Brassica napus* (Neuhaus *et al.* 1987), using the pioneering advances made by Koop *et al.* (1983) on the individual culture of selected single cells and protoplasts in microdroplets of defined media. The recent report of genetically transformed maize plants from protoplasts treated with chimeric plasmid (Rhodes *et al.* 1988) is a major stimulus to the use of cereal protoplasts for the production of transgenic plants. Also, transgenic rice plants have been produced recently by electroporation-mediated plasmid uptake into protoplasts (Zhang *et al.* 1988). It was previously established that electroporation was the most efficient procedure for the production of kanamycin-resistant rice tissues following plasmid uptake into protoplasts (Yang *et al.* 1988); a combination of optimum conditions for transformation with those for plant regeneration resulted in the production of transgenic rice plants resistant to kanamycin.

## LEGUME PROTOPLASTS

There has been extensive work on the regeneration of plants from protoplasts of forage legumes, laying a foundation for reproducible plant regeneration from the world's major forage legumes (Davey and Kumar 1983). The recent report of somatic

hybridization of Birdsfoot Trefoil *(Lotus corniculatus)* and *Lotus coimbrensis* (Wright *et al.* 1986) illustrates a useful application of protoplast technology in the forage legumes. The recent report of plant regeneration from protoplasts of *Glycine canescens*, a wild relative of soybean, will stimulate research on the fusion of soybean with wild species of *Glycine*, with the expectation that any somatic hybrids will be capable of plant regeneration (Hammatt *et al.* 1987).

Several improvements in protoplast culture protocol have aided legume protoplast studies. Plant regeneration from cotyledon protoplasts was obtained in *Medicago difafcata*, *M. falcata*, *M. glutinosa*, *M. hemicycla*, and *M. varia*; co-cultivation of freshly isolated cotyledon protoplasts with protoplasts from an albino *M. sativa* cell suspension was beneficial in promoting division (Gilmour *et al.* 1987).

## TOMATO PROTOPLASTS

Protoplasts of tomato (*Lycopersicon esculentum*) can be readily isolated and can be induced to undergo sustained division to form callus in a wide range of cultivars. However, reproducible regeneration of plants from such protoplast-derived calluses is restricted to a few cultivars. In contrast, the wild tomato species (*L. peruvianum*) is readily capable of regeneration into plants from protoplast-derived callus (Zapata *et al.* 1977). Fusions between protoplasts *L. esculentum* and *L. peruvianum* have resulted in the regeneration of fertile somatic hybrid plants (Kinsara *et al.* 1986), demonstrating that plant regeneration capability is required only in one of the parent species. This result indicates that protoplast fusion may be useful for the introgression of desired genes from the '*peruvianum*' complex into the cultivated '*esculentum*' complex.

## SOLANUM PROTOPLASTS

*Solanum dulcamara* (woody nightshade) is an important alkaloid-producing plant and there is currently interest in developing a protoplast system for this plant similar to those already developed for *Solanum viarum* (Kowalczyk *et al.* 1983). Encouragingly, it has been observed that electroporation stimulates division of cell suspension protoplasts of *S. dulcamara* and enhances shoot formation from protoplast-derived tissues (Chand *et al.* 1988b). These effects may be correlated with the fact that electroporation is known to increase DNA synthesis in cultured plant protoplasts (Rech *et al.* 1988).

## CONCLUSION

In this survey, emphasis is placed on recent advances in novel approaches to improvement in the culture of crop protoplasts. Several exciting technological developments have taken place recently (Davey and Power 1988) with major implications in relation to plant genetic manipulation and our ability to apply protoplast technology to crops.

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## RESUMEN

Los protoplastos son necesarios en muchos aspectos de la manipulación fitogenética. La regeneración eficiente de plantas a partir de los protoplastos es necesaria para aplicar esta tecnología de protoplastos en la agricultura. En el caso de los cereales, es posible regenerar plantas a partir de protoplastos de maíz y arroz. En la actualidad se pueden obtener sin dificultad plantas transgénicas de maíz y arroz mediante la interacción directa de protoplastos y plasmidios. Los protoplastos de las legumbres también responden, dando por resultado la producción de híbridos somáticos y de plantas transgénicas. Tanto en el tomate como en las legumbres, la fusión de los protoplastos, aunada a una división y regeneración adecuada de las plantas, se utiliza para incorporar los genes deseados de especies silvestres en especies cultivadas.

# Microculture, microfusion, and microinjection of defined plant cells

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Due to the high degree of heterogeneity in, for example, the morphology, chromosome number, and structure and the biosynthetic and morphogenic potential of higher plant cells in mass culture, the development of methods for individually culturing defined plant cells seems to be useful and necessary for particular experimental purposes. The use of individual cell culture allows for studies on the physiology of different cell types, the analysis of cell-to-cell interactions, and for the performance of genetic micromanipulations. This paper describes an improved microculture system, based on a computer-controlled setup, for the selection, transfer, and individual culture of defined higher plant cells in nanodroplets of culture medium. Also presented are other micromanipulation possibilities for genetically altering higher plant cells under controlled conditions, such as electrofusion of defined pairs of protoplasts and subprotoplasts, cell reconstitution, and intranuclear microinjection of protoplasts and karyoplasts. In addition, for plant species that cannot be readily regenerated from protoplasts, but for which anther or isolated microspore cultures are available, we show an alternative gene transfer method by using micromanipulation techniques based on microinjection into multicellular microspore-derived embryoids.

The improved microculture system originally developed for protoplasts of *Nicotiana tabacum* and suspension cells of *Datura innoxia* (Koop *et al.* 1983a, Koop and Schweiger 1985a) uses a fully automated setup (Spangenberg 1986, Schweiger *et al.* 1987). It allows for the microscopic selection of single protoplasts and their *ab initio* individual culture in nanodroplets of unconditioned culture medium with regeneration of cells, microcolonies, and, subsequently, plants at similar frequencies as in a mass culture.

On the basis of this useful tool, many interesting experimental questions can be addressed under strictly defined conditions. These include studies on developmental physiology and cell-to-cell interactions and the performance of genetic manipulations at the single cell level. We discuss some examples regarding: 1) the individual selection and microculture of plant protoplasts, and 2) different categories of gene transfer by individual manipulation of plant protoplasts, including electrofusion of defined pairs of protoplasts, transfer of partial genomes by electrofusion of protoplast-subprotoplast pairs, and monofactorial transformation via microinjection.

## METHODS AND RESULTS

### Setup for selection and experimental manipulation of single protoplasts

The instrument setup (Fig. 1) is based on the original system developed by Koop and Schweiger (1985a) with additional full automation of the positioning functions. The system, assembled in a flow cabinet, consists of: 1) an inverted microscope with a stepmotor-driven, programmable stage, 2) a microprocessor-controlled pump, allowing for the delivery and withdrawal of volumes in the nanoliter range, connected by a hydraulic system filled with mineral oil to a microcapillary, which is fixed to a holding device vertically driven by a programmable z-axis stepmotor, and 3) a microprocessor for the control of the positioning electronics of the three stepmotors as well as for the nanoliter-pump (Spangenberg 1986, Schweiger *et al.* 1987).

In this way, it is possible to inject nanodroplets of culture medium into 1-µl microdroplets of mineral oil for the fully automatic preparation of microculture chambers described by Koop and Schweiger (1985a). This setup also allows microscopic selection of defined plant cells and their transfer to individual cultures.

This microculture system has been successfully applied for the individual culture of protoplasts of *N. tabacum* (Koop and Schweiger 1985a), *Brassica napus* (Spangenberg *et al.* 1986a), and the moss *Funaria hygrometrica* (Mejia *et al.* 1988). It has allowed the establishment of defined cell clones and regeneration of plants with frequencies ranging from 1 to 60%, depending on the plant species. In addition, it has proven useful for the analysis of cell-to-cell interactions and conditioning effects at the single cell level (Spangenberg *et al.* 1985).

### One-to-one electrofusion of defined pairs of protoplasts

Koop and Schweiger (1985b) first showed the feasibility of somatic hybridization via controlled electric field-induced fusion of defined preselected pairs of protoplasts for homofusions of tobacco protoplasts. It was accomplished with an instrumental setup previously reported (Koop *et al.* 1983b). In this way, perfect control of the number and type of protoplasts involved in the fusion event is possible. The further development of the micromanipulation system allowed the fixing of different microtools in the vertically-driven holding device. For example, microelectrodes, connected to commercially available electrofusion equipment, made it possible to achieve a more accurate and fast electrofusion of individually selected pairs of protoplasts (Fig. 2). By using this system, up to 50 preselected pairs of protoplasts can be fused; the fusion products obtained can be transferred into the microculture within 1 hour.

Depending on the type of protoplast to be fused, the yield (no. of fusants x 100/no.

of selected pairs) of the one-to-one electrofusion varies between 45% and 60% for rapeseed hypocotyl protoplasts (Spangenberg and Schweiger 1986), 11% and 25% for protonema protoplasts of *F. hygrometrica* (Mejia *et al.* 1988), and up to 90% for tobacco mesophyll protoplasts (Koop and Schweiger 1985b). The fusants obtained can be microcultured, producing hybrid cell clones and regeneration of whole plants. No significant difference has been found regarding the behavior in individual culture between fusion products and control nonfused protoplasts for all cases so far reported.

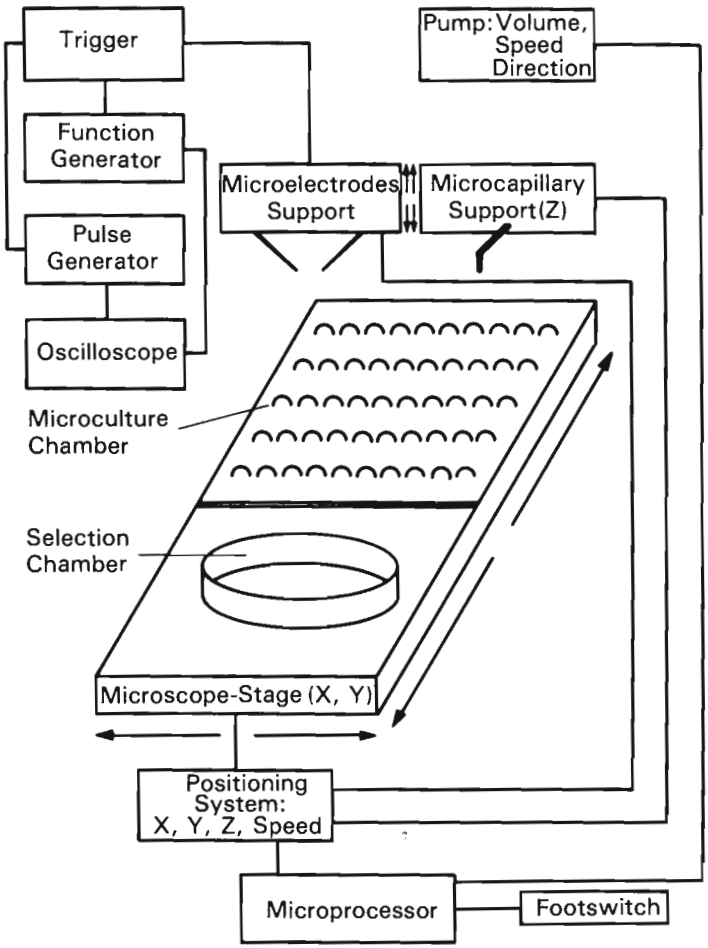


**Figure 1. Setup for microprocessor-controlled selection, transfer, and electrofusion of plant cells.**

## Microfusion of preselected protoplast-subprotoplast pairs and cell reconstruction

The use of protoplast fusion for transfer of alien cytoplasm has been shown to be an important way to increase the genetic diversity of extranuclear genomes of plants, mainly because of its potential to allow for biparental inheritance of cytoplasmic genophores.

Particularly, the transfer of partial genomes by using subprotoplasts (cytoplasts and karyoplasts) has attracted some attention in the one-to-one electrofusion technique. The subcellular compartments, that would be involved in the fusion event, are defined *a priori*. This is not the case in experiments dealing with organelle transfer by mass fusion of protoplast-cytoplast populations because: 1) all the protocols available so far

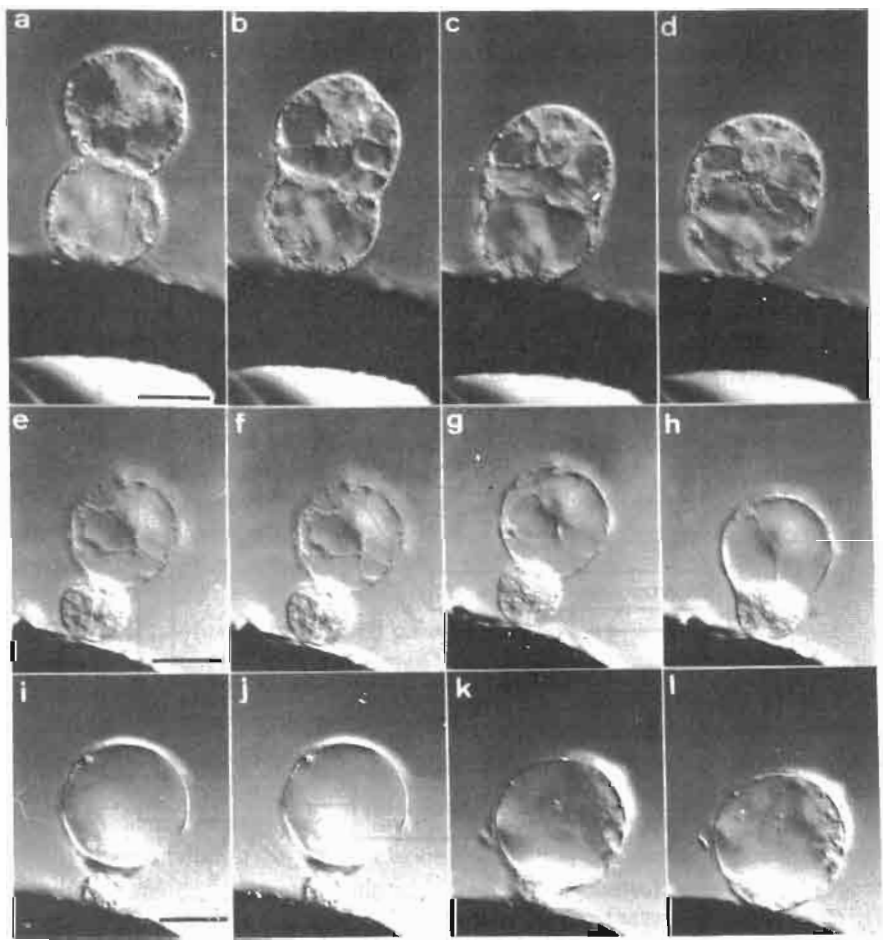


**Figure 2. Electrofusion of *B. napus* protoplasts and subprotoplasts (Spangenberg *et al.* 1986): a-d) fusion of two type B protoplasts; e-h) fusion of a type A and a type B protoplast; i-l) cell reconstitution by karyoplast-cytoplast fusion.**

for subprotoplast isolation do not yield pure subprotoplast preparation and 2) protoplasts that commonly contaminate (for example, cytoplast preparations) are known to be more stable and efficient in the fusion process than the cytoplasts, therefore, they preferentially participate in the fusion events.

Production and culture of fusants arising from a single cytoplast and protoplast, a karyoplast and a protoplast, or a karyoplast and cytoplast (cell reconstitution) by using the one-to-one microfusion approach and setup. In this case, fusion yields ranging from 5 to 30%, mainly depending on the fusion combination, were achieved (Fig. 2e-h). Similar behavior in microculture regarding cell division and microcallusing was observed in subtroplast-protoplast fusions or reconstructed cells compared with nonfused protoplasts.

**Transformation via microinjection of DNA into higher plant cells**

Studies on transformation of defined plant cells were performed by using micromanipulation techniques. This was accomplished mainly by combining the microculture system previously described or other alternatives for culturing a small number of cells in microchambers with a microinjection method. The microinjection technique involves the use of a holding capillary, pneumatically connected to a micrometer-driven syringe. This achieves a smooth fixing of the plant cells to be injected to the tip of the holding capillary. An injection needle, containing DNA solution, is connected to a commercially available microinjector. Either the microinjection needle or the holding capillary is positioned by commercially available micromanipulators and fixed to the movable stage of an inverted microscope that is assembled in a flow cabinet.

*Microinjection into protoplasts and karyoplasts.* The combination of the microinjection technique (Neuhaus *et al.* 1986) with micromanipulation methods for cell reconstruction and microculture of manipulated protoplasts allows an assessment of the transient expression of microinjected foreign genes and stable transformation of rapeseed and tobacco cells (Spangenberg *et al.* 1986b, Neuhaus *et al.* 1987a).

Intranuclear microinjection of chimeric Npt II gene constructions (pSV2neo and pLGV23neo) into hypocotyl protoplasts of *B. napus* (Fig. 3a,b) and karyoplasts (Fig. 3c) as well as cell reconstructions with microinjected karyoplasts (Fig. 3d,e) could be performed at a rate of up to 100 preselected cells per hour. Up to 90% of the microinjected protoplasts or karyoplasts could be transferred into a microculture. A survival rate in the range of 10 to 20% was achieved after cell reconstitution of microinjected karyoplasts that were electrofused to cytoplasts (Fig. 3f-h). Up to 70% of the microinjected cells, independent of the cell type, showed transient expression of the microinjected gene; this was assessed by an indirect immunofluorescence test. After individual culture of microinjected hypocotyl protoplasts of rapeseed, calluses could be established and integration of the foreign DNA was detected by Southern blot analysis of plant genomic DNA (Fig. 4a).

It can be concluded that microinjection extends the range of techniques available for plant cell transformation and shows no host range limitation. This has been demonstrated for protoplast-derived cells, karyoplasts, and suspension cells. In addition, in spite of the requirements for experienced hands and a micromanipulation instrumental setup, the microinjection of protoplasts can be usefully applied in cases where exclusively nonselectable marker genes (but not a large number of transformants) are

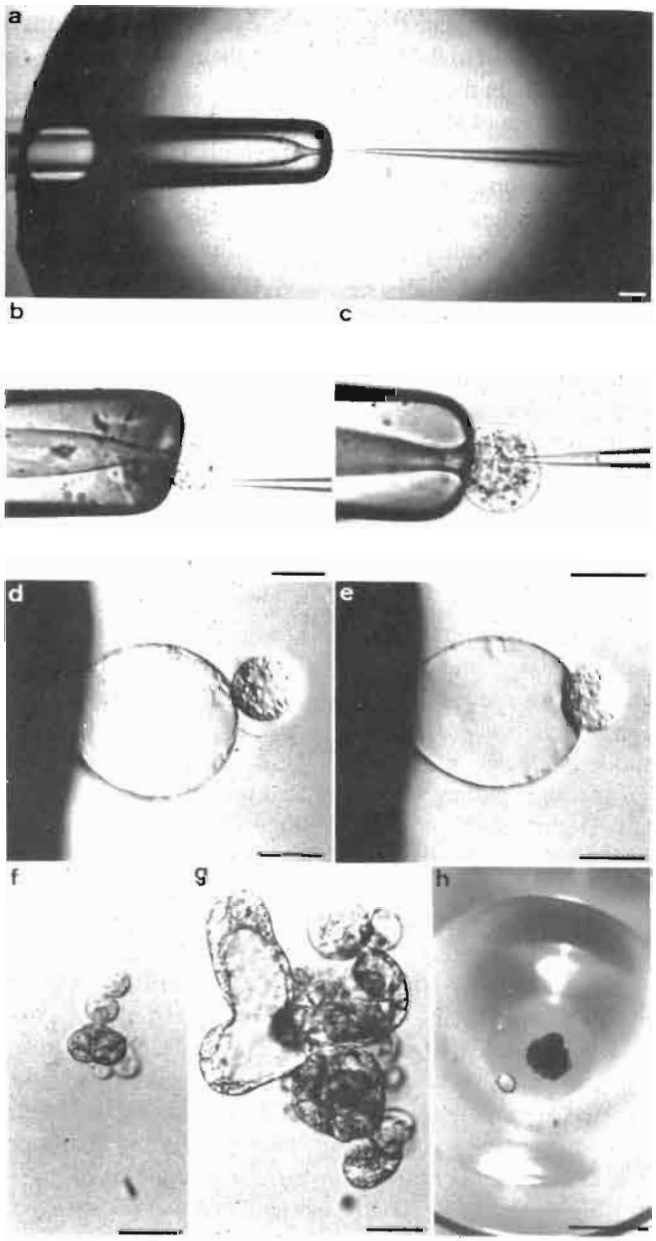


Figure 3. Microinjection, electrofusion-mediated cell reconstruction, and callus formation: a, b) intranuclear microinjection of a hypocotyl protoplast (bars: 200 and 100 $\mu$m); c) microinjection of a karyoplast (bar: 10 $\mu$m); d, e) electrofusion-mediated cell reconstitution from a microinjected karyoplast and a cytoplast (bars: 10 $\mu$m); f-h) development of microinjected reconstructed cells in microculture (bars: 100 $\mu$m, 50 $\mu$m, and 1 $\mu$m, respectively). (Schweiger *et al.* 1987).

required and where present protocols for direct gene transfer to protoplasts fail. An additional promising application of microinjection as a tool for gene transfer is presented in the next section.

*Microinjection into microspore-derived embryoids.* There is now a novel method for gene transfer by microinjection into multicellular structures that have a high competence for plant regeneration through embryogenesis. It was developed to aid in the search for alternatives to *Agrobacterium*-mediated transformation and methods suitable for plant species that cannot readily be regenerated from protoplasts.

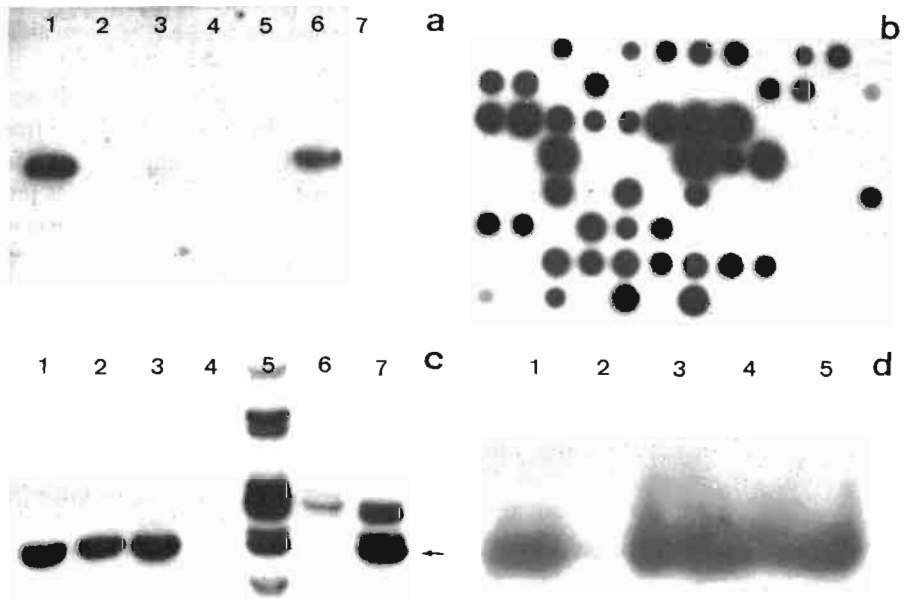


**Figure 4. a) Southern blot analysis of digested (BamHI and Bgl I) genomic DNA isolated from calluses of *B. napus* regenerated after microinjection of SV40 DNA into single protoplasts; lane 1: digested SV40 DNA (0.05 ng) insert containing the T-antigen gene (2.7 kb), lanes 2-5: genomic DNA from not transformed calluses of *B. napus*, lane 6: genomic DNA from a transformed callus clone of *B. napus* after microinjection, lane 7: negative control genomic DNA of *B. napus*; the BamHI/Bgl I insert of SV40 DNA was used as a probe.**
**b) Dot blot analysis of genomic DNA (10 $\mu$g) isolated from primary regenerate after pSB2neo microinjection of microspore-derived embryoids of *B. napus*, probed with the npt II sequence.**
**c) Southern blot analysis of digested (Hind III) genomic DNA isolated from regenerants after microinjection of K5 into microspore-derived embryoids of *B. napus*; lanes 1-3 and 5-7: DNA from transformed regenerant, lane 4: DNA from nontransformed control regenerant. The plasmid insert (1 kb) and the integrated full length insert are marked with an arrow.**
**d) Assay for Npt II activity in plants regenerated after K5 microinjection; lane 1: positive control, bacterial enzyme, lane 2: regenerant from microinjected but no hybridization with Npt II DNA and lanes 3-5: Npt II activity from plants with integrated Npt II sequence.**

Microspore-derived embryoids of *B. napus* were individually selected, microinjected with Npt II gene constructions (pSV2neo and K5), and microcultured for haploid plant regeneration through embryogenesis (Fig. 5) (Neuhaus *et al.* 1987b). Transformation efficiencies in the range of 30 to 50% were determined by DNA dot blot analyses of primary regenerants (Fig. 4b).

Integration of the full length Npt II gene as well as rearranged copies of the microinjected foreign gene was proven by Southern blot analysis of genomic DNA isolated from regenerated plants after induction of secondary embryogenesis from the primary microinjected microspore-derived embryoids (Fig. 4c). The expression of the transferred gene could be assessed by neomycin phosphotransferase II enzyme assay (Fig. 4d).

In addition, the *in vitro* segregation of the putative chimeras obtained after multiple injections into 8 to 20 cell-staged embryoids was attempted by plant regeneration from secondary embryoids of a stem-embryo system. In this way, experimental evidence for stable transformation via microinjection into microspore-derived embryoids is provided. Thus an attractive alternative is opened for gene transfer to those plant species

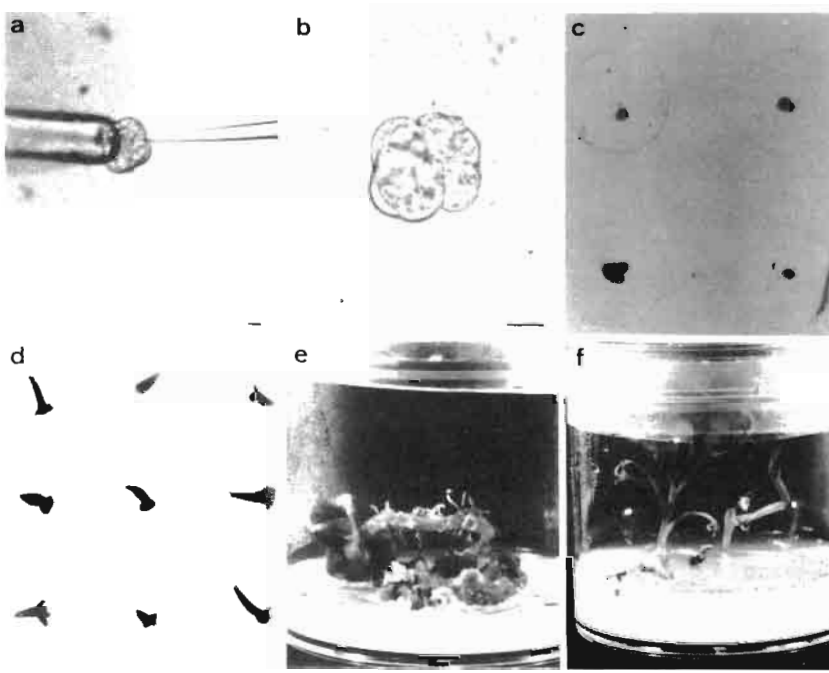


**Figure 5. a) Microinjection into individual cells of a multicellular embryoid derived from 6-day-old microspore culture of *B. napus*, bar: 50 $\mu$m.**
**b) Detailed view of embryoid immediately after microinjection, bar: 50 $\mu$m.**
**c) Microculture of microinjected embryoid in 0.5 $\mu$l medium droplets.**
**d) Individual culture of microinjected embryoids in 5-$\mu$l medium droplets.**
**e) Induction of secondary embryogenesis from the primary regenerants for segregation of putative chimeras.**
**f) Regenerated haploid plants 8 weeks after microinjection of the microspore-derived embryoids. (Neuhaus *et al.* 1987).**

that cannot be easily transformed by currently available transformation systems (*Agrobacterium*-mediated transformation and direct gene transfer to protoplasts). The use of this technique for cereal transformation seems feasible, as analogous anther culture or isolated microspore culture systems are available for rice, wheat, barley, and maize. In this respect, selectable marker genes have been injected into microspore-derived embryoids of wheat and barley (in collaboration with Experimental Station Eschikon, ETH-Lindau and Carlsberg Laboratory, Denmark, respectively). The microinjected embryoids were cultured until plant regeneration and progeny setting. Molecular analysis of the progeny from putative transformed chimeras is in progress.

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## RESUMEN

A causa del elevado grado de hetereogeneidad en la morfología, el número y la estructura de los cromosomas y el potencial biosintético y morfogénico de las células de las plantas superiores en el cultivo en masa, la creación de métodos para el cultivo individual de células vegetales definidas parece ser útil y necesaria para ciertos fines experimentales. El empleo de cultivos de células individuales hace posible la realización de estudios sobre la fisiología de los diferentes tipos de células, el análisis de las interacciones que se producen entre las células y la ejecución de micromanipulaciones genéticas. En este artículo se describe un sistema mejorado de microcultivo, basado en un proyecto controlado por computadora, para la selección, transferencia y cultivo individual de células definidas de plantas superiores en cantidades minúsculas de medio de cultivo. Asímismo, se presentan otras posibilidades de micromanipulación para la modificación genética de células de plantas superiores en condiciones controladas, tales como la electrofusión de pares definidos de protoplastos y subprotoplastos, reconstitución celular y microinyección intranuclear de protoplastos y carioplastos. Por otra parte, en el caso de especies vegetales que no se pueden regenerar fácilmente a partir de los protoplastos, pero para las cuales existen cultivos de anteras o de microsporas aisladas, se presenta también un nuevo método de transferencia de genes que utiliza técnicas de micromanipulación basadas en la microinyección de embrioides multicelulares derivados de microsporas.

# *In vitro* manipulation of cereal crops

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Prerequisites for the application of *in vitro* manipulation of cereal crops are efficient and reproducible methods for plant regeneration, especially from isolated protoplasts to plants. Efficient standards for *in vitro* regeneration and multiplication of barley, maize, rice, and triticale have been established. Plants have been regenerated from protoplasts in rice and maize; whereas in barley, and triticale, albino plantlets and embryoids, respectively, have been obtained from cultured protoplasts. Different methods have been developed in recent years to transform higher plants directly with DNA and to produce genetically engineered plants by means of somatic cell genetics. While these techniques have been successful in dicotyledonous species, success in direct genetic transformation and somatic hybridization of cereals has been limited. Reproducible and efficient regeneration of fertile plants from somatic cells and from isolated protoplasts is a major problem. Thus, gene transfer methods that are independent from *in vitro* cultures are of special interest in cereal biotechnology. Besides direct DNA transfer to protoplasts, injection of DNA into somatic embryos, microspore-derived embryoids, or young floral tillers; incubation of mature embryos with DNA, pollen-mediated gene transfer, and *Agrobacterium*-mediated transformation are being studied intensively. Preliminary positive results have been obtained.

Cereals and grasses are by far the most important plants for human and animal nutrition. Thus, it is not only an academic challenge, but also of great economic relevance to apply the techniques of plant biotechnology to these agriculturally important crop species. Most, if not all, of the new techniques were developed first with *Solanaceae* species; for a long time the *Gramineae* species were difficult to work with in terms of

*in vitro* culture and genetic manipulation (Vasil 1987, Lörz *et al.* 1988). Recent progress and present limitations in cell culture and genetic manipulation of the major cereal crops are discussed.

## PLANT REGENERATION FROM *IN VITRO* CULTURES

*In vitro* plant regeneration from multicellular explants has now been achieved with all the major cereal crops (Bright and Jones 1985, Vasil and Vasil 1987). Although immature embryos and young inflorescences are used most commonly as explants, other tissues, such as the base of young leaves, the mesocotyl and, in the case of rice, root tips, have been used successfully. The procedure of *in vitro* plant regeneration from multicellular explants and regeneration via somatic embryogenesis or shoot-root morphogenesis provides a suitable means for rapid multiplication, *in vitro* selection, and induction of somaclonal variation. For practical applications though, it is important to establish cultures with long-term regenerating capabilities. Such cultures consist of a specific type of friable callus which forms somatic embryos and which, in turn, gives rise to single cell-derived regenerants. Regeneration studies with *Zea mays*, *Oryza sativa*, *Hordeum vulgare*, *Triticum aestivum*, *Secale cereale*, and *X Triticosecale* have consistently shown that the efficiency of culture initiation, the long-term maintenance of totipotent cultures, and the efficiency of plant regeneration are influenced by genotype, physiological stage of the donor plant, and, to a minor extent, by media composition and physical culture conditions (Stolarz and Lörz 1986, Lúhrs and Lörz 1987).

## VARIABILITY IN TISSUE CULTURE-DERIVED PLANTS

Not all tissue culture-derived plants exhibit a normal phenotype and so-called somaclonal variation is found, not only among the regenerants ($R_1$), but also in the progeny ($R_2$, $R_3$...) of such regenerants. The major factors affecting the extent of somaclonal variation in tissue culture-derived plants include the genotype explant, medium composition, and time it takes to culture nondifferentiated tissue. Whether or not the pathway of regeneration (somatic embryogenesis versus shoot-root morphogenesis) influences the extent of variability is not yet clear. Polyploidy, aneuploidy, and chromosomal rearrangements are likely main causes for somaclonal variation and many examples of karyological abnormalities have been found in tissue culture-derived plants (Larkin *et al.* 1984, Lörz *et al.* 1988). In addition to chromosomal changes, a wide range of molecular changes have been shown, including alterations in nuclear, chloroplast and mitochondrial DNA through changes in DNA methylation pattern or single base pair composition, and activation of transposable elements.

Most of the somaclonal variants obtained and described to date have no direct value in crop improvement. Obtaining variation in the gene pool of seed crops, such as cereals, is easily accomplished by crossing different genotypes of the cultigen or by crossing with wild relatives of the cultivated species. Therefore, somaclonal variability will be of most use in vegetatively propagated species. Still, tissue culture-derived variability detected as a response to *in vitro* selection may be of use for seed crops. In principle, *in vitro* selection is possible for all stress factors that can be applied in the petri dish or in the Erlenmeyer flask. *In vitro* selection has been successful in maize for

disease resistance, herbicide resistance, and amino acid overproduction (Green *et al.* 1983).

Genetic manipulation (e.g., somatic hybridization, transformation) often involves the culture of either protoplasts, single cells or multicellular explants, and their regeneration to whole plants, with the possibility of the induction of variability. It is therefore important to understand somaclonal variation. This may help us to reduce the frequency of such variability and to produce more uniform tissue culture-derived plants.

## CEREAL PROTOPLASTS

Culture and regeneration of protoplasts are prerequisites for the use of higher plant cells in somatic cell genetics and for direct DNA-mediated gene transfer. Mesophyll protoplasts are most commonly isolated and cultured in the case of dicotyledonous species. However, culture and regeneration experiments with mesophyll protoplasts isolated from *Gramineae* have so far failed. It has been concluded that most (if not all) cereal mesophyll cells are irreversibly differentiated and have lost their totipotency and capacity to undergo cell divisions in culture. Protoplasts isolated from meristematic tissue are seen as an alternative, but experimentally this material is not easily accessible.

Instead of isolating protoplasts directly from the plant, *in vitro* cultures can also be used as a source for the preparation of cereal protoplasts. Since it is assumed that protoplasts express the same ability in respect to regeneration as the cells from which they have been isolated, embryogenic suspensions appear to be the most promising material. During the process of establishing a cell culture suitable for the isolation of protoplasts, the original embryogenic cultures frequently lose their embryogenic capacity. With the exception of recent reports describing plant regeneration from rice protopasts (Abdullah *et al.* 1986, Kyozuka *et al.* 1987), sterile plants from maize (Rhodes *et al.* 1988), and publications describing "plantlet" regeneration (Lu *et al.* 1981, Lúhrs and Lörz 1988), the culture of protoplasts has led, in the most cases, only to callus formation. However, protoplast culture systems regenerating to the callus stage, established for maize, rice, barley, wheat, and triticale, are reproducible and sufficiently efficient to be used for experiments in somatic cell genetics.

## DIRECT GENE TRANSFER INTO PLANT CELLS

A high efficiency of direct gene transfer into plant cells is achieved by microinjection. DNA is injected directly into the nucleus of protoplasts about 24 hours after isolation (Crossway *et al.* 1986, Schweiger *et al.* 1987). At present, the application of this technique is still limited to a few protoplast systems and there are several technical obstacles to overcome before it can be applied to other protoplast systems. The limitation with respect to cereals is the difficulty to culture cereal protoplasts. In addition to using protoplasts as starting material, cereal plants can be regenerated reproducibly from isolated microspores (Köhler and Wenzel 1985, Datta and Wenzel 1987). Regenerating tissues, such as meristematic cell clusters, somatic embryos, immature zygotic embryos, and unfertilized ovaries or ovules, are considered to represent potent recipient cells for microinjection as shown recently with microspore-

derived embryos of *Brassica* (Neuhaus *et al.* 1987). To succeed with this approach, one has to develop a microinjection procedure capable of penetrating the cell wall, delivering the DNA into the nucleus, and which does not cause irreversible damage to the majority of the treated cells.

Another technique that allows efficient delivery of DNA directly into intact cells and tissues was described recently as transformation by particle bombardment or gene delivery by high velocity microprojectiles (Klein *et al.* 1987). It has been shown that small tungsten particles can be accelerated to velocities that permit their penetration of intact cells. By coating these microprojectiles with DNA, they can be used as a means for the transfer of genetic material into intact cells. Cultured cells of maize have been transformed this way with plasmid DNA carrying the β-glucuronidase as a marker gene (Klein *et al.* 1988). This transformation procedure is extremely attractive because genes could be transferred to regenerable cells of many different cereal species. Preliminary results indicate that DNA can also be delivered to cell organelles, namely to the nucleus, to chloroplasts, and to mitochondria (cited by Klein *et al.* 1988).

The incubation of freshly isolated protoplasts with naked DNA is another direct way of gene transfer, first described for the transformation and regeneration of tobacco protoplasts. DNA-uptake into protoplasts is stimulated either by PEG-treatment or induced by electroporation. Transformation of *Gramineae* protoplasts was first achieved in 1985 with *Triticum monoccoccum* and *Lolium multiflorum* and, since then with maize, rice, barley *Pennisetum* and *Panicum* (for references see Table 1). In all cases, plasmids have been used that contain a selectable chimeric gene coding for antibiotic resistance. The direct gene transfer methods described above provide transformation systems that are independent of the commonly used *Agrobacterium tumefaciens* or functions of the Ti-plasmid. In principle, the methods are applicable to all protoplast systems. However, their applicability to cereal species is still limited by the difficulty of regenerating plants from protoplasts.

De la Peña *et al.* (1987) presented an alternative transformation method for rye. They demonstrated that it is possible to introduce a foreign gene by injection of DNA into young floral tillers, resulting in seeds and seedlings in which the foreign gene is integrated into the genome and is actively expressed.

Another simple method of introducing genes directly into cereal cells involves incubating mechanically isolated wheat embryos in DNA solution (Töpfer 1987). Mature embryos isolated from dry seeds inbibed the DNA solution and transient expression of chimeric genes was demonstrated by assaying NPT- or CAT-activity in embryos of wheat, barely, rye, triticale, oats, and maize. Embryos treated this way can be efficiently and easily regenerated to plants. Whether the foreign genes are stably integrated and transmitted to the progeny is presently under investigation.

## VECTOR-MEDIATED TRANSFORMATION TECHNIQUES

Foreign genes are transferred to dicotyledonous plants efficiently and routinely with the natural vector system of the Ti-plasmid from *Agrobacterium tumefaciens* (Fraley *et al.* 1986). The host range of *Agrobecterium* includes numerous dicotyledonous species, but until now only a few monocotyledonous species have been found to be transformable by *A. tumefaciens* (reviewed by Hooykaas and Schilperoort 1987).

**Table 1. Direct gene transfer and vector-dependent DNA transfer to cereals.**[a]

| | |
|---|---|
| **DNA transfer to protoplasts** | |
| *Hordeum vulgare* | (Lazzeri and Lörz, in prep.) |
| *Lolium multiflorum* | (Potrykus *et al.* 1985) |
| *Oryza sativa* | (Uchimiya *et al.* 1986) |
| *Panicum maximum* | (Hauptmann *et al.* 1988) |
| *Pennisetum americanum* | (Hauptmann *et al.* 1988) |
| *Sorghum purpurem* | (Ou-Lee *et al.* 1986) |
| *Triticum monococcum* | (Lörz *et al.* 1985) |
| *Zea mays* | (Fromm *et al.* 1986, Rhodes *et al.* 1988) |
| **Microinjection of DNA into embryoids** | |
| *Hordeum vulgare* | (Potrykus, pers. comm.) |
| *Triticum aestivum* | (Potrykus, pers. comm.) |
| **High-velocity microprojectiles** | |
| *Zea mays* | (Klein *et al.* 1988) |
| **Injection of DNA into floral tillers** | |
| *Secale cereale* | (De la Peña *et al.* 1987) |
| **Pollen-mediated transformation** | |
| *Triticum aestivum* | (Hess 1988, Picard *et al.* 1988) |
| *Zea mays* | (De Wet *et al.* 1985, Ohta 1986) |
| **DNA uptake into embryos** | |
| *Avena sativa* | (Töpfer 1987) |
| *Hordeum vulgare* | (Töpfer 1987) |
| *Oryza sativa* | (Töpfer 1987) |
| *Secale cereale* | (Töfper 1987) |
| *Triticosecale* | (Töpfer 1987) |
| *Triticum aestivum* | (Töpfer 1987) |
| *Zea mays* | (Töpfer 1987) |
| **Agrobacterium as vector** | |
| *Triticum aestivum* | (Dale *et al.* 1988) |
| *Zea mays* | (Graves and Goldman 1986, Grimsley *et al.* 1987) |
| **Viruses as vector** | |
| *Hordeum vulgare* | (French *et al.* 1986) |
| *Triticum monococcum* | (Matzeit 1987) |
| *Zea mays* | (Matzeit 1987) |

[a] The table summarizes attempts towards transformation of cereal crops. Stable genetic transformation and integration of the foreign gene has not been confirmed in all cases.

*Agrobacterium*-mediated transformation of *Gramineae* has been reported for maize (Graves and Goldman 1986), and these results have been discussed critically (Christou *et al.* 1986). Other interesting results concerning the infection of cereals with *Agrobacterium* were described for maize (Grimsley *et al.* 1987) using an Agrobacterium strain carrying a plasmid with the cloned maize streak virus (MSV) DNA, and for wheat with cloned wheat dwarf virus (WDV) DNA (Dale *et al.* 1988). After "agroinfection," replication of the virus and disease symptoms in treated plants was found.

The suitability of viruses as a vector system for plant transformation has been demonstrated previously with Cauliflower Mosaic Virus (Gronenborn 1987). A system providing a plant gene vector and a self-replicating system for transformation of cereals is seen in the gemini viruses such as Wheat Dwarf Virus or Maize Streak Virus. Cloned copies of the WDV genome have been used to incubate *Triticum* protoplasts and double-strained replicative forms of the viral DNA, as well as to detect a chimeric gene within 3 to 7 days after protoplast treatment. This indicates the potential usefulness of a viral vector system for cereals (Matzeit 1987).

Pollen-mediated transformation has been discussed for many years as an alternative gene transfer procedure not restricted by any host range or tissue culture limitations. The method of incubating pollen with total genomic donor DNA or recombinant DNA plasmids, followed by pollination and seed production, has been applied to *Petunia*, *Nicotiana*, maize (De Wet *et al.* 1985, Ohta 1986), and wheat (Hess 1988, Picard *et al.* 1988). While in the earlier reports, evidence for transformation was based mostly on phenotypic changes and formal genetic analyses, the recent publications have also included preliminary molecular evidence. Even so, further and more detailed molecular proof and evidence for reproducibility of the system need to be provided.

## CONCLUSIONS

Significant progress has been made in the last few years in cell culture and genetic manipulation of cereals. Multicellular explants of different tissues or organs have been used for the initiation of totipotent or morphogenic (regenerating) callus cultures. Plants have been regenerated from such cultures of all major cereal species either via somatic embryogenesis or organogenesis. However, serious limitations still exist in the use of single cells, protoplasts or microspores. Culture and regeneration of protoplasts isolated from nonmorphologenic cell suspension cultures of numerous cereal species have resulted in callus formation, but not in plant regeneration. Until now, fertile plants have been regenerated only from protoplasts of rice, but progress is expected soon for other *Graminaceous* species.

Several routes are utilized in the genetic manipulation of cereals. The present difficulties in generating plants from protoplasts clearly limit the applied aspects of somatic cell genetics, e.g. somatic hybridization and cybridization. However, protoplasts have already been used for direct gene transfer experiments, giving rise to stably transformed cell lines.

Alternative gene transfer methods applicable to cereals are designed primarily to circumvent the difficulties of plant regeneration from cultured somatic cells. These approaches include the microinjection of DNA into cells, injection of genetic material directly into plants, the use of pollen or embryos for DNA uptake, and the use of vector

systems based on *Agrobacterium* or viruses. In the long term, and for applied aspects of crop improvement, only highly efficient and reproducible methods are of interest for the transfer of defined traits (in the form of isolated genes) from one plant to another. Such transfer could be within species, or between species to overcome natural incompatibility barriers.

## ACKNOWLEDGMENTS

The work described on *in vitro* culture and transformation of cereals in our laboratory has been supported by grants from the Bundesminister für Forschung und Technologie, Bonn (BMFT, BCT 0390-2); the European Community Research Program, Brussels (BAP-0013-D); and the Rockefeller Foundation Rice Biotechnology Program, New York (RF 8659#52).

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## RESUMEN

La existencia de métodos eficaces y reproducibles de regeneración de plantas, en especial de protoplastos aislados a plantas, es un requisito indispensable para la aplicación de la manipulación *in vitro* de los cereales. Se han establecido normas eficaces para la regeneración *in vitro* y la multiplicación de cebada, maiz, arroz y triticale. En el caso del arroz y del maíz, se estudiaron plantas derivadas de protoplastos, en tanto que en cebada y triticale se obtuvieron plántulas albinas y embroides, respectivamente, de los protoplastos cultivados. En años recientes se han creado diferentes métodos para transformar plantas superiores directamente mediante el ADN y producir plantas genéticamente manipuladas por medio de la genética de célula somática. Si bien esas técnicas han tenido éxito en las especies dicotiledóneas, ha sido limitado el logrado en la transformación genética directa y la hibridización somática de los cereales. La regeneración reproducible y eficaz de plantas fecundas a partir de células somáticas y de protoplastos aislados plantea un grave problema. Por tanto, los métodos de transferencia del gen que son independientes de los cultivos *in vitro* son de especial interés para la biotecnología de los cereales. Además de la transferencia directa del ADN a los protoplastos, en la actualidad se estudian en forma intensiva la inyección del ADN en embriones somáticos, embriodes derivados de microsporas o en macollos florales tiernos, así como la incubación de embriones maduros usando ADN, la transferencia de genes por medio del polen y la transformación mediante *Agrobacterium*. Se han obtenido resultados preliminares positivos.

# A protoplast approach to obtain transgenic rice plants, and nodulation of rice plants by rhizobia

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The production of transgenic cereals is at present only possible by direct interaction of DNA with isolated protoplasts. And until recently, only transgenic maize plants have been produced. Success in this approach depends on efficient delivery of plasmids to the recipient protoplast system, the use of a suitable chimeric plasmid construct, and efficient plant regeneration from selected transformed colonies. Transgenic rice plants have now been produced using a combination of efficient plasmid delivery by electroporation coupled with efficient plant regeneration from protoplasts by somatic embryogenesis. The present lack of interaction of rhizobia and agrobacteria with cereals is limiting the use of these bacteria in various aspects of genetic manipulation. Following our finding that a barrier to *Rhizobium* specificity could be removed by enzymatic degradation of the cell wall at the apices of legume root hairs, we have investigated whether such enzymatic treatment of cereal root hairs would enable novel interactions with rhizobia. Very interestingly, nodular structures have been produced on rice roots following enzyme treatment and incubation with rhizobia in the presence of polyethylene glycol. It is suggested that these nodular structures may, with further study, provide an inroad into the effective nodulation of cereals by rhizobia; and perhaps also to interaction with agrobacteria.

Although some dicotyledonous crop plants respond well to the natural gene transfer system of the soil bacterium, *Agrobacterium rhizogenes*, the cereals, which constitute the most important group of plant for human nutritional needs, have so far remained unresponsive. This failure to achieve *Agrobacterium*-induced transformation in cereals has led to increased interest in assessing other transformation methods, especially the direct uptale of DNA (Cocking and Davey 1987). In this review the use of a protoplast approach to obtain transgenic rice plants is surveyed. There is also general

interest in the interaction of bacteria, including agrobacteria and rhizobia, with cereals, and in particular whether any tumor or nodular response can be elicited.

## PRODUCTION OF KANAMYCIN-RESISTANT RICE TISSUES FOLLOWING DNA UPTAKE INTO RICE PROTOPLASTS

The first report of the use of kanamycin to select transformed tissues of rice (*Oryza sativa*) utilized polyethylene glycol (PEG)-induced DNA delivery to rice protoplasts (Uchimiya *et al.* 1986). When reproducible plant regeneration from rice protoplasts became possible utilizing the japonica variety Taipei 309 (Abdullah *et al.* 1986), work was done using this system to obtain optimal transformation of protoplasts of Taipei 309, and, coupled with this, reproducible regeneration of transformed plants. Comparisons were made of two plasmid delivery procedures—electroporation and PEG, plus a combination of these two (Yang *et al.* 1988).

Protoplasts were isolated from established cell suspensions of *Oryza sativa* Taipei 309 line LBI (Abdullah *et al.* 1986). The plasmid pCaMVNEO, carrying a chimeric gene consisting of the CaMV35S promoter, the NPTII gene from Tn5, and the *nos* polyadenylation region, was isolated from *Escherichia coli*. For PEG-mediated DNA uptake, 1 ml containing 1.4 x $10^6$ rice protoplasts was mixed with 20 μg of sheared calf thymus DNA and 5 μg of unrestricted pCaMVNEO followed by dropwise addition of 40% PEG 6000. For electroporation, 1ml containing 4.25 x $10^6$ rice protoplasts was mixed with 50 μg of sheared calf thymus DNA and 10 μg pCaMVNEO and given three pulses, at 10-sec. intervals, of 500 to 2500 V with 20 to 50-nF capacitance. The rice protoplasts were transformed to kanamycin resistance following uptake of pCaMVNEO induced by electroporation, PEG, and PEG combined with electroporation. Protoplast-derived colonies were selected on a medium containing 100 μg/ml of kanamycin-expressed NPTII activity and contained DNA that hybridized to a 1.0 Kb BamHI fragment of pCaMVNEO carrying the NPTII gene. Expression of the transformation frequency in relative terms (number of kanamycin-resistant colonies compared to the number of colonies on kanamycin-free medium) gave frequencies of 26%, 8.5%, and 2.9% following electroporation, PEG, and PEG with electroporation, respectively. Having established that electroporation was the superior procedure, work was conducted to couple the production of transformed callus with the regeneration of transformed rice plants.

## TRANSGENIC RICE PLANTS PRODUCED BY ELECTROPORATION-MEDIATED PLASMID UPTAKE INTO PROTOPLASTS (ZHANG *ET AL.* 1988)

Protoplasts isolated from suspension cultures of Taipei 309 rice were electroporated with pCaMVNEO as previously described. Protoplasts were also electroporated in the absence of plasmids. Protoplasts were then cultured in a semi-solid medium with 1.2% agarose in the presence of 100 μg/ml of kanamycin. Careful attention was paid to the timing of the addition of kanamycin to the electroporated protoplasts and when kanamycin-resistant calluses had been selected, kanamycin was omitted from the rice regeneration medium so that plant regeneration would not be inhibited. Green plants were regenerated and analyzed for NPTII activity and the presence of the NPTII gene.

DNA isolated from these plants hybridized to the 1.0 Kb BamHI fragment of pCaMVNEO containing the NPTII gene. This confirmed the presence of foreign DNA in the regenerated plants. NPTII enzyme activity was also detected.

Thus, transgenic rice plants can be readily obtained by combining the most efficient procedures for the production of transformed kanamycin-resistant rice tissues, that is, by electroporation of rice protoplasts with sequenced selection using kanamycin, coupled with the established plant regeneration protocol from Taipei 309 protoplasts.

## NODULAR RESPONSES ON RICE SEEDLINGS ELICITED BY RHIZOBIA

Cell and molecular biologists have been interested for a long time as to whether any tumor or nodular response can be elicited in cereals by the interaction of agrobacteria or rhizobia. The observation that an added mixture of cellulase and pectolyase is able to degrade the cell wall at the apices of root hairs from a wide range of crop species, including the cereals, has provided the opportunity for such an assessment (Cocking 1985).

The host range specificity of rhizobia has long been an intriguing problem in the nodulation of legumes, and it has been suggested that an understanding of the mechanisms of this specificity might indicate ways in which the interaction of *Rhizobium* and plant species might be manipulated to increase the range of nodulated plants. When the root hairs of white clover (*Trifolium repens*) seedlings were treated with a mixture of cellulase and pectolyase, followed by inoculation with *R. loti* in the presence of polyethylene glycol, nodules were produced on the white clover seedlings (Al-Mallah *et al.* 1987). This treatment had removed a barrier to *Rhizobium*-plant host specificity since white clover is not naturally nodulated by *R. loti*. This result further stimulated interest in the interaction of rhizobia with cereals. For instance, would a similar enzymatic treatment of cereal seedlings remove a barrier to *Rhizobium* specificity that was preventing the interaction of rhizobia?

The root systems of rice seedlings were treated with the cellulase pectolyase enzyme mixture and then inoculated with *R. loti* and *R. trifolii* in the presence of polyethylene glycol. Within 1 month, nodular structures appeared on the treated roots. Recently these roots were examined in detail and it is clear that a nodular response was initiated (Al-Mallah *et al.* 1988).

## CONCLUSIONS

In this review, two approaches to the manipulation of plants were described. At first glance, the use of rice protoplasts interacting with plasmids, resulting in the production of transgenic rice plants and the interaction of rice plants in which the protoplast surface at the tips of root hairs has been exposed enzymatically to rhizobia to form nodular structures, may seem unconnected. In this procedure, one sees that the production of transgenic plants is required for the regeneration of plants from protoplasts. Nodular structures produced on rice seedlings by *in situ* interaction at the protoplast surface may provide an inroad into the effective nodulation of cereals by rhizobia as well as enable a new approach to agrobacteria interactions. For instance, would agrobacteria interacting with enzyme-treated cereal root systems produce tumor-like nodules from which

transgenic cereals might be obtained without having to interact plasmids with protoplasts?

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## RESUMEN

Hoy día sólo es posible la producción de cereales transgénicos mediante la interacción directa del ADN con protoplastos aislados; por otra parte, hasta hace poco tiempo sólo se habían podido producir plantas transgénicas de maíz. El éxito de este método depende de la entrega eficaz de plasmidios al sistema receptor de protoplastos, del uso de un constructo quimérico adecuado de plasmidios y de la regeneración eficaz de plantas a partir de colonias transformadas seleccionadas. Ya se han producido plantas transgénicas de arroz utilizando una combinación de entrega eficaz de plasmidios mediante la electroporación, aunada a una regeneración eficaz de plantas a partir de protoplastos por medio de la embriogénesis somática. La actual falta de interacción de rizobios y agrobacterias con los cereales limita el uso de estas bacterias en varios aspectos de la manipulación genética. De acuerdo con nuestro descubrimiento de que la barrera a la especificidad de *Rhizobium* podría eliminarse mediante la degradación enzimática de la pared celular en los ápices de los pelos radicales de las leguminosas, hemos investigado si semejante tratamiento enzimático de los pelos radicales haría posible novedosas interacciones con los rizobios. Es interesante notar que se han producido estructuras nodulares en las raíces del arroz después del tratamiento enzimático e incubación con rizobios en presencia de glicol polietileno. Se sugiere que estas estructuras podrían, con un estudio más profundo, permitir una nodulación eficaz de los cereales con rizobios, y quizá también la interacción con agrobacterias.

# Transformation of rice by direct gene transfer

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Stems of rice plants containing immature inflorescenses were injected with recombinant DNA plasmid solutions. A total of 3683 selfed seeds was harvested from DNA-injected stems at maturity; 239 seeds from five control plants were injected with buffer only. Initial screening for transformation was conducted through germination of seed on medium containing 150 µg/ml kanamycin. Among 3922 tested seeds, 37 seedlings remained green despite the presence of kanamycin. Thirty of the 37 plants showed delayed reaction to the kanamycin exposure, slowly turned albino, and eventually died. Seven plants survived to maturity and were evaluated with Southern blot analysis for transformation. Hybridization of DNA isolated from leaf tissue of the resistant plants with whole plasmid probe DNA indicated the presence of a ca. 2.4 kb HindIII fragment in one plant.

Several gene transfer systems have been developed for plants, including: 1) electroporation (Fromm *et al.* 1985, 1986; Shillito *et al.* 1985, Langridge *et al.* 1985, Morikawa *et al.* 1986, Riggs and Bates 1986, Ou-Lee *et al.* 1986, and Rhodes *et al.* 1988); 2) chemically mediated DNA uptake using PEG or high calcium concentrations (Krens *et al.* 1982, 1985; Paszkowski *et al.* 1984, Potrykus *et al.* 1985 a,b,c; Shillito *et al.* 1985, and Uchimiya *et al.* 1986); 3) use of *Agrobacterium* and *Escherichia coli* spheroplasts (Baba *et al.* 1986, Hain *et al.* 1984, 1985); and, 4) by direct injection (Zhou *et al.* 1983, de la Peña *et al.* 1987).

Cereal crops such as rice and wheat are resistant to infection by *Agrobacterium tumefaciens* and have, therefore, not proved to be good subjects for Ti-mediated DNA transfer using *Agrobacterium* as the biological vector. An alternative to the use of biological vectors is direct DNA transfer. By using spheroplasts, Baba *et al.* (1986) introduced plasmids into rice protoplasts and obtained rapidly growing colonies. Uchimiya *et al.* (1986) obtained stable transformed calluses from protoplasts using polyethylene glycol and a gene consisting of the nopaline synthase promoter, the aminoglycoside phosphotransferase II structural gene from TN 5, and the terminator

region from cauliflower mosaic virus. Ou-Lee *et al.* (1986) demonstrated gene expression in rice protoplasts following the introduction of a chloramphenicol acetyl transferase gene through electroporation. Though genetic transformation has been achieved at the level of protoplast-derived calluses, the production of transformed rice plants has not been reported. This report describes the successful transformation of rice by DNA injection into floral tillers.

## MATERIALS AND METHODS

### Rice cultivar

The long grain rice cultivar 'Lemont' was used as the DNA recipient. 'Lemont' is a leading U.S. rice cultivar that has high yields, high quality, and excellent resistance to lodging. Foundation seed of 'Lemont' was supplied by the Rice Research Station, Louisiana State University. Field-grown plants were transferred to greenhouse pots the day before DNA injection.

### Plasmid DNA

Four DNA plasmids were used for injection, all of which contained a neomycin phosphotransferase II (NPT II) gene under the control of the nopaline synthase promoter. The plasmids were described as follows: 1) C-9, a construct of vector pMON 200 with a ca. 6.5 kb insert containing the genomic gene of cecropin B from the giant silk moth (*Hyalophora cecropia*)—the insert was obtained as an EcoRI-XhoI fragment from plasmid pW5/12 provided by Dr. K. Xanthopoulos; 2) 37-3, a constructor of vector pMON 530 with a 120 bp BglII-EcoRI fragment containing the coding sequence of SB-37 (Jaynes *et al.* 1988); 3) 3, a pMON 237 vector with a ca. 2.7 kb insert which codes for a protein containing elevated levels of essential amino acids (Wier *et al.* 1988); and 4) 237, the pMON 237 vector with no insert. Plasmids pMON 200, pMON 237, and pMON 530 were provided by Dr. Stephen Rogers, Monsanto Corporation.

### DNA injection

Stems of rice plants containing immature inflorescenses were injected with solutions containing DNA plasmids (Table 1). Stems were injected with 0.1 to 0.4 ml of a sterile buffer solution containing 0.1 μg of plasmid DNA per μl using a tuberculin syringe (25G 3/8). Plasmid solutions were injected into or at various distances above the uppermost stem node. Most injections were made 2 cm above the node at maturity stages corresponding to immature panicle lengths of 1 to 5 cm. Sufficient solution was injected to fill the stem, as indicated by the formation of droplets at the stem tip.

### Test for kanamycin resistance

Initial screening for transformation was conducted through germination of seed on medium containing kanamycin. Seeds were heat treated at 50 °C for 5 days to eliminate dormancy, then dehulled, surface sterilized, and aseptically plated on half strength MS (Murashige and Skoog 1962) medium containing 15 g/L sucrose and 150 μg/ml kanamycin. This concentration of kanamycin induces 'Lemont' rice seedlings to turn and remain albino without otherwise inhibiting germination.

### Southern blotting

Rice genomic DNA was isolated from leaf tissue according to Shure *et. al.* (1983) and HindIII digests were fractionated on 1% agarose gels. Southern blot analysis was conducted using whole plasmids as probes.

## RESULTS

Of the four plasmids injected into rice, treatment with the C-9 and 237 plasmids resulted in production of kanamycin-resistant plants. Among a total of 3922 seeds germinated on medium containing 150 µg/ml kanamycin, 3683 seedlings were kanamycin-sensitive and turned fully albino within 10 days of culture. Two hundred and two seedlings showed various degrees of chlorosis and 37 seedlings remained green despite exposure to kanamycin. However, all but seven of these plants slowly turned albino and eventually died in the greenhouse. The seven surviving plants were analyzed with Southern blots.

The presence of introduced DNA was confirmed using the C-9 plasmid as the probe. No hybridization was found in DNA isolated from control 'Lemont' plants while DNA isolated from one of the seven kanamycin-resistant plants yielded a ca. 2.4 kb hybridizing band, corresponding to a partial HindIII fragment of C-9. The successful transformation occurred with an injection of 20 µg of DNA at a point 2 cm above the uppermost node into a stem in which the tip of the flag leaf had emerged 1.5 cm past the penultimate leaf collar.

## DISCUSSION

Transformation of rice by direct injection into stems occurred when the recipient tiller contained a 2-3 cm panicle at the maturity stage approximately 2 weeks before meiosis. This is the same stage at which rye was found sensitive to injections of caffeine and colchicine into floral tillers (de la Peña *et al.* 1981, Puertas *et al.* 1984), and is the stage at which transformation was successfully achieved for rye through injections of DNA (de la Peña *et al.* 1987).

**Table 1. DNA injection into 'Lemont' floral tillers.**

| Plasmid DNA injected | Amount of DNA injected (µg) | No. of stems injected | Distance of injection above uppermost node (cm) | Flag leaf emergence (cm) |
|---|---|---|---|---|
| 237[a] | 10-40 | 17 | 0-7 | 0-12 |
| 3[a] | 10-40 | 19 | 0-4 | 1-12 |
| 37-3[a] | 10-40 | 48 | 0-15 | 1-12 |
| C-9[a] | 10-50 | 19 | 2 | 0-12 |
| Buffer | 0 | 8 | 0-2 | 1-10 |

[a]237—pMON 237 with no insert, 3—pMON 237 vector with insert for high essential amino acid content, 37-3—pMON 530 vector with SB-37 insert, C-9—pMON 200 vector with genomic cecropin B insert.

A method for increasing disease resistance in plants has recently been proposed through gene transfer of insect antibacterial genes which code for lytic peptides (Jaynes *et al.* 1987). Numerous published reports describe peptides or proteins capable of lysing organisms or cells (Bhakadi and Tranum-Jensen 1984, Donovan *et al.* 1981, Kehoe and Timmis 1984). Boman and colleagues were the first to delineate the humoral defense system utilized by *H. cecropia* as a protective mechanism against bacterial infection (Hultmark *et al.* 1980, Boman and Steiner 1981, Hultmark *et al.* 1983, v. Hofsten *et al.* 1985, Boman *et al.* 1985, Andreu *et al.* 1985). Unique proteins, found in the insect's hemolymph after induction by either live or heat-killed bacteria, are capable of membrane perturbation resulting in bacterial cell lysis. Among this family of inductive proteins are a type known as the cecropins. Three principal cecropins have been described (A, B, and D) which are highly homologous small basic proteins each containing a comparatively long hydrophobic region (Hultmark *et al.* 1985). Their primary mode of action appears to be one of membrane disruption and subsequent lysis due to the target cell's loss of osmotic integrity (Jaynes *et al.* 1987). Similar types of lytic proteins have been found which may play key roles in providing protection from disease in other organisms. For example, peptides isolated from amphibians (Gibson *et al.* 1986, Giovannini *et al.* 1987, Zasloff 1987) appear to possess antibacterial activity.

Cecropins are potent antimicrobial peptides and are active against a broad spectrum of plant pathogenic bacteria and fungi. Incorporation and expression of these genes in rice plants might increase their level of resistance to diseases caused by bacteria and fungi. The C-9 plasmid, which was successful in transforming rice, contained an insert of the cecropin B gene. The ca. 2.4 kb portion of the original plasmid incorporated is sufficient to include the kanamycin resistance gene, but insufficient to also include more than a small part of the ca. 6.5 cecropin B insert. Evaluations are underway to more specifically characterize the incorporated plasmid DNA.

The results of this study indicate that direct gene transfer by DNA injection into floral tillers is a method by which foreign genes can be introduced into rice. This relatively simple technique may prove useful for transforming a broad range of plant species.

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**RESUMEN**

Se inyectaron vástagos de plantas de arroz que contenían inflorescencias inmaduras con soluciones de ADN recombinante proveniente de los plasmidios. En el momento de la madurez, se cosechó un total de 3,683 semillas autofecundadas de los vástagos inyectados con ADN; a 239 semillas de cinco plantas testigo se les inyectó tan solo una solución tampón. La selección inicial para determinar la transformación se efectuó mediante la germinación de la semilla en un medio que contenía 150 µg/ml de canamicina. De las 3,922 semillas sometidas a prueba, 37 plántulas conservaron el color verde a pesar de la presencia de la canamicina; 30 de las 37 plantas presentaron una reacción tardía a la exposición a la canamicina, ya que se volvieron albinas poco a poco y terminaron por morir. Siete plantas sobrevivieron hasta la madurez y se las evaluó con la prueba de manchas Southern para determinar la transformación. La hibridización del ADN aislado del tejido foliar de las plantas resistentes con una sonda ADN de plasmidios completos, indicó la presencia, en una de las plantas, de un fragmento de ca. 2.4 kb HindIII.

# Wide crossing and gene transfer methods between species

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The aim of wide crossing is to transfer useful genes from the wild relatives of crops into cultivars. The wild relatives of modern cultivars possess many desirable traits such as resistance to diseases and insects, tolerance to abiotic stresses, high seed protein content, and sources of male sterility. This paper describes achievements in using genes from wild relatives of rice, barley, wheat, and cotton. It shows the success in hybrid rice production in China and how basic sources of male sterility are derived from wild rice. The techniques available for sexual and parasexual gene transfer are also analyzed.

The collection and study of native crop cultivars enable breeders to use the wide genetic diversity these cultivars contain for crop improvement and provide considerable benefits for plant breeding and agriculture. In some cases appropriate genes are not available within the crop species themselves and hence the wild species represent an alternative source for traits such as disease and insect resistance, abiotic stress tolerance, and male sterility. In recent years, the wild relatives of rice, wheat, barley, and cotton have been used successfully in the improvement of these crops.

## RICE IMPROVEMENT USING WILD SPECIES

The development of hybrid rice is a great technological innovation in rice breeding. It has the potential to markedly increase rice yields on a large scale. Development of suitable cytoplasmic male sterile, maintainer, and restorer lines will be a critical aspect of making hybrid rice a commercial success. In the course of collecting wild rice germplasm for distant crosses, a male sterile wild rice (*Oryza sativa* f. *spontanea* L.) was found on Hainan Island in 1970. It was named "wild abortive" (WA). This type of male sterile wild rice was used as the female parent in crosses with cultivated rice (male parent). The $F_1$ was backcrossed with cultivated rice and, from this combination, more

than 200 male sterile lines derived from WA cytoplasm were created. Some of them, such as Zhe-Shan 97A and V20A, are widely used in hybrid rice production and have been backcrossed 30 to 35 times and still show stable male sterility under various environmental conditions.

Many excellent hybrids with early maturity, multiple resistance to diseases and insects, and good grain quality have been developed. The cumulative production increase due to cultivation of hybrid rice in China, from 1976 to 1985, has been estimated at 94 million tons (Yuan 1986, 1987).

## BARLEY IMPROVEMENT USING WILD RELATIVES

Interspecific and intervarietal crosses of wild and cultivated barley collected from the Qingzang Plateau have been made in the Institute's experimental station since 1979. Hybrid vigor was observed in the $F_1$ hybrids of some combinations. Male sterile plants were found among the $F_2$s and $F_3$s of several crosses. Complete and partially (90% or above) male sterile forms were obtained in 1982. Male sterile plants were derived from a cross between two-rowed wild barley (*H. spontaneum*) and the six-rowed cultivated form (*H. vulgare* var. *nudum*). The male sterility was controlled by a recessive gene. Two male-sterile types were identified. One was two-rowed with naked grains and a tough rachis; the other had covered grains and a tough rachis. Both types had small degenerated anthers with dried and shrivelled pollen grains. Pollen abortion occurred between the tetrad and microspore stages, indicating that both types exhibited sporophytic sterility.

Field observation in 1983 showed that five backcross combinations maintained sterility and 16 test cross combinations restored fertility. To obtain stable maintainer and restorer lines, test crosses and backcrosses are in progress (Shao *et al.* 1986).

In addition to these, attempts have been made to solve two practical problems in hybrid barley production: 1) to look for mutants with a more open glume structure at pollination; and 2) to incorporate the tough rachis character into some wild species having good combining ability and glume opened pollination. The first effort has been successful; the second is still underway.

## SOURCES OF MALE STERILITY IN THE WILD RELATIVES OF WHEAT

Semi-wild wheat and *Aegilops squarrosa* could serve as a source of male-sterility for wheat. Semi-wild wheat was discovered during a scientific expedition to the Qingzang Plateau in 1974 and large numbers of seed and plant samples were collected and cytologically and genetically analyzed. Semi-wild wheat grows as a weed in barley and wheat fields. The mature spikelets freely shatter and fall to the ground, thus being naturally sown. Identification of genetically stable lines of semi-wild wheat showed that the majority had the stable, brittle rachis trait. The semi-wild wheat showed considerable polymorphism and it could be grouped into types. Because of its distinctness from other species of wild wheat, we classified it into a new subspecies, *Triticum aestivum* ssp. *tibetanum* (Shao *et al.* 1983). In the spring of 1979, a cross was made between 'Zon-kan-ai', a cultivar of bread wheat, with many recessive straits, and a semi-wild wheat. Reciprocal crosses were also made and the $F_1$ hybrids were studied.

The mature $F_1$ hybrid plants also had a brittle rachis with red and hairy glumes and red grains. These traits come from the *tibetanum* parent, indicating that the broken rachis of the semi-wild wheat is dominant and that the stable rachis of common wheat is recessive. The reciprocal cross gave the same results. At the same time, the data show the existence of something like a primitive interspecific barrier. It is one reason why semi-wild wheat from Tibet was separated as a new subspecies, *tibetanum*.

Crosses between bread wheat and semi-wild wheat are usually easy to do. The $F_1$ hybrid for such a combination is usually fertile, but occasionally it is possible to obtain sterile lines from such hybrid combinations. Semi-wild wheat can therefore serve as a source of male sterility for bread wheat.

*Aegilops squarrosa* cytoplasm can be used as a source of male sterility in common wheat, as shown by Zhang (1984). Zhang has succeeded in producing hybrids between *Ae. squarrosa* line No. 11, as the female parent, and the bread wheat cultivar 'Kenya' as the male parent. Using this cross combination, an alloplasmic substitution line was obtained. Examination of the root tips revealed that the BC2 plants had a normal chromosome number (2n=42). Their morphological appearance was similar to that of the nuclear donor, suggesting that the nuclear genotype of the nucleo-cytoplasmic hybrid was homologous to that of the pure line 'Kenya'. In most cases, the substitution line had complete pollen and seed fertility. However, male sterile plants were obtained among the BC4 self-pollinated progenies.

Ten of the total 1200 plants grown were either completely or highly male sterile. Anthers of all completely sterile plants were smaller and more slender than those of the fertile plants. This material has been used for breeding male sterile lines of wheat.

## COTTON IMPROVEMENT THROUGH INTERSPECIFIC HYBRIDIZATION

Liang *et al.* 1985 successfully obtained interspecific hybrids between cultivated cotton and several species of wild cotton. Many useful characters have been introduced into cultivated cotton from the wild relatives.

Interesting results were obtained from crosses with the wild cotton, *Gossypium bickii*, an Australian wild species belonging to the G1 genome. It has many desirable characteristics, such as insect resistance (due to its excessive hairiness), a high-gossypol stalk, and gossypol-free seed. However, due to difficulties in interspecific hybridization, gene transfer into cultivated cotton did not succeed for a long time. Only in recent years were hybrids successfully obtained by using new techniques such as embryo rescue, $F_1$ grafting, and backcrossing (Liang *et al.* 1985).

Hybrids of *G. hirsutum*, *G. bickii*, and *G. herbaceum*/*G. bickii* were obtained by phytohormone spraying and embryo culture. When the former $F_1$ is grafted onto upland cotton and then backcrossed with upland cotton under cool-night and short-day conditions, backcross I (BCI) seeds can be harvested, and BCI, and later BCII, plants are grown. Incompatibility of interspecific hybridization may be overcome to a certain degree by this technique.

There is still a great potential for using wild relatives for crop improvement. Many desirable traits from wild species could be transferred into crop plants and techniques for transferring these genes have also been developed.

## SEXUAL GENE TRANSFER

The above-mentioned examples of gene transfer into rice, wheat, barley, and cotton were achieved using sexual methods. Triticale (*X Triticosecale*) is an excellent example of a crop that combines whole genomes together. However, in this case, some negative traits of rye were also transferred into triticale.

In addition to the combination of the whole genomes, there are several other methods of sexual gene transfer. These may take the form of the transfer of single genes, or single chromosomes, either as substitution lines or as addition lines. In a number of cases, such lines have given desirable results.

## PARASEXUAL GENE TRANSFER

Parasexual gene transfer techniques can be divided into two basic groups. One uses a vector, and the other does not. The nonvector techniques are DNA injection, pollen tube-mediated transformation, chromosome-mediated transformation, and protoplast fusion.

Vector-mediated techniques utilize vectors such as the Ti-plasmid, virus, ct-DNA, and mt-DNA transposable elements. Among these vectors, the Ti-plasmid is the most useful. The t-DNA of the Ti-plasmid is easy to insert into nuclei of the tranformants and is stably inherited in the offspring. The Ti-plasmid has been widely studied by many molecular biologists to transfer many genes into dicots, such as tomato, tobacco, brassicas, and some legumes. However, this approach has not yet succeeded with cereals.

Quite recently, our laboratory obtained data that show *Agrobacterium tumefaciens* can transform *Triticum aestivum* and *Hordeum vulgare* (Deng *et al.* 1988). This paper first reports that *A. tumefaciens* strains T37, A208, and B6 can transform some cultivars of *T. aestivum* and *H. vulgare* to form swellings and tumors. There is some indication that the phenolic compound acetosyringone may promote this transformation process. Besides this, inoculating agrobacteria on the appropriate plant tissues has also proven to be the key step in achieving successful transformation.

## TUMOR-INDUCING EFFECTS OF AGROBACTERIUM

### On barley

Five barley cultivars tested had thick straw and were of late maturity when grown in Beijing. Our experiments showed that the base of the leaf sheath of all five barley cultivars was susceptible to *A. tumefaciens*, at differing levels. Among them, the two-rowed, short-awned 'Chingko' cultivar was the most susceptible. All three *A. tumefaciens* strains (A208, T37, and B6) could infect the base of the leaf sheath of the barley materials to form visible swellings, and even crown galls (Fig. 1). The diameter of the biggest tumor was 3.5 mm. The tumor-inducing effect of *A. tumefaciens* on the two-rowed, short-awned 'Chingko' barley is shown in Table 1.

### On wheat

Similar to the experiments on barley, injection was carried out on flowering wheat ('Maoying No. 139') plants. The tissues of all these plants were relatively tender, and moreover the spike stems of some were not empty in the center at the beginning. In

addition to making injections from the base to the leaf sheath, some spike stems were also injected with agrobacteria. Results showed that the internode and leaf sheath were not susceptible to agrobacteria. similar to the situation in barley. Young, full spike stems were found to be susceptible and some of them formed large tumors (2-3 mm in diameter) 3 weeks after injection (Fig. 2), while older, empty spike stems did not respond to *A. tumefaciens*. The tumor-inducing effect of *A. tumefaciens* on wheat is shown in Table 2.

## CONCLUSION

This paper shows that more and more techniques are becoming available for transferring desirable genes from the wild relatives of crops through both sexual and parasexual means.

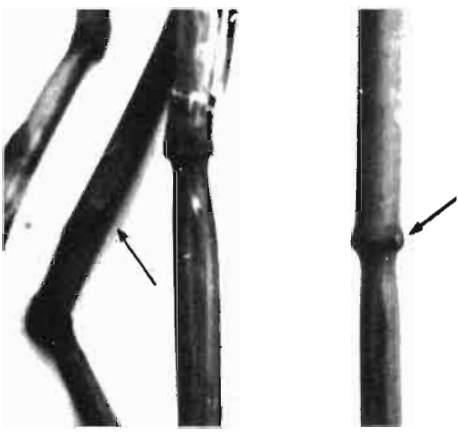

**Figure 1. Tumor-formation (arrows) on barley in base of leaf sheath induced by *A. tumefaciens*, strain A20B.**

**Figure 2. Tumor-formation (arrows) on wheat in spike stem induced by *A. tumefaciens*, strain T37.**

**Table 1. Tumor-inducing effect of *A. tumefaciens* on the two-rowed, short-awned 'Chingko' cultivar of barley.**

| *A. tumefaciens* Strains | Culture conditions[a] | Injected sites | Tumor-inducing frequency[b] | Tumor diameter (mm) |
|---|---|---|---|---|
| T37 | YEB + As | Base of leaf sheath | 6/15 | 1-2 |
| B6 | YEB + As | Base of leaf sheath | 7/17 | 2-3.5 |
| A208 | YEB | Base of leaf sheath | 3/16 | 0.5-1.5 |
| A208 | YEB + As | Base of leaf sheath | 10/21 | 1-2 |

[a] YEB: Yeast extract broth; AS: Acetosyringone.

[b] No. of tumors/No. of injected sites.

**Table 2. Tumor-inducing effect of *A. tumefaciens* on wheat cultivar 'Maoying No. 139 .**

| *A. tumefaciens* Strains | Culture conditions[a] | Injected sites | Tumor-induction frequency[b] | Tumor diameter (mm) |
|---|---|---|---|---|
| T37 | YEB | Base of leaf sheath | 3/13 | 0.5-1.5 |
| T37 | YEB | Spike stem | 2/ 6 | 1.0-1.5 |
| T37 | YEB + As | Base of leaf sheath | 8/21 | 1.0-2.0 |
| T37 | YEB + As | Spike stem | 6/11 | 2.0-3.0 |
| B6 | YEB + As | Base of leaf sheath | 4/10 | 1.0-1.5 |
| B6 | YEB + As | Spike stem | 3/ 8 | 1.0-1.5 |
| A208 | YEB + As | Base of leaf sheath | 7/15 | 1.0-1.5 |

[a] YEB: Yeast extact broth; As: Acetosyringone.

[b] No. of tumors/No. of injected sites.

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## RESUMEN

El objetivo de las cruzas amplias consiste en transferir genes útiles de los parientes silvestres de los cultivos a las variedades cultivadas. Los parientes silvestres de las variedades cultivadas poseen gran número de características positivas, tales como resistencia a insectos y enfermedades, tolerancia a los factores abióticos adversos, alto contenido de proteína en la semilla y fuentes de esterilidad masculina. En este artículo se describen los progresos logrados en la utilización de genes de parientes silvestres del arroz, cebada, trigo y algodón. Se muestran los adelantos en la producción de arroz híbrido en China y la forma en que se derivan las fuentes básicas de esterilidad masculina del arroz silvestre; por otra parte, se analizan las técnicas existentes para la transferencia sexual y parasexual de genes.

# Genetic resources of *Tripsacum* and gene transfer to maize

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Interest in maize x *Tripsacum* hybrids and gene transfer into maize is not new. Since the first successful cross between maize and *Tripsacum*, gene transfer from *Tripsacum* to maize has been shown to be possible. Many traits may be useful, such as insect and disease resistance. However, the lack of information about *Tripsacum* genetic diversity and resources is evident. Clearly, it is a bottleneck in the exploitation of these resources. Although the systematics of *Tripsacum* have been studied, a large scale evaluation of genetic resources of *Tripsacum* has never been conducted. Many accessions from previous surveys in various parts of America and especially in Mexico are available for such an evaluation. This project primarily focuses on a survey and evaluation of *Tripsacum* genetic diversity within and among species using modern tools, such as molecular and cytogenetic markers. A special effort will be made not only to detect desirable traits within the species, but also to understand the corresponding genetic control. Up to now, exploitation of *Tripsacum* genetic resources has been hindered by lack of basic knowledge on the genetic control of favorable traits. Results of the proposed research will offer the much needed information for gene transfer. These results will allow choice of the most efficient ways of transfer, either through conventional breeding or genetic engineering. Proposals of cooperative research efforts will be welcome.

Maize is a crop with a great adaptability. Originally from the tropics, its range has been continuously extended into temperate zones through breeding and selection. However, there is a need for new genes and wider genetic diversity. A first possibility is inclusion of genes from teosinte (*Zea mays* ssp. *mexicana*) in the maize gene pool. Several studies have already documented this potential. A second possibility is offered by the genus *Tripsacum*.

Previous studies of maize x *Tripsacum* hybridization (Mangelsdorf and Reeves 1931, Galinat 1973, Harlan and De Wet 1977, Bernard and Jewell 1985) have shown that hybrids can be obtained and gene transfer achieved. Therefore, the genus *Tripsacum* is a potential source of valuable characters for maize breeding. To use *Tripsacum* advantageously, we need to have better knowledge of the genus. Taxonomic studies have already been published (Randolph 1970, De Wet *et al.* 1976, Brink and De Wet 1983). Useful traits have been detected (resistance against pathogens and insects), but there is still a need for a comprehensive genetic resource evaluation. Interesting agronomic traits must be characterized and their genetic control studied. Also genetic relationships among and within species of *Tripsacum* must be studied. Our proposal is directed to fill this need. *Tripsacum* species belong to the secondary gene pool of maize as defined by Harlan and De Wet (1971). We would like to genetically transfer them to the primary gene pool of maize (species and forms that hybridize quite easily with maize).

Apomixis, i.e. vegetative propagation through seeds, is a common phenomenon in the genus *Tripsacum*, and especially in *T. dactyloides* (Farquharson 1955). In our genetic resource analysis, this aspect will be considered and attempts to transfer this trait into maize will be conducted.

This review considers the germplasm base of *Tripsacum*, its current status, and studies in progress. We summarize methods used for gene transfer from *Tripsacum* to maize, and indicate those most effective. We then develop our research project, focusing on points which could be done under cooperative agreement.

## *TRIPSACUM* BOTANY

### Systematics of *Tripsacum*

This genus has been studied recently by several authors (De Wet *et al.* 1976, 1981; Brink and De Wet 1983; Doebley 1984). Two sections, *Fasciculata* and *Tripsacum*, have been defined based on inflorescence morphology. Sixteen species are currently recognized. The range of this New World genus is impressive: from 42 °N to 24 °S latitude (Fig. 1). Mesoamerica has the largest concentration of species—11. The two common chromosome numbers are 36 and 72, but other numbers (2n=90, 108) occur in *T. peruvianum* (De Wet *et al.* 1981). The distribution of 36- and 72-chromosome plants or species does not conform to a special geographical pattern. Ecological adaptation of species or forms extends from wet tropical forest (*T. maizar*, *T. laxum*) to xeric habitats (*T. zopilotense*, *T. lanceolatum*).

### Surveys and collections

Surveys have covered most of the areas where *Tripsacum* occurs. Main *Tripsacum* collections are:

- United States: One collection (approximately 200 accessions) of *T. dactyloides* at Urbana, Illinois; one larger collection of *T. dactyloides* at Woodward, Oklahoma; one collection of approximately 500 accessions of tropical *Tripsacum* at Homestead, Florida.
- Mexico: one collection for species from Mexico and Guatemala in a clonal garden at CIMMYT's Tlaltizapan station.

Although only a small fraction of the total genetic diversity of this genus is represented in these collections, this amount is still large and is a useful basis for evaluation of *Tripsacum* genetic diversity. Several studies have already been conducted on this material. Morphology of the different species has been studied (De Wet *et al.* 1981, Brink and De Wet 1983). Cytological studies have been limited mostly to chromosome counts, although a few interspecific *Tripsacum* hybrids have been obtained. The *Tripsacum* collections can provide material for further genetic studies and may become of great value in breeding schemes, including those using molecular techniques.

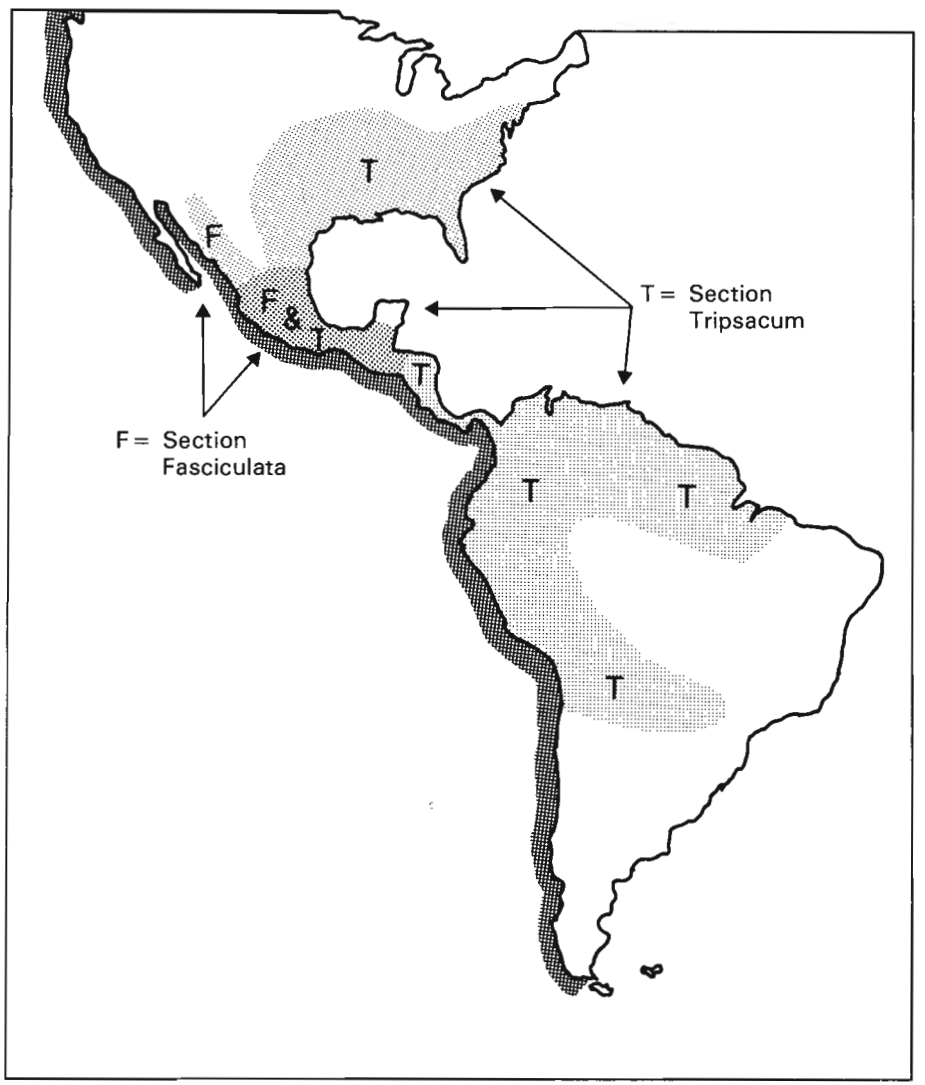


**Figure 1. Distribution area of the genus *Tripsacum* in The Americas.**

## WIDE CROSSES IN MAIZE

### General approach

We discuss only maize x *Tripsacum* hybridization. We do not cover maize x teosinte, as these are very close relatives (Wilkes 1967).

The ability to cross maize and *Tripsacum* was demonstrated by Mangelsdorf and Reeves (1931, 1939); their publications now are classic references on the subject. Hybridization was achieved with the diploid *T. dactyloides* (2n=36) as the male parent. The silks of the maize maternal parent were cut to permit successful pollination. Hybrids had 28 chromosomes (10 Zm + 18 Tr) and were male sterile. The first backcross to maize gave 38-chromosome plants, and the second backcross, 20-chromosome plants that are phenotypically identical to maize. This scheme of producing plants successively with 28, 38, and 20 chromosomes has been followed for several decades by many other researchers, (Randolph 1955, Maguire 1960, Chaganti 1965, Simone and Hooker 1976). Galinat (1973) studied the intergenomic relationships between maize and *Tripsacum* chromosomes in similar hybrids. The method he developed is summarized in Figure 2. A maize line with recessive markers for the 10

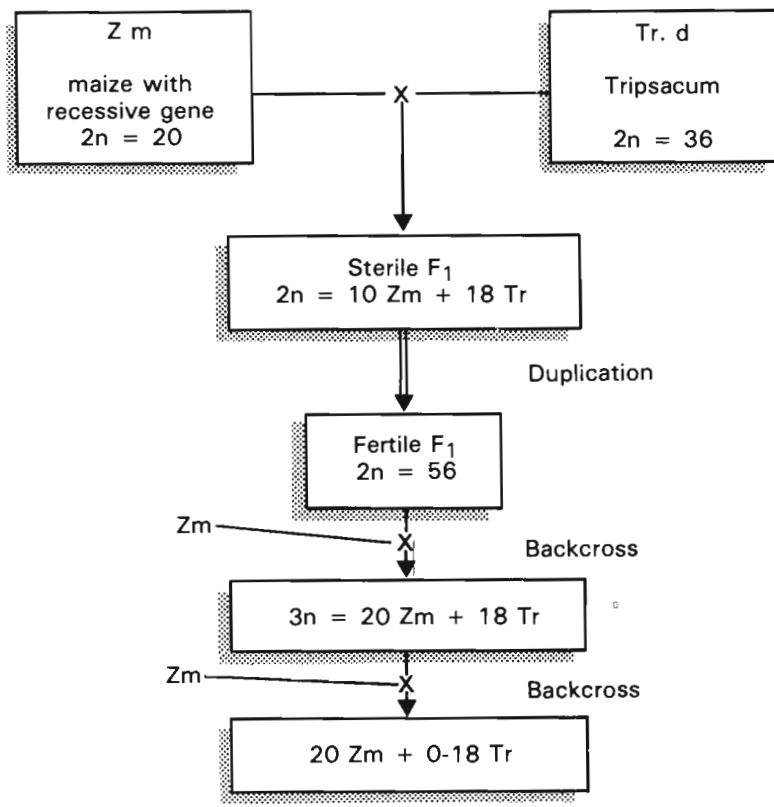


**Figure 2. Breeding chart illustrating the crosses involved in developing the various addition monosomic stocks of maize that carry extra cytogenetically identified chromosomes from *Tripsacum* (Adapted from Galinat 1973).**

chromosomes, developed by Mangelsdorf, was crossed with a diploid *T. dactyloides*. The sterility of 28-chromosome hybrids was overcome by chromosomic duplication. Two backcrosses led to recovered maize lines with 20 *Zea* chromosomes plus 0 to 18 *Tripsacum* chromosomes. The presence of *Tripsacum* chromosomes in recovered maize lines has permitted assessment of their effects on expression of the maize marker genes. Homoeology has been detected between maize chromosomes #2, #4, #7, and #9 and *Tripsacum* chromosomes. Genes homoelogous to those on one maize chromosome may be scattered on different *Tripsacum* chromosomes. This is the case especially for gene controlling characters responsible for the domestication syndrome on maize chromosome #4. Maize chromosome #4 is homologous with *Tripsacum* chromosomes #7 and #13. The data suggest a chromosome repatterning between these two genera (Galinat 1973).

Petrov *et al.* (1984) followed another cytogenetic pathway in Siberia. They have crossed tetraploid maize (2n=40) with a tetraploid *T. dactyloides* (2n=72). Their hybrids (2n=56), when backcrossed to maize, give 38- and 28-chromosome plants. Some of these plants are apomictic.

**Another approach**

We discuss next the different hybridization pathways tested at the University of Illinois. Figure 3 is reproduced from Harlan and De Wet (1977). The first important result is that hybridization success depends on the particular combination of maize and *Tripsacum* genotypes. De Wet (1979) stated that only 1% of studied *Tripsacum* collections crossed with maize. One major reason for this incompatibility was the presence of a Ga$^s$ gene (De Wet *et al.* 1973, 1978). The effects of different crossing schemes on *Tripsacum*-maize gene transfer have been studied. The shortest path for maize line recovery, the 28-38-20 pathway, gives little evidence of gene transfer.

Study of a second, longer scheme (46-56-38) was initiated with a tetraploid *Tripsacum* (2n=72). Some of the 46-chromosome hybrids produced by De Wet *et al.* (1970) were female-fertile and 56-chromosome offspring were obtained. Further backcrosses gave 38-chromosome plants and 20-chromosome recovered maize lines. *Tripsacum* chromosomes are lost more slowly than in the previous pathway, but the genetic exchange between chromosomes of the two species remains limited.

A third, "irregular pathway" has been tried. The 46-chromosome stock can be maintained for several generations. At each generation, the maize-chromosome stock is lost during meiosis and a new maize stock reincorporated through fertilization (De Wet *et al.* 1970). Backcrossing a maizoid BC2 plant with recovered maize gave a BC3 56-chromosome plant, which in turn produced progeny of extreme cytological variability. Plants with chromosome numbers from 28 to 70 were observed. In further backcrosses, within 2 to 4 generations, recovered maize lines were obtained. Genetic transfer in these lines has been evident.

The key distinction of the third pathway from the previous two is the presence of several generations of 46-chromosome plants. This allows genetic contamination of *Tripsacum* chromosomes by maize; thus, apparently, if one wishes to contaminate maize with *Tripsacum*, one should first contaminate *Tripsacum* with maize (Harlan and De Wet 1977). These authors also noted that only one accession of *T. dactyloides* gave these results. Even with this limitation, recovered maize lines with genetic

elements from *Tripsacum* could be the most suitable parents for new cycles of intercrossing between maize and *Tripsacum*.

In conclusion, we believe these hybridization pathways will eventually permit genetic transfer of interesting characters from *Tripsacum* to maize.

## A RESEARCH PROJECT

Evidence cited in the review shows that genetic transfers are possible between *Tripsacum* and maize. To take a full advantage of this possibility, we need better knowledge of *Tripsacum* genetic diversity. This means studies of:

- Genetic diversity *per se*.
- Interesting agronomic traits (including apomixis) that occur in *Tripsacum*; distribution and genetic control of these traits, especially when placed into a new genetic background such as maize-*Tripsacum* hybrids.
- Possibilities of gene exchanges within *Tripsacum*, and between *Tripsacum* and maize, including the possible "bridges" that newly recovered maize lines may offer.

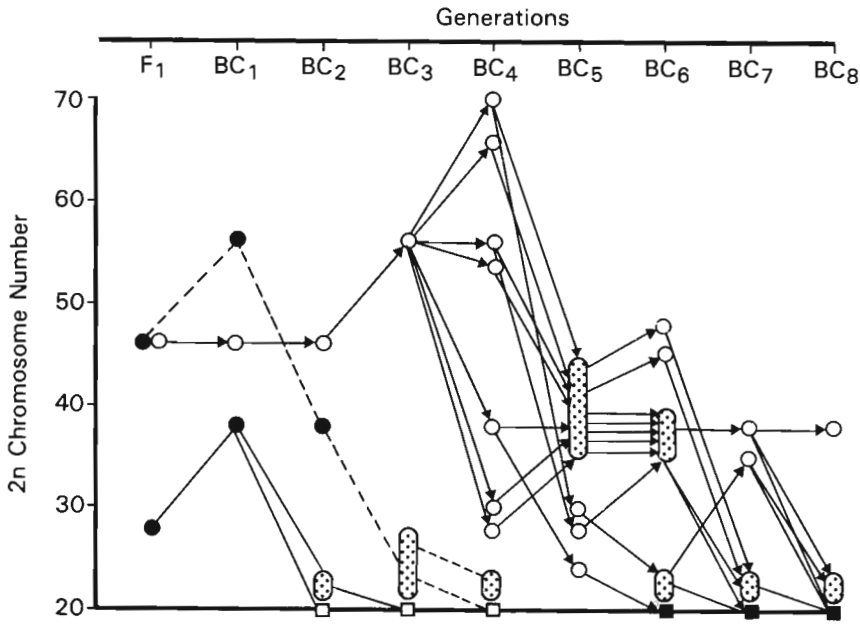


**Figure 3. Simplified diagram of some of the pathways to recovered maize from *Zea/Tripsacum* hybrids. The stippled ovals represent populations with the indicated range of chromosome numbers varying from plant to plant within families (From Harlan and De Wet 1977).**

□ recovered maize (usually pure)
■ recovered maize (often tripsacoid);
●——● 28 → 38 → 20 pathway;
●- -● 46 → 56 → 38 → 20 pathway;
○ → ○ irregular pathways

Our project concentrates on three main points:

- Conservation of *Tripsacum* resources.
- Evaluation of *Tripsacum* species.
- Studies of ways of transfer between *Tripsacum* and maize.

**Conservation of *Tripsacum* resources**

Conservation can be effective *in situ* and *ex situ*. We plan to localize wild populations of *Tripsacum* in Mexico for *in situ* studies and for conservation. Samples of these populations will be established in a living collection. A major living collection will permit a comparative observation of the different species of *Tripsacum*, using morphometric traits. This living collection will also supply researchers with the needed material for the genetic experiments.

**Evaluation of *Tripsacum* species**

We will conduct three types of studies:

- Evolutionary.
- Evaluation of agronomic traits.
- Evaluation of genetic controls.

*Evolutionary studies.* Relationships between species can be assessed through various techniques:

- Cytogenetics: "Knobs." In maize, "knobs" are good chromosome markers and they allow an estimation of the relationship between the many maize races. Studies on maize were conducted by McClintock and her students. DNA probes and *in situ* hybridization could be a new source of information on chromosome structure and differentiation.
- Isozymes analyses. Electrophoresis has not yet been used on *Tripsacum* material. Adaptations of techniques used with maize (Goodman and Stuber 1983) may be possible. Correspondence between alleles described for maize and those seen in *Tripsacum* will have to be established. Once markers in *Tripsacum* are made available, they will be utilized for describing diversity of the various *Tripsacum* species obtained from living collections or from *in situ* populations. Markers are also useful for interspecific hybridization.
- DNA analyses. The RFLP technique has given very interesting results when applied to maize (see Helentjaris *et al.* 1985). Now we can apply this technique to the *Tripsacum* species.

With the help of a specialized laboratory, we plan to test several types of probes for their effectiveness in describing *Tripsacum* diversity (probes from nonrepeated sequences, repeated sequences, and highly repeated sequences). Another approach will compare the sequences of interesting genes (genes coding for seed proteins) between maize and *Tripsacum* species.

*Evaluation for agronomic traits.* It is reasonable to assume that the wider range of adaptations found in *Tripsacum* species is related in part to *Tripsacum*'s having more resistance traits than maize and teosinte. The ensemble of *Tripsacum* species should be a good reservoir of interesting genes. Currently, it is difficult to give many such examples because very little is known about resistances in *Tripsacum*. Our research proposal is aimed to fill this gap.

Resistance traits found in *Tripsacum* species and already transferred to maize are monogenic resistance against fungi or bacteria (Bergquist 1977, 1981). But new research projects, as well as projects already started at CIMMYT and in some seed companies, are focused on resistances against virus or insects. Resistance sources should be determined within populations and species, and their nature described. These resistance sources should be the starting point for gene transfer from *Tripsacum* to maize.

Protein quality can be considered as a another kind of agronomic trait. The quality of seed proteins will be assessed, especially as some genes involved in seed storage protein production are under study using molecular biology tools.

Apomixis will be studied. In forage grasses, this mechanism is common (Brown and Emery 1958). Genetic control is based on one or a few genes (Harlan *et al.* 1964, Savidan 1980). Transfer of this trait to maize would have an impact on breeding schemes.

*Evaluation of genetic controls and ways of transfer.* At present, gene transfer is only possible if species can be crossed. But crossability is highly diverse from one genotype to another one. Sterility genes are known for maize and suspected for *Tripsacum.* Hybridization techniques need to be improved for crossing *Tripsacum* species. In conjunction, a study of the nature of these genetic barriers will be carried out.

Before starting maize x *Tripsacum* hybridizations, we must understand the genetic controls working in *Tripsacum* species for the selected traits. How many genes are involved? What is their chromosomic localization? Genes will be enumerated and located by crossing the most convenient *Tripsacum* progenitors from the same species or from different species. This collected information should lead to a policy for genetic transfer between *Tripsacum* and maize. At the moment, possibilities are quite open. The ultimate aim is to move *Tripsacum* species from a secondary gene pool to the primary gene pool of maize.

## EXPANDING THE PROJECT

This project appears to be quite ambitious. It can only be fully operational on a cooperative basis. We have already contacted several laboratories interested in the project and we would like to broaden our network to combine more expertise in different fields of research to have a comprehensive approach on documenting the genetic diversity of *Tripsacum* and on formulating ways to transfer it to maize.

Once a very comprehensive collection of *Tripsacum* species is established and well documented, this genetic material will be made available for molecular biology experiments. Such experiments comparing genes from *Tripsacum* and maize should give information on the diversity, evolution, and regulation mode of these genes. The new tools offered by molecular biology will be included in our studies when it is possible. We think that cytology will benefit a lot from these tools. Expertise in this field is welcome.

We also need more expertise on immature embryo rescue. It will be a crucial point for the gene transfer program. With the technique they developed, Laurie and Bennett

(1988) were able to rescue 2-day-old wheat x maize hybrid embryos. Such a technique, once available for maize x *Tripsacum* hybrid spikelet culture, could help in getting more interspecific hybrids and more genes transferred.

We can get more technical expertise. We also feel that, in the long run, complementary genetic material should be included in these studies. We should study the relationships between maize, teosinte, and *Tripsacum* and compare the possible gene flows between these species, their effectiveness, and usefulness for maize breeding.

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**RESUMEN**

El interés en los híbridos de maíz x *Tripsacum* y la transferencia de genes al maíz no es nada nuevo. Desde que se tuvo éxito por primera vez en el cruzamiento de maíz y *Tripsacum*, se ha convertido en posible la transferencia de genes del *Tripsacum* al maíz. Muchas características pueden ser de gran utilidad, tales como la resistencia a insectos y enfermedades; no obstante, es evidente la falta de información acerca de la diversidad y recursos genéticos del *Tripsacum*, y no cabe duda de que constituye un verdadero cuello de botella en la explotación de estos recursos. Si bien se han estudiado las características sistemáticas del *Tripsacum*, nunca se ha realizado una evaluación a fondo de sus recursos genéticos. Para llevar a cabo una evaluación semejante, se dispone de un gran número de accesiones recolectadas en encuestas anteriores realizadas en distintas partes de América y, sobre todo, en México. Nuestro proyecto se concentra fundamentalmente en la encuesta y evaluación de la diversidad genética del *Tripsacum* en diferentes especies, empleando para ello herramientas modernas, tales como marcadores moleculares y citogenéticos. Se llevará a cabo un esfuerzo especial, no sólo para detectar las características más convenientes dentro de una misma especie, sino también para comprender el control genético correspondiente. Hasta la fecha, la explotación de los recursos genéticos del *Tripsacum* se ha visto obstaculizada por la falta de conocimientos básicos sobre el control genético de sus características favorables. Los resultados de la investigación propuesta ofrecerán la información tan necesaria para la transferencia de genes. Estos resultados permitirán la elección de los medios más eficaces de transferencia, ya sea mediante el fitomejoramiento convencional o la ingeniería genética. Se recibirán con agrado todas las propuestas para cooperar en la investigación.

# Marker-assisted introgression of alien chromatin into wheat

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The study involved assessment of some schemes for increasing the probability of transfers of alien chromatin into the wheat genome in wheat-alien hybrids. Backcross I (BCI) seed set, earlier a significant constraint, has been obtained from *ph1b* or N5BT5-/Alien sp. $F_1$ hybrids involving Aegilops species and rye cultivars making alien introgression through allosyndetic pairing at $F_1$ a viable procedure. Another scheme deals with topcrossing the *Triticum aestivum* or *T. turgidum* based $F_1$ hybrids with *T. turgidum* or *T. aestivum*, respectively, in order to induce centromeric breakage and fusion of chromosomes. It has been successfully implemented in attaining advanced derivatives from the initial cross. Modifications of these major schemes were also initiated. Monitoring of the alien chromosomes in the progenies was through application of cytological, morphological, and biochemical markers. These have assisted in the production of several alien chromosome additions of *Haynaldia villosa*, *Agropyron junceum*, and *Elymus giganteus* in a *T. aestivum* background.

The wild relatives of wheat and other Triticeae have long been recognized as valuable reservoirs of useful genes for the improvement of this important cereal (Feldman and Sears 1981, Dewey 1984). Some of the barriers to interspecific hybridization have been overcome, thus permitting exploitation of a small but significant part of this gene source (Sharma and Gill 1983, Mujeeb-Kazi and Kimber 1985, Brar and Khush 1987, Mujeeb-Kazi and Asiedu 1988a,b). Apart from the hybridization barriers, the major limitation to introgression of alien genes into wheat has been the lack of pairing and recombination between wheat and alien chromosomes (ter Kuile *et al.* 1987). While pairing in wheat is influenced by a number of suppressor and promoter genes on chromosomes 2, 3, 4, and 5 (Sears 1976, Kimber and Feldman 1987), the most potent of the genes is well documented as the suppressor of homoeologous pairing (*Ph*) on 5BL of *Triticum aestivum* and *T. turgidum* (Okamoto 1957, Riley and Chapman 1958) although its mode of action is rather controversial (Feldman 1966, Driscoll 1979).

In order to induce allosyndesis, it has been necessary to remove the 5B chromosome (Lacadena 1967, Koebner and Shepherd 1986), suppress the gene with an alien genome, or use mutant lines of wheat (Sears 1984). Accessions of *Aegilops speltoides*, *Ae. mutica*, and *Ae. longissima* have been reported to show variability for ability to suppress the *Ph* gene (Dover and Riley 1972, Chen and Dvorak 1984). Similar promotion of heterogenetic pairing in wheat backgrounds has been reported for barley (Fedak 1977) and rye (Lelley 1976, Dhaliwal *et al.* 1977, Dvorak 1977, Naranjo *et al.* 1979). Studies of a number of polyploid *Aegilops* species have not revealed *Ph*-like genes in these species (Abu Baker and Kimber 1982, McGuire and Dvorak 1982, Sharma and Gill 1986) even though the pairing systems in these species must be under some genetic control (Gupta and Fedak 1985). In the absence of the *Ph* genes in wheat/*Aegilops* hybrids, the *Aegilops* species may or may not influence homoeologous chromosome pairing (Abu Baker and Kimber 1982, McGuire and Dvorak 1982). Driscoll and Quinn (1970) reported variability among cultivars of *T. aestivum* for the level of heterogenetic pairing following hybridization to *Ae. variabilis*.

The methods used for wheat-alien transfers include irradiation to induce translocations (Driscoll and Jensen 1964, Sharma and Knott 1966) induction of centromeric breakage and fusion (May and Appels 1982, Lukaszewski and Gustafson 1983) and induction of allosyndesis (Riley *et al.* 1968, Joshi and Singh 1978, Koebner and Shepherd 1985, 1986). Driscoll (1968) proposed a combination of irradiation and meiotic control for increased rate and specificity of wheat-alien transfers. Darvey (1984) later suggested the use of the *ph* mutant in direct hybridization with alien species for the construction of an alien gene bank, but attempts by Sharma and Gill (1986) in this direction were unsuccessful, while those of ter Kuile *et al.* (1987) were promising.

Many of the important traits desired from the alien species are not easy or cheap to select for in the segregating populations, especially during early generations when the populations need to be screened to avoid carrying too many unwanted families later. Some of the complications are the lack of knowledge of the biochemical and genetic control of the characters, heterogeneity of the breeding materials, sporadicity of natural disease epiphytotics, environmental influence, the expense associated with artificial infestations, and the large number of samples or plants required for efficient screening. It is in this context that heritable and more easily identifiable characteristics, termed markers, associated with the desired agronomic traits become an important consideration (see Diagnostic Markers in Wheat Wide Crosses in the Poster Section of these Proceedings). Cusick and McIntosh (1987) and Hart and Gale (1987), respectively, have provided a linkage map of wheat and a listing of biochemical/molecular markers currently available for wheat research. Miller and Reader (1987) have compiled many of the morphological markers associated with specific homoeologous groups in the Triticeae. Furthermore, the use of differential chromosome staining for recognition of transferred alien segments has been demonstrated (Gill and Kimber 1977, Lukaszweski and Gustafson 1983).

Our objectives were to evaluate and test several hybridization schemes for the introduction of useful alien chromatin into wheat and to track such chromatin in the wheat background by using different markers in segregating populations.

## MATERIALS AND METHODS

The alien species used are listed in Table 1. The *Aegilops* accessions were originally obtained from the Plant Breeding Institute (PBI), Cambridge. Seeds of *Haynaldia villosa* and *Thinopyrum distichum* were obtained from E.R. Sears (Missouri, USA) and R. Pienaar (South Africa), respectively. The *Leymus* and remaining *Thinopyrum* species were supplied by D.R. Dewey (Logan, Utah, USA). In addition to the foregoing, several cultivars of *T. aestivum* including 'Chinese Spring', its *ph1b* mutant, and nullisomic 5B tetrasomic 5A (or 5D) stocks and cultivars of *T. turgidum* var. *durum* were used. Seeds of the *ph1b* mutant were provided by K.W. Shepherd (Adelaide, Australia). All other cultivars used were from CIMMYT. Most of the alien species used are known to have useful traits for wheat improvement.

Four crossing schemes were employed:

A. Hybrids were made between *ph1b* or CSN5BT5- with all the *Aegilops* species, the *S. cereale* cultivars, *Thinopyrum bessarabicum*, and *Thinopyrum junceiforme*. Embryo rescue onto standard artificial medium was performed for the hybrids involving the *Thinopyrum* species. The $F_1$ hybrids were backcrossed to CS, *ph1b*, and/or cultivars of *T. aestivum* and *T. turgidum* var. *durum*. All backcross I (BCI) plants obtained were backcrossed to cultivars of *T. aestivum*.

**Table 1. Species of *Triticum* (*Aegilops*), *Haynaldia*, *Leymus*, *Secale*, and *Thinopyrum* used in this study.**

| Species | Acc. No.[a] | Genome[b] | Ploidy Level |
|---|---|---|---|
| *T. macrochaetum* (*Ae. biuncialis*) | 10A | UM | 4X |
| *T. dichasians* (*Ae. caudata*) | 16A | C | 2X |
| *T. cylindricum* (*Ae. cylindrica*) | 17G | CD | 4X |
| *T. cylindricum* (*Ae.cylindrica*) | 18A | CD | 4X |
| *T. juvenale* (*Ae. juvenalis*) | 45A | DMU | 6X |
| *T. tripsacoides* (*Ae. mutica*) | 24D | Mt | 2X |
| *T. ovatum* (*Ae. ovata*) | 4E | UM | 4X |
| *T. neglecta* (*Ae. triaristata*) | 5F | UM | 4X |
| *T. triunciale* (*Ae. triuncialis*) | 15X | UC | 4X |
| *T. peregrinum* (*Ae. variabilis*) | 13E | US | 4X |
| *T. syriacum* (*Ae. vavilovii*) | 41A | DMS | 6X |
| *H. villosa* | (ex Sears) | V | 2X |
| *L. racemosus* | PI313965 | JN | 4X |
| *S. cereale* (cv. Elvon) | - | R | 2X |
| *S. cereale* (cv. Prolific) | - | R | 2X |
| *S. cereale* (cv. Semi-dwarf) | - | R | 2X |
| *Thinopyrum distichum* | (ex Pienaar) | $E_1E_2$ | 4X |
| *Thinopyrum bessarabicum* | Jaaska-11 | J | 2X |
| *Thinopyrum junceiforme* | PI414667 | $J_1J_2$ | 4X |
| *Thinopyrum scirpeum* | (ex Dewey) | $E_1E_2$ | 4X |

[a] Accession number.
[b] After Kimber and Feldman (1987) and Dewey (1984).

B. $F_1$ hybrids of CS with *H. villosa* (Mujeeb-Kazi and Bernard 1985) and *Thinopyrum bessarabicum* (Mujeeb-Kazi *et al.* 1984, 1987) were backcrossed to *ph1b* and spikes of the progenies were bagged to ensure selfing (*T. aestivum*/Alien//*ph1 b* ①.

C. Cultivars of *T. turgidum* var. *durum* were used as pollen parents for crosses to $F_1$ hybrids of CS with *Th. bessarabicum* (Mujeeb-Kazi *et al.* 1984, 1987), *H. villosa* (Mujeeb-Kazi and Bernard 1985) and *L. racemosus* (Mujeeb-Kazi and Rodríguez 1981), respectively. The resulting BCI progenies were backcrossed to cultivars of *T. aestivum*. An $F_1$ hybrid, *T. turgidum* cv. Memo/Mexicali//*Th. junceiforme* (Mujeeb-Kazi and Bernard 1985), was also backcrossed to *T. aestivum* cv. Alondra/Pavon, i.e. *T. aestivum*/Alien//*T. turgidum*/3/*T. aestivum* and *T. turgidum*/Alien//*T. aestivum*.

D. An $F_1$ hybrid, *T. turgidum* cv. Altar 84/*Th. scirpeum* (Mujeeb-Kazi, unpublished), was topcrossed with *ph1b* and the progeny was backcrossed to cultivars of *T. aestivum* directly or after one cycle of selfing, i.e. *T. turgidum*/Alien//*ph1b*/3/*T. aestivum*.

These approaches were designed to: 1) promote homozygosity for the *ph1b* locus after selfing the BCI in order to enhance recombination and 2) induce D genome and alien genome translocations (Mujeeb-Kazi 1984).

All plants were grown in the greenhouse and authenticity of hybrids was checked by root-tip counts (Mujeeb-Kazi and Miranda 1985) sometimes supported by Giemsa C-banding and isozyme analyses. Spikes were fixed in Carnoy's solution and the anthers were stained in alcoholic carmine and squashed in 2% acetocarmine for obtaining data on the meiotic configuration at metaphase I. For analyses of isozymes (Table 2), standard procedures for polyacrylamide gel isoelectricfocussing (PAG.IEF) and conventional electrophoresis on cellulose acetate or polyacrylamide gel were used. Esterase and malate dehydrogenase were stained according to Brown *et al.* (1978), aconitate hydratase according to Jung *et al.* (1986), and the remaining enzymes according to Vallejos (1983). All progenies were observed for characteristic morphological features.

**Table 2. List of enzymes analyzed.**

| Enzyme | E.C. No. |
|---|---|
| Acid phosphatase | 3.1.3.2 |
| Aconitate hydratase | 4.2.1.3 |
| Alcohol dehydrogenase | 1.1.1.1 |
| Aspartate aminotransferase | 2.6.1.1 |
| Esterase | 3.1.1.2 |
| Glucosephosphate isomerase | 5.3.1.9 |
| Glucose-6-phosphate dehydrogenase | 1.1.1.49 |
| Malate dehydrogenase | 1.1.1.37 |
| Phosphoglucomutase | 2.7.5.1 |
| Phosphogluconate dehydrogenase | 1.1.1.43 |
| Shikimate dehydrogenase | 1.1.1.25 |

## RESULTS

The $F_1$ hybrids involving *Aegilops* species more resembled their *Aegilops* parents in spike morphology, although a phenotypic co-dominance was apparent. All the $F_1$ hybrids with the CS stocks were self-sterile, but many produced a few BCI seeds (Table 3). Backcross II (BCII) seed set was generally much higher than BCI seed set—3.6%, 10.6%, 20.5%, and 22.9% for *ph1b*/*Ae. variabilis*, N5BT5A/*Ae. biuncialis*, N5BT5/*Ae. variabilis*, and N5BT5A/*Ae. cylindrica*, respectively. Generally, chromosome pairing was higher in *ph1b* or N5BT5- x Alien hybrids than expected in the normal respective wheat x Alien hybrids (Table 4). The only direct comparison involving hybrids of *Ae. variabilis* with CS, *ph1b*, and N5BT5-, respectively, attests to this. The chromosome numbers of the BCI plants were generally lower than those expected when complete meiotic restitution of the female gametes is assumed (Table 5); a variation trend consistent with earlier observations of BCI derivatives among several intergeneric hybrids in the Triticeae (Jewell and Mujeeb-Kazi 1982; Mujeeb-Kazi and Bernard 1985). As shown for the rye hybrids (Table 6), the BCI plants may have different numbers of alien chromosomes. BCII or backcross III (BCIII) plants are currently available for all hybrids that were successfully backcrossed. Selfed progenies from CS/*H. villosa*//*ph1b* and CS/*Th. bessarabicum*//*ph1b* will soon be screened for selection of *ph1b* homozygotes.

BCI progenies from topcrossing $F_1$ hybrids of scheme C had the expected chromosome numbers assuming complete restitution of the female gametes. Those for *T. aestivum*/*T. bessarabicum*//*T. turgidum* had 42 chromosomes associated as 14

**Table 3. Backcross I (BCI) seed set on $F_1$ hybrids.**

| $F_1$ hybrid | No. of florets pollinated | No. of BCI seeds | Percentage seed set |
|---|---|---|---|
| *ph1b*/*Ae. caudata* | 250 | 0 | 0.0 |
| *ph1b*/*Ae. juvenalis* | 2736 | 68 | 2.5 |
| *ph1b*/*Ae. mutica* | 2954 | 11 | 0.4 |
| *ph1b*/*Ae. ovata* | 612 | 0 | 0.0 |
| *ph1b*/*Ae. triuncialis* | 414 | 4 | 1.0 |
| *ph1b*/*Ae. vavilovii* | 1918 | 134 | 7.0 |
| *ph1b*/Elvon rye | 608 | 2 | 0.3 |
| *ph1b*/Prolific rye | 302 | 2 | 0.7 |
| *ph1b*/Semidwarf rye | 476 | 0 | 0.0 |
| *ph1b*/*Th. bessarabicum* | 152 | 0 | 0.0 |
| *ph1b*/*Ae. variabilis* | 2058 | 18 | 0.8 |
| CS/*Ae. variabilis* | 1112 | 22 | 2.0 |
| N5BT5-/*Ae. variabilis* | 3452 | 10 | 0.3 |
| N5BT5A/*Ae. biuncialis* | 582 | 2 | 0.3 |
| N5BT5A/*Ae. cylindrica* (18G) | 1900 | 9 | 0.5 |
| CS/*Ae. cylindrica* (17A) | 228 | 9 | 4.0 |
| N5BT5A/Elvon rye | 3560 | 18 | 0.5 |
| N5BT5A/*Ae. triaristata* | 1628 | 8 | 0.5 |
| N5BT5A/*T. junceiforme* | 608 | 0 | 0.0 |

**Table 4. Mean meiotic metaphase I chromosome association.**

| Combination | Plant Number | Chromosome Number | I | II[a] | II[b] | III | IV | V | VI |
|---|---|---|---|---|---|---|---|---|---|
| Nulli 5B Tetra 5A/Elvon | 87-3290 | 28 | 8.5 | 3.7 | 2.7 | 1.75 | 0.25 | 0.25 | |
| Nulli 5B Tetra 5D/ *Ae. variabilis* | 87-3313 | 35 | 8.8 | 2.7 | 7.0 | 0.85 | 0.35 | | |
| *ph1b*/*Ae. variabilis* | 87-77-8 | 35 | 9.5 | 1.6 | 7.2 | 2.1 | 0.25 | 0.08 | 0.04 |
| Ald/Pvn//*Ae. variabilis* | 87-247 | 35 | 27.6 | 0.1 | 3.3 | 0.2 | | | |
| Nulli 5B Tetra 5A/ *Ae. triaristata* | 87-274-1 | 35 | 11.2 | 0.7 | 5.6 | 2.8 | 0.5 | 0.13 | 0.04 |
| *ph1b*/Prolific rye | 87-3343 | 28 | 7.7 | 3.0 | 3.2 | 2.2 | 0.33 | | |
| *ph1b*/*Ae. vavilovii* | 87-3868 | 42 | 17.9 | 0.05 | 7.4 | 1.83 | 0.38 | | |
| *ph1b*/*Ae. juvenalis* | 87-3887 | 42 | 19.2 | 1.6 | 5.0 | 2.32 | 0.4 | 0.12 | 0.08 |
| *ph1b*/*Ae. umbellulata* | 87-3385 | 28 | 7.5 | 1.8 | 4.2 | 0.09 | 0.45 | 0.09 | |
| *ph1b*/*Ae. mutica* | 87-3352 | 28 | 6.7 | 1.9 | 3.0 | 1.81 | 0.90 | 0.36 | 0.09 |
| *ph1b*/*Ae. ovata* | 87-3349 | 35 | 10.0 | 1.9 | 4.8 | 2.22 | 0.90 | 0.18 | 0.04 |
| Nulli 5B Tetra 5A/ *Ae. biuncialis* | 87-3378 | 35 | 4.5 | 1.0 | 7.5 | 4.5 | | | |

[a] II= Ring bivalent.
[b] II= Rod bivalent.

**Table 5. Number of chromosomes of some backcross I (BCI) plants.**

| Pedigree | Number of Plants | Chromosome number | | |
|---|---|---|---|---|
| | | Range | Mean | Expected |
| CS/*Ae. variabilis*//*T. aestivum* | 15 | 41-58 | 51.5 | 56 |
| N5BT5A/*Ae. variabilis*// *T. aestivum* | 17 | 36-59 | 40.9 | 56 |
| *ph1b*/*Ae. variabilis*// *T. aestivum* | 9 | 37-42 | 39.0 | 56 |
| *ph1b*/*Ae. vavilovii*//*T. aestivum* | 56 | 40-48 | 42.8 | 63 |
| *ph1b*/*Ae. juvenalis*//*T. aestivum* | 25 | 41-47 | 43.2 | 63 |

**Table 6. Number of rye chromosomes in backcross I (BCI) plants.**

| Pedigree | Chromosome number | |
|---|---|---|
| | Total | Rye |
| N5BT5A/Elvon//CNO | 41 | 7 |
| N5BT5A/Elvon//CS | 49 | 7 |
| *ph1b*/Elvon//CS | 40 | 5 |

bivalents + 14 univalents (Mujeeb-Kazi 1984). Plants with different somatic chromosome numbers resulted from the subsequent backcross to *T. aestivum* cultivars; e.g., 95 plants of CS/*Th. bessarabicum*//*T. turgidum*/3/*T. aestivum* had a mean of 41.8 chromosomes with a range of 35-50. There was a significant level of necrosis among progenies of some of these crosses, the worst combinations of *T. turgidum*/*T. aestivum* for this being Altar 84/Yaco, Altar 84/Pavon, and Chen/Goshawk. Necrosis-free combinations included Altar 84/Ciano 79, Yavaros//Mirlo/Buckbuck, and Yavaros//Buckbuck/Bluejay. Progenies from another cycle of backcrossing to *T. aestivum* cultivars are being analyzed. Owing to low self-fertility only four plants resulted from selfing of the BCI plants of *T. turgidum* cv. Altar 84/*Th. scirpeum*//*T. aestivum* cv. Chinese Spring *(ph1b)*. The BCII plants produced directly have been bagged for selfing.

Backcross derivatives from all schemes showed some segregation for characters such as spike morphology, pubescence, presence of anthocyanin, isozyme patterns, and the number of alien chromosomes when differential C-banding chromosome staining was used for derivatives involving *S. cereale*, *H. villosa*, and *L. racemosus*.

## DISCUSSION

These preliminary results show some of the alternative ways in which introgression of alien genes into wheat may be accomplished. The demonstration of BCI seed set in $F_1$ interspecific and intergeneric hybrids involving the *ph1b* mutant means wheat alien recombination at $F_1$ is a viable route for genetic exchanges. Indeed, when coupled with embryo rescue techniques, more BCI progenies may be obtained as BCI seeds of hybrids with *Ae. ovata* and *Ae. caudata* shrivelled and died on the parent plants. The only accurate comparison of percent seed set involves *Ae. variabilis* where the CS/*Ae. variabilis* $F_1$ set more BCI seeds than *ph1b*/*Ae. variabilis*. This supports the hypothesis of Sharma and Gill (1986) that there may be an influence of the *ph* mutant status on restitution in female gametes. BCI seed set on N5BT5-/*Ae. variabilis* and a rather rough comparison for the situation of *Ae. cylindrica* where two different accessions were involved suggest a similar role of the nullisomic 5B status. However, the number of somatic chromosomes of some of the BCI plants seems to indicate that, for these plants, BCI seed setting resulted not from restitution but from a process akin to a reduction division in the female gametes possibly owing to the high level of chromosome association at metaphase I. In the case of *T. aestivum*/*Ae. juvenalis* and *T. aestivum*/*Ae. vavilovii*, this may have been coupled with some similarity of the D genomes of the alien species with that of *T. aestivum* (Chapman and Miller 1978).

The low number of chromosomes in these plants may result in reduction in the number of alien chromosomes at BCI. However, as shown in the BCI plants from N5BT5A/Elvon, the total complement of alien chromosomes could be present even when the total number of chromosomes for the hybrid is less than expected. Reduction in the number of alien chromosomes may or may not be disadvantageous depending on the number of plants obtained and whether or not the critical alien chromosome or its segment is transmitted. So long as the critical alien segment is present in some of the BCI progenies, the wheat aneuploidy can be corrected with backcrossing without having to worry about disposing of many alien chromosomes. In the case of the hybrids for which BCI seed set was unsuccessful, scheme B may be the alternative as proposed

by Sharma and Gill (1986). However, it may be helpful to backcross the selected *ph1b* homozygotes, as well as the current progenies available from scheme B, to a cultivar of *T. turgidum* var. *durum*, which would introduce univalency of at least the D genome chromosomes in order to enhance allosyndetic pairing before backcrossing again to *T. aestivum*, unless there is sufficient desynapsis already.

Naturally cultivated wheat would be the most accessible source of genes for improvement of existing cultivars. When alien sources become necessary, the ease of hybridization to wheat, possibilities for wheat-alien chromosome recombination, and ploidy level should be taken into account. Generally, these imply giving priority to species that have genome similarities with cultivated wheat. Lower ploidy levels would normally speed up the transfer as there would be less unwanted chromatin to dispose of. Two further considerations in the decision to use an alien source are the genetic control of the trait and its expression in a wheat background (Mujeeb-Kazi *et al.* 1987, Mujeeb-Kazi and Asiedu 1988a). Single genes are most suitable for transfer from alien sources since only a small fraction of the alien genome is involved, thus limiting inclusion of deleterious alien chromatin. Characters under complex or multigenic inheritance may however be transferred if some of the genes have significant major effects leading to a reasonable expression of the trait after introduction of a small section of the alien genome or by avenues of interspecific hybridization, i.e. *T. turgidum* (AABB) or *T. aestivum* (AABBDD) x *T. tauschii* (DD) (Mujeeb-Kazi and Asiedu 1988a,b).

## CONCLUSIONS

The efficiency of introgressing alien genes could be increased with judicious use of markers. For this purpose correlations should preferably be made as soon as practicable between the presence of the various markers and the desired traits in the progenies. Some of these may reveal close linkages between trait and marker loci or trait loci flanked on both sides by marker loci. Once such reliable associations have been established, screening for the markers may be substituted for screening for the agronomic traits at some generations or may be used to reduce the populations to be screened. This is especially so with markers that can be identified using endosperm portions or sampling for analyses during early seedling stages. It must be remembered, however, that just as there is an inverse relationship between genetic distance and ease of hybridization, the application of markers is easier with wider hybrids.

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**RESUMEN**

El estudio incluyó la evaluación de algunos esquemas para incrementar la posibilidad de transferir cromatina extraña al genoma de trigo en híbridos de trigo y especies extrañas. La producción de semillas en la generación retrocruzada I (BCI), que antes constituía una limitación importante, se obtuvo de *phlb* o de los híbridos $F_1$ de N5BT5-/especies extrañas, que incluyen especies de *Aegilops* y variedades de centeno, con lo cual la incorporación de genes extraños mediante el apareamiento alosintético en la generación F1 se convirtió en un procedimiento viable. Otro esquema se refiere al mestizaje de híbridos $F_1$ basados en *Triticum aestivum* o *T. turgidum* con *T. turgidum* o *T. aestivum*, respectivamente, con el fin de inducir la ruptura centromérica y la fusión de los cromosomas. Se tuvo éxito en la obtención de derivados avanzados del cruzamiento inicial; asímismo, se pusieron en marcha modificaciones necesarias de estos esquemas principales. La vigilancia de los cromosomas extraños en las progenies se llevó a cabo mediante la aplicación de marcadores citológicos, morfológicos y bioquímicos, que han participado en la producción de varias acumulaciones de cromosomas extraños de *Haynaldia villosa*, *Agropyron junceum* y *Elymus giganteus* en una base de *T. aestivum*.

# Towards the introgression of rye genes into wheat

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Rye possesses genes for a number of desirable traits which could be utilized for the improvement of wheat for yield, quality, and adaptability. However, the direct incorporation of the rye genes into wheat through wheat x rye hybridization is problematic because of the lack of pairing of the chromosomes of the two crop plants, although their genomes are homoeologous. Chromosomal manipulations, such as suppression of homoeologous pairing (*Ph*) activity, could be exploited for transferring rye genes to wheat. Triticale x wheat hybridization has proven to be a potential method for incorporating genes or small segments of rye chromatin into wheat. Through this approach, rye genes/small chromatin segments for traits such as resistance to Karnal bunt, stripe rust, and leaf rust; tolerance to drought; long grains; and intense waxy bloom have been introgressed into bread wheat. Some of the wheat lines with rye traits also showed high yield potential.

Rye (*Secale cereale* L.) is a rich source of genes for improving wheat (*Triticum aestivum*) grain yield, quality, and adaptability. These traits include resistance to wheat rusts—particularly stripe rust (*Puccinia striiformis*), Karnal bunt (*Tilletia indica*), powdery mildew (*Erysiphe graminis*), and barley yellow dwarf virus (BYDV); tolerance to cold, drought (mainly due to a profuse and deeper root system), and soil acidity (aluminum tolerance); low input and cultural requirements; long spike with a larger number of spikelets; high lysine and protein content in grains; and desirable novel traits resulting from intergenomic interactions. Other morphological and physiological characters of rye (e.g., hairy peduncle, intense waxy bloom on leaves and other plant parts, anthocyanin in coleoptile and shoot-base, pubescent glume, long floret and anther, lax ear and prominent apical hairy tuft on grains) have also been found to express in the wheat background. The direct or indirect contribution of these traits to yield and other economic traits needs to be determined.

For the traits showing homologous genetic variation, rye chromatin can bring about diversification and broadening of the genetic base of wheat and may give trans-

gressive segregation for biomass, grain yield, and grain quality. Some rye traits—such as its cross-pollinated nature, no terminal spikelet on the spike, usually two-flowered spikelet, absent or inconspicuous auricles, and tapered and laterally compressed grains—do not express in the wheat background and, therefore, can not be exploited in wheat improvement. At the same time, rye has some undesirable traits—such as inferior bread making quality, poor feed quality, sensitivity to high temperatures, susceptibility to ergot (*Claviceps purpurea*), soft red grain, low yield potential, poor harvest index, and poor response to inputs—that must be avoided when working for wheat improvement.

The seven chromosomes of rye are known to be homoeologous to the corresponding chromosomes of the three wheat genomes. However, the rye chromosomes generally do not pair with their wheat homoeologues in wheat x rye hybrids, even in the absence of 5B activity (Riley and Kimber 1966), for which the excessive heterochromatization of telomeres of rye in the course of evolution might be responsible (Roupakias *et al.* 1983). Even if wheat or rye allosyndetic pairs are rarely formed, their crossover frequency is very low, restricted, and localized. Therefore, the chances of gene exchange in the wheat-rye hybrids, as such, are very meager and it was not possible to resort to conventional breeding procedures for transferring desirable traits from rye to wheat (Joshi 1985). The wheat-rye amphiploids (i.e., triticale, *X Triticosecale* Wittmack) show some unfavorable effects, including meiotic instability, partial sterility, grain shrivelling, etc. The chromosome addition lines, although useful in understanding the contribution of individual rye chromosomes, are of no commercial value because of their instability, which causes disturbances in the genetic balance. The following cytogenetic manipulations appear to be practicable for transferring rye chromatin to wheat.

### Chromosome substitutions by hybridizing monosomics with chromosome addition lines or through other techniques

The chromosome substitution lines, in general, are of little practical value. This is because several undesirable genes in the substituting chromosome are brought in along with the few desirable ones. In addition, several desirable genes in the substituted chromosome are lost. Even so, some spontaneous 1R(1B) substitutions, which are disease resistant and high yielding, have done very well in Europe (Zeller 1973).

### Induced translocations by irradiating chromosome substitution or addition lines

Driscoll and Jensen (1965) transferred rye chromatin as a translocation, carrying genes for resistance to leaf rust and powdery mildew following irradiation of the seed of an addition line. However, this technique is very laborious and it cannot be assured that each transfer will be made to the most favorable wheat chromosome.

### Induced homoeologous recombination using *phlb* or 'nulli-5B tetra- 5D' or 'mono-5B'stock

This is a very desirable method of transferring genes from rye to wheat. Singh and Joshi (1983) transferred rust resistance in conjunction with exceptionally large seed after

crossing monosomic 5B of 'Chinese Spring' with a rust-resistant rye and backcrossing the 27-chromosome hybrid (lacking 5B) with an improved wheat cultivar 'Sonalika'. The transfer occurred presumably as a homoeologous recombination between the chromosomes of wheat and rye. However, this technique has the limitation of crossing rye directly with only those wheat genotypes devoid of the crossability inhibitors (*Kr1*, *Kr2*, and *Kr3*) and is monosomic for 5B as well. Moreover, the wheat homoeologues of the three genomes might still pair among themselves rather than with the rye chromosomes even in the absence of 5B.

**Triticale (AABBRR) x wheat (AABBDD) hybridization to obtain chromosome substitutions, whole-arm as well as small translocations, and genic transfers**

Without much difficulty, useful genetic materials of rye can be incorporated into wheat via triticale through D- and R-genomic chromosome substitutions (Merker 1975, May and Appels 1980, Sethi and Plaha 1988) and whole-arm translocations resulting from centric breakage and fusion (Driscoll and Sears 1965, May and Appels 1978, 1980, Lukaszewski and Gustafson 1983, Sethi and Plaha 1988) as well as small translocations and possibly genic transfers (Lukaszewski and Gustafson 1983, Sethi and Plaha 1988). Unlike in the wheat x rye hybridization, triticale can be crossed with any wheat cultivar irrespective of the presence or absence of the crossability inhibitors (Sharma and Sethi 1983, Wanjari 1986). Moreover, the rye chromosomes will get the maximum opportunity to pair with the wheat D-genome chromosomes in the univalent state because the latter do not have the homoeologues as univalents from the other wheat genomes (exceptions ignored) for preferential pairing. This technique provides an additional advantage in a wheat improvement program in that it allows recombination between chromosomes of the A and B genomes from the tetraploid wheat involved in the synthesis of the triticale and those from the hexaploid wheat, which have undergone considerable differentiation in the course of evolution (Kerber 1964).

In view of the potential of triticale x wheat hybridization for introgressing rye genes into wheat, a program following this approach was undertaken at Palampur, India with encouraging results.

## MATERIALS AND METHODS

Ten diverse and agronomically promising triticales (TL68, TL161, UPT72142, UPT7440, UPT74303, UPT76001, DTS138, JNK6T-230, JNK6T-231, and Rahum) were crossed reciprocally with five improved amber-grained wheat cultivars ('Sonalika', 'Shailja', 'Girija', 'HS74', and 'VL421'). Only the $F_1$s of female triticale x male wheat crosses attained field emergence (Sharma and Sethi 1983). Some of these $F_1$s were backcrossed with their respective wheat parents and the others were selfed. Subsequently, pedigree breeding was followed, making selections for the hexaploid wheat-like phenotypes under low to moderate natural epiphytotic conditions for stripe rust and powdery mildew. A total of 100 $F_6$-derivatives (RL lines) were field-evaluated under drought-stressed conditions, and 29 of these were characterized with some traits presumably contributed by rye (Sethi 1985).

The 100 derivatives in the $F_7$ generation were screened for seedling reaction against stripe rust and leaf rust (*Puccinia recondita*) in 1985 at the AFRC, Institute of

Plant Science Research, Cambridge Laboratory, Cambridge, England, in collaboration with Dr. Roy Johnson. For the stripe rust screening, the seedlings were inoculated with races 104E137(1) and 232E137. For leaf rust, seedlings were inoculated with races WBR 82/1 and WBR 80/1. The derivatives had also been screened against Karnal bunt (Sethi *et al.* 1988).

The rye chromatin introduced into the derivatives was identified using two techniques (Sethi and Plaha 1988). In one, chromatin homology analysis was done on 23 of the derivatives from crosses with three improved bread wheat cultivars ('CPAN1922', 'HB618', and 'HD2323') that showed one or more identifiable rye traits. Meiotic analysis of the $F_1$s was also done. In the other, 12 of the derivatives were C-band analyzed using the PBI technique.

## RESULTS AND DISCUSSION

Of the 100 wheat-like derivatives from triticale x wheat hybridization evaluated for various morphological and agronomic traits and for reaction to different diseases, 70 showed the presence of some traits possibly contributed by rye. The parentage of these isolated lines is given in Table 1.

The practical utilization of the introgressed rye chromatin may be limited to only those traits that express in the wheat background to a reasonable extent. Often, a character is governed by two or more oligogenes or a number of polygenes. The introgressed rye chromatin in a wheat line may carry along only a few of the genes

**Table 1. Parentage of 70 isolated lines resulting from crossing 10 triticale lines with improved, amber-grained wheat cultivars.**

| Triticale/wheat | Line(s) |
|---|---|
| TL68/Sonalika | RL4 |
| TL68/Shailja | RL1,RL22 |
| TL68/Girija | RL16 |
| TL68/HS74 | RL5,RL8,RL10,RL25,RL77,RL78, RL79,RL100 |
| TL161/Sonalika | RL23,RL24,RL35,RL36,RL83 |
| UPT7214/Shailja | RL6,RL12,RL13,RL15,RL28,RL29,RL30 RL31,RL32,RL33,RL34,RL43,RL44, RL47,RL48,RL49,RL50,RL51,RL52, RL53,RL54,RL56,RL57,RL58,RL59, RL61,RL62 |
| UPT72142/Girija | RL7 |
| UPT 72142/HS74 | RL84,RL85,RL87,RL88,RL89,RL95, |
| UPT74303/Sonalika | RL9,RL11,RL20,RL68,RL69.RL74,RL75, RL76,RL99 |
| UPT74303/Girija | RL2,RL3,RL67 |
| UPT74303/HS74 | RL21,RL66 |
| UPT7440/Girija | RL14,RL17 |
| DTS138/VL421 | RL37,RL41,RL42 |

governing the character, which may lead to the dilution of the character expression. Moreover, the expression of the alien character may be modified due to intergenomic interaction. Therefore, character expression and the mode of inheritance of a few oligogenic rye traits that expressed in the wheat background (hairy peduncle, intense waxy bloom, red grain color, long anthers, and the presence of prominent hairy tuft on the distal end of the grains) were studied by Plaha and Sethi (1987). The inheritance of hairy peduncle was found to be monofactorial and dominant, but with variable expressivity, whereas the intense waxy bloom was conditioned by one gene exhibiting partial dominance. Red grain color was probably governed by two or three genes in different lines, having similar and cumulative effects. Two dominant complementary genes were probably responsible for the manifestation of long anthers and the presence of prominent hairy tufts on the grains.

The results of the transfer of some rye traits of agricultural value to wheat are briefly described in the following sections.

**Karnal bunt resistance**

The alien introduction of Karnal bunt (KB) resistance into bread wheat is of a special significance because of the nonavailability of confirmed sources of resistance in wheat to this a disease. KB is difficult to manage due to the perpetuation of the pathogen in the soil and is spreading to new areas through infected seed. Most of the rye genotypes are known to be resistant to KB and this resistance expresses in the wheat background (Aujla *et al.* 1985, Gill 1985). Of 98 triticale x wheat derivatives screened against KB under artificial inoculation at the boot-leaf stage in the present study, 14 exhibited no incidence of the disease, whereas seven others showed up to 5% incidence (Sethi *et al.* 1988). These lines, along with the nature of the rye chromatin introgressed into them, are listed in Table 2. Nine of these lines were rye (wheat) chromosome substitutions and one (RL25) was a rye/wheat arm translocation, whereas four lines (RL7, RL75, RL2, and RL14) showed no evidence of rye chromatin and thus, rye introgression into them was at a genic or very small chromatin level. The cytological analysis of the remaining seven lines was yet to be done at this writing.

**Table 2. Karnal bunt resistant lines from triticale x wheat with the nature of the rye chromatin introgressed into them in parentheses.**

| |
|---|
| *No incidence*: |
| RL1 (3R or 6R or 7R), RL4 (1R),RL7 (NE), RL8 (1R or 6R), |
| RL20 (-), RL21 (1R or 6R), RL23 (-), RL24 (6R), |
| RL25 (arm translocation), RL39 (-), RL75 (NE), RL82 (5R,?), |
| RL83 (7R), RL87 (-) |
| *Up to 5% incidence*: |
| RL2 (NE), RL10 (6R), RL14 (NE), RL18 (-), RL22 (6R), |
| RL38 (-), RL97 (-) |

NE = no evidence of rye chromatin; - = not studied;
? = not certain (Sethi *et al.* 1988).

The substituting rye chromosome was established to be 1R in RL4, 6R in RL10 and RL24, and 7R in RL83, indicating that chromosomes 1R, 6R, and 7R carry genes for KB resistance and any of them singly may confer resistance to the disease (Sethi *et al.* 1988). However, based on the reaction of 'Chinese Spring' wheat-'Imperial' rye addition lines, it has been shown that chromosomes 4R and 6R are associated with KB resistance. It might be that the 1R and 7R of 'Imperial' rye do not carry resistance genes or their resistance was suppressed by the homoeologous D-genome chromosomes in the addition lines, as the epistatic genes in the D-genome chromosomes suppressing the expression of genes on the chromosomes of other genomes has been demonstrated by Kerber (1983). At the same time, two substitution lines, RL16 with 6R and RL78 with 7R, were not resistant to KB in this study, indicating the existence of variability for KB resistance in rye. None of the cytologically-analyzed reconstituted lines in this study showed the substitution of chromosome 4R, lending no support to the association of this chromosome with KB resistance. However, it cannot be ensured that there was no co-transfer of unidentifiable rye segments from other chromosomes in the present substitution lines. Thus, further studies are required to arrive at a definite conclusion in this regard.

### Stripe rust resistance

The derivatives under study were screened against stripe rust race 104E137(1), which overcomes wheat resistance genes *Yr2*, *Yr3*, and *Yr4*, and race 232E137, which overcomes rye resistance gene *Yr9* and the three wheat genes just mentioned. Only one line, RL37, showed seedling resistance to 104E137(1) and susceptibility to 23E137. This indicates the presence of rye gene *Yr9* in RL37 (Table 3). As shown in Table 3, nine other lines showed resistance to both races and this may indicate that these lines

**Table 3. The reaction to stripe rust of resistant triticale x wheat lines inoculated with races 104E137(1) and 232E137.**

| Line | Field reaction |
|---|---|
| RL4 (1R)[a] | ts |
| RL5 (NE)[a] | ts |
| RL10 (6R)[a] | ts |
| RL34 (-)[a] | ts |
| RL37(arm translocation) *Yr9*[a] | F |
| RL68(NE)[a] | ts |
| RL78(7R)[a] | F |
| RL79(-) | ts |
| RL85(-) | ts |
| RL100 (1R)[a] | - |

[a]Lines also showing seedling resistance to leaf rust.
The nature of the introgressed rye chromatin is given in parentheses:
NE = no evidence of rye chromatin; - = not studied.

carry either *Yr9* in conjunction with some other resistance gene(s) or a new transferred resistance gene other than *Yr9* from rye or wheat resistance gene(s) not overcome by the two races used for seedling inoculation in the study.

In the field test under natural epiphytotic conditions, only two lines, RL37 and RL78, remained free from infection, whereas all the others (except RL100 which was not subjected to the field test) showed trace severity with susceptible field response at the adult plant stage.

RL37, found to be carrying rye resistance gene *Yr9*, is a rye/wheat whole-arm translocation. Since *Yr9* is known to be present in 1RS, it is inferred that the rye arm in this translocation is 1RS. This arm is very desirable because, in addition to *Yr9*, it carries *Lr26* for leaf rust resistance, *Sr27* for stem rust resistance, *Pm7* for powdery mildew resistance, and several other desirable genes. Rajaram *et al.* (1983) have developed high-yielding, widely adapted cultivars (Veery'S', Bobwhite'S', and Alondra'S'), which carry the 1B/1R translocation derived from three-way crosses between Mexican spring semidwarfs and the winter wheat cultivars Kavkaz and Aurora from the USSR and Weique Redmace from the USA as the sources of the translocation.

RL4 and RL100 were the chromosome substitutions for 1R; RL10 for 6R; and RL78 for 7R. RL5 and RL68 showed no evidence of identifiable rye chromatin and are, thus, the most desirable if they have derived the rust resistance from rye. The remaining three lines, RL34, RL79, and RL85, have yet to be analyzed cytologically as of this writing. Interestingly, all the stripe rust-resistant lines, except RL79 and RL85, showed seedling resistance to leaf rust as well.

**Leaf rust resistance**

Of the lines under test, only four (RL2, RL3, RL37, and RL87) showed resistance to race WBR 82/1 (which does not overcome rye *Lr26* resistance, but does for several wheat genes). These lines were susceptible to race WBR 80/1 (which overcomes the rye resistance, besides some of the wheat resistance). This indicates the presence of *Lr26* (Table 4). Since, like *Yr9*, *Lr26* is known to be located in 1RS, these results lend further support to this idea that the rye arm in RL37 (a rye/wheat whole-arm translocation) is 1RS. RL2 and RL3 (sister lines separated in the $F_5$) showed no evidence of rye chromatin, indicating that *Lr26* might have been transferred in these lines at the genic or small chromatin level. RL87 is yet to be studied cytologically.

As many as 51 other derivatives showed resistance to both the races under test. Although several of these are likely to have only wheat resistance that can be screened out after subjecting them to inoculation with the other known races, a few may carry resistance acquired from rye. Of these lines, RL5, RL7, RL13, RL14, RL30, RL42, and RL68 showed no evidence of rye chromatin and are, therefore, very desirable if they carry rye resistance. RL5 and RL68 were resistant to stripe rust as well.

**Some other desirable rye traits showing evidence of alien gene introgression**

Two lines, RL7 and RL68, with no evidence of rye chromatin, exhibited a high degree of drought tolerance. Unlike other lines, they showed distinctly superior growth under drought-stressed conditions. They were highly tolerant to frost as well. RL5, with no evidence of rye chromatin, yielded distinctly long and heavy grains with a 1000-grain

weight of about 60 g compared to 45 g for 'Sonalika', a heavy-grained commercial cultivar. It appears that this line combines the grain length of rye with the grain thickness of wheat. Singh and Joshi (1983) developed a similar rust-resistant line with very high grain weight after crossing mono-5B of 'Chinese Spring' wheat with a rust-resistant rye and then backcrossing the 27-chromosome hybrid (lacking 5B) with 'Sonalika'.

Six lines (RL7, RL16, RL21, RL42, RL68, and RL84) exhibited intense waxy bloom on leaves and other plant parts. Of these, RL16 and RL21 were substitutions for 6R and RL84 was a substitution for 1R or 6R, whereas the remaining three lines showed no evidence of rye chromatin and, therefore, introgression of the rye trait at the genic or cryptic translocation level appears to have occurred in them.

Using triticale as the bridge, this study has identified the introgression of several desirable rye traits into wheat at the genic or very small translocation level. Some of these transfers might have resulted from meiotic recombination that occurred between the wheat and rye homoeologues. As in the $F_1$ of one of the triticale-wheat hybrids, from which the lines under study were derived, Sharma *et al.* (1983) reported a heteromorphic bivalent (possibly paired D and R chromosomes). Sánchez-Monge and Sánchez-Monge (1977) and Sánchez-Monge (1980) have also advocated the occurrence of the homoeologous pairing between the D and R chromosomes. Some of the transfers might have resulted from somatic recombination as well, as Feldman *et al.* (1966) demonstrated somatic association among homologous and homoeologous chromosomes in dividing root-tip cells of wheat. Another possibility of such transfers could be transposable elements, as Flavell (1988) reported that some of the families of repeated DNA sequences studied in wheat had structures characteristic of transposable elements.

**Table 4. The source of resistance to leaf rust in triticale x wheat lines inoculated with races WBR82/1 and WBR80/1.**

*Rye resistance (Lr26):*
RL2 (NE), RL3 (NE), RL37 (arm translocation), RL87 (-)
*Resistant to both races:*
RL4 (1R), RL5 (NE), RL7 (NE), RL8 (-), RL10 (6R),
RL12 (-), RL13 (NE), RL14 (NE), RL15 (-), RL16 (6R),
RL17 (-), RL20 (-), RL21 (6R), RL24 (6R or 6D),
RL28 (-), RL29 (-), RL30 (NE), RL32 (-),
RL33 (-), RL34 (-), RL35 (1R or 6R), RL36 (-),
RL41 (-), RL42 (NE), RL43 (-), RL44 (-), RL47 (-),
RL48 (-), RL49 (-), RL50 (-), RL51 (-), RL52 (-),
RL53 (-), RL54 (-), RL56 (-), RL57 (-), RL58 (-),
RL59 (-), RL61 (-), RL62 (-), RL67 (-), RL68 (NE),
RL69 (-), RL75 (-), RL76 (-), RL77 (-), RL78 (7R),
RL84 (1R or 6R), RL89 (-), RL95 (-), RL100 (1R)

The nature of introgressed rye chromatin is given in parentheses:
NE = no evidence of rye chromatin; - = not studied.

Of the lines having introgressed desirable rye traits at the genic or cryptic translocation level (except RL37, a 1RS unidentified wheat chromosome arm translocation), the following were found to be high yielding:

RL2: highly resistant to KB and leaf rust, but red grained.

RL5: stripe and leaf rust resistant; very long, large, and hard amber grains.

RL7: KB resistant, frost and drought tolerant, profuse tillering and waxy, but susceptible to stripe rust, late maturing and red grained.

RL20: KB resistant, dwarf.

RL37: Stripe and leaf rust resistant; good early growth, but poor later growth; dwarf, very long lax tapering rye-like spike.

RL68: Stripe and leaf rust resistant, drought and frost tolerant.

RL75: KB resistant, dwarf.

These lines are being intercrossed for pyramiding the desirable rye genes as well as for obtaining the superior recombinants for wheat genes. Being stripe and leaf rust resistant (the foremost requirement in cultivars for hilly areas of northern India), RL5 and RL68 have been entered in the All-India Coordinated Initial Evaluation Trials for 1988-89 in the Northern Hill Zone of India.

Several of the other lines having chromosome substitutions or whole-arm translocations were crossed with the improved *T. aestivum* cultivars for shortening the rye chromatin involved in transfer and involving the lines in the second cycle of breeding. The segregating material is presently in the $F_5$ generation.

In conclusion, the present study has established hexaploid triticale x bread wheat hybridization as a simple, but effective technique for introgressing the desirable genes of rye into wheat.

## ACKNOWLEDGMENT

Thanks to Dr. R. Johnson for conducting the seedling test of the lines against stripe and leaf rusts at the AFRC, Institute of Plant Science Research, Cambridge Laboratory, Cambridge, UK.

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**RESUMEN**

El centeno posee genes responsables de diversas características positivas, que podrían utilizarse para mejorar el rendimiento, la calidad y la adaptabilidad del trigo. No obstante, la incorporación directa de los genes del centeno en el trigo mediante la hibridización de trigo x centeno resulta muy problemática a causa de la falta de apareamiento de los cromosomas de ambas especies, a pesar de que sus genomios son homoeólogos. Se podría explotar la manipulación de los cromosomas (como la supresión de la actividad del *Ph*) para transferir genes del centeno al trigo. La hibridización de triticale x trigo que se analiza en este estudio ha demostrado ser un método posible para incorporar genes o pequeños segmentos de cromatina de centeno en el trigo, además de las sustituciones de cromosomas y de las translocaciones de brazo completo. Con este método, se ha efectuado la incorporación de genes y pequeños segmentos de cromatina del centeno, responsables de características tales como resistencia al carbón parcial, roya lineal, roya de la hoja y tolerancia a la sequía, granos largos, intensa floración cerosa, etc., en el trigo harinero. Algunas de las líneas de trigo que poseen características del centeno demostraron también una elevada capacidad de rendimiento.

# The use of chromosome banding and *in situ* hybridization for the study of alien introgression in plant breeding

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In wide crosses produced by sexual and somatic cell hybridization, there is an urgent need to monitor the amount and location of alien chromatin at the chromosome level. In wide hybridizations, the plant will tolerate only a small amount of alien chromatin without adverse effects on agronomic performance. The monitoring of the amount and location of alien chromatin can now be done with a degree of sensitivity and efficiency that was difficult to imagine just a few years ago. These new techniques are indispensable in genetic engineering for crop improvement. This paper describes work at the Kansas State University laboratory on the use of chromosome banding, *in situ* hybridization, and molecular markers to monitor alien introgression in plant breeding.

Over the past 10 years, we have been working with hybrids of bread wheat (*Triticum aestivum*) with the perennial grasses *Elymus trachycaulus* (syn. *Agropyron trachycaulum* 2n=28, genomes $S^tS^tH^tH^t$) and *Agropyron ciliare* 2n=28, genomes $S^cS^cY^cY^c$). Both grasses have resistance to several diseases and pests that attack wheat (Sharma *et al.* 1984). In addition to the transfer of resistance genes, we have been interested in 1) gaining insights into the polyploid nature of genome evolution in the genus Elymus, and 2) understanding the cytogenetic relationships of the S, Y, and H genomes of Elymus with the A, B, and D genomes of wheat.

Our research also has implications on strategies to be employed for the management and utilization of homoeologous gene pools of wheat. The homoeologous gene pool encompasses those genes that ordinarily do not pair and recombine with A, B, and D genome chromosomes in the presence of the pairing regulator *Ph* gene in wheat.

Hence, genetic material from alien taxa cannot be transferred by chromosome pairing and crossing-over in backcross generations. Additionally, genetic control and expression of the trait in a wheat background must be worked out before a successful transfer can be made. These and other topics pertaining to our research on wide crosses in wheat are discussed.

## APPROACHES FOR UTILIZATION OF THE HOMOEOLOGOUS GENE POOL

Three approaches for handling hybrids have been suggested. The classical approach involves the production of an $F_1$ hybrid followed by the isolation of a complete set of individual chomosomal disomic addition lines. Lukaszewski (1988) has elegantly demonstrated the feasibility and relative efficiencies of different schemes for the isolation of addition lines. As discussed later, the classical approach remains the most desirable method for the management and utilization of the homoeologous gene pool of wheat.

More recently, with the increased use of embryo culture techniques, a wide array of intergeneric hybrids have been produced (Mujeeb-Kazi *et al.* 1989). It has been suggested that $F_1$ hybrids can be successively crossed with wheat to obtain monosomic chromosome additions that can be selfed to produce disomic chromosome additions. This method is called the wheat-alien direct hybridization backcrossing (DHB) approach.

A variation of this technique has been to use wheat with the *ph* gene as a parent to produce intergeneric hybrids to promote homoeologous pairing. Thus, successive backcrosses can be made to recover desirable wheat-alien chromosome translocations. This scheme is called the *ph* mutant wheat-alien DHB approach.

Our experience indicates that most alien chromosomes are rapidly lost in a wheat-alien DHB approach. Certain chromosomes, however, are preferentially transmitted and a vast majority of the monosomic addition lines contain these chromosomes (Sharma and Gill 1984). Moreover, the alien chromosomes undergo frequent translocations among themselves and as a result intact individual chromosome addition lines are hard to recover (Morris 1988).

The most serious drawback to the *ph* mutant wheat-alien DHB approach is the severe sterility of the $F_1$ hybrid. Thus, it may be impossible to obtain $BC_1$ seed (Sharma and Gill 1986). Moreover, most translocation chromosomes will be lost by the time fertility has been restored in the backcross derivatives.

Therefore, in a wide crosses program, the production of an amphidiploid should receive a very high consideration. Not only is a true breeding amphidiploid a first step for the isolation of a complete set of disomic addition lines, but it is also useful for evaluation for a variety of qualitative and quantitative traits. Since the alien chromosome set is essentially fixed in the amphidiploid, it represents a valuable resource for long-term observations and exploitation.

It should be stressed that there are perhaps several dozen distinct genomes in the homoeologous gene pool (both in the annual and perennial Triticeae). One major objective of germplasm enhancement workers worldwide should be the isolation of a complete set of addition lines (and substitution or translocation lines) in wheat of each

one of the genomes of the Triticeae. These sets of addition lines can be used for the dual purpose of basic cytogenetic information and applied breeding. Moreover, each taxa has a rich gene pool and the addition lines can be repeatedly used as 'probes' to extract additional genes from the new accessions of the original taxa. Obviously such an approach will require coordination and cooperation among the wheat cytogeneticists.

## CHROMOSOME BANDING ANALYSIS OF ALIEN CHROMATIN IN ADDITION AND TRANSLOCATION LINES

Once a basic breeding approach for the isolation of alien chromosome addition lines has been established, the foremost task is the isolation of a complete set of disomic alien addition chromosomes. In this respect, chromosome banding techniques (C-banding and N-banding) provide important tools for the identification of the alien chromosomes in single, double, or triple monosomic additions. Using these methods, it is possible to select a minimum number of plants among the backcross derivatives that must be analyzed for isolating a complete set of alien addition lines. Furthermore, these methods are invaluable for monitoring the structural integrity of individual alien chromosomes by comparing their structure with the chromosomes in the original parent (Morris *et al.*, submitted).

Another application of banding analysis is genome allocation of individual addition line chromosomes derived from polyploid species containing two or more genomes. Thus, Morris and Gill (1987) were able to determine the genomic affinities of individual chromosomes in polyploid *Elymus trachycaulus* (2n=28, $S^tH^t$ genomes) based on chromosome banding patterns of S-genome and H-genome diploid progenitor species. Based on these results, genome allocation of individual *Elymus* chromosomes added to wheat was determined and further verified by analysis of genome-specific repeated DNA sequences (Gill *et al.* 1988).

Finally, and perhaps of greater plant breeding significance, banding techniques are useful for the detection of spontaneous wheat-alien chromosome translocations during analysis of backcross derivatives. Apparently, such translocations are quite frequent and, because they have undergone gametic selection, may involve homoeologous arms. Thus, Morris (1988) recovered spontaneous translocations involving transfer to wheat of *Elymus* chromatin conditioning resistance to leaf rust (*Puccinia recondita* f.sp. *tritici*), barley yellow dwarf virus, and wheat streak mosaic virus.

## *IN SITU* HYBRIDIZATION OF ALIEN CHROMATIN IN WHEAT

There are many instances where bands do not mark the entire arm of a chromosome. This shortcoming may lead to difficulties in the identification of the alien chromosome, especially if only a part of the alien chromosome has been translocated to a chromosome of wheat. For such cases, *in situ* hybridization offers a powerful technique for the detection of the amount, breakage-point, and location of alien chromatin on a wheat chromosome.

The most useful DNA probes are those that give a contrasting type of hybridization patterns with chromosomes of the two parents. Lapitan *et al.* (1986) used a rye repeated DNA probe (pSc119) that gave a dispersed type pattern and labeled the rye chromosomes that could be individually identified (Rayburn and Gill 1985). When a 1B.1R

translocation was analyzed with this probe, it indicated that the entire short arm of 1R (1RS) with the breakage-point in the centromeric region was translocated to the 1BL arm of wheat (Lapitan *et al.* 1986, Gill and Sears 1988).

Other kinds of repeated DNA probes that are genome specific, and virtually found to be absent in the crop parent, are also useful for monitoring of alien chromatin transfers, both by *in situ* hybridization and by Southern blot analysis (Gill *et al.* 1988, Fig. 1). More importantly, these types of repeated DNA sequences can be used to monitor agronomically useful alien chromatin transfers in a large number of breeding populations as suggested by Flavell (1982).

## MOLECULAR MARKERS FOR ANALYSIS OF GENE SYNTENY RELATIONSHIPS

Apart from the cytological monitoring of alien chromatin, another important step in the utilization of alien chromatin is the determination of its genetic relationships with wheat chromosomes. Because gene synteny relationships have been conserved among

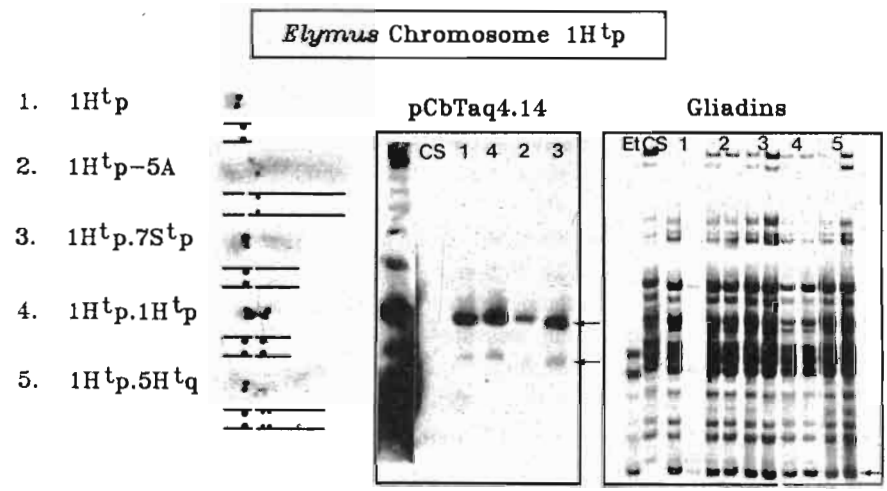


**Figure 1. Cytological and molecular analysis of *Elymus trachycaulus* chromosome 1H$^t$p added to wheat. Left panel: N-banded chromosomes of 1H$^t$p (1), translocation chromosome 1H$^t$p-5A (2), 1H$^t$p.7s$^t$p (3), isochromosome 1H$^t$p.1H$^t$p (4), and translocation chromosome 1H$^t$p.5h$^t$q (5). In the 1H$^t$p-5A translocation, the breakage point in the 1H$^t$p arm is distal to the heterochromatic band and was identified by the gliadin marker (right panel). Middle panel: Southern blot analysis using *E. trachycaulus*-specific probe pCbTaq4.14. The $^{32}$p labeled probe was used to assay TaqI digested genomic DNAs of *E. trachycaulus* (Et), Chinese Spring (CS), and other translocation chromosomes shown in the left panel. It can be seen that repeated DNA that marks the Et genome is absent in CS. Et-specific bands (arrows) can be seen in the addition line chromosomes. Right panel: The Et-specific gliadin protein band absent in CS can be seen in all the addition line chromosomes (arrow). Gliadin is a group 1 short arm marker; hence 1H$^t$p arm must be homoeologous to the group 1 short arm of wheat.**

the various genera of the Triticeae, protein markers such as isozymes, storage proteins (Fig. 1), and unique DNA sequences can be used to determine the homoeologous relationship of unknown alien chromosomes with chromosomes of wheat. Figure 2 shows the different protein markers that can also be used for the analysis of gene synteny relationships of Elymus chromosomes (Raupp et al. 1987).

Recently, Sharp *et al.* (1988) have developed a set of cDNA markers that can also be used for the analysis of gene synteny relationships in the Triticeae.

## CONCLUSIONS

The management and utilization of the homoeologous gene pool presents both a challenge and an opportunity for wheat improvement. The initial objective should be the transfer and stabilization of genomes added to wheat in the form of wheat-alien taxa amphidiploids. The amphidiploids or partial amphidiploids (Sharma *et al.* 1987) will be most suitable for analysis of the expression of various resistance and agronomic and physiological traits in a wheat background. A combination of classical and molecular cytogenetic approaches should then be used for isolating a complete set of individual chromosome addition and substitution lines from each of the distinctive genomes of the Triticeae. Chromosome banding and *in situ* hybridization are most suitable for recognition of alien-wheat chromosome translocations. Molecular markers should be

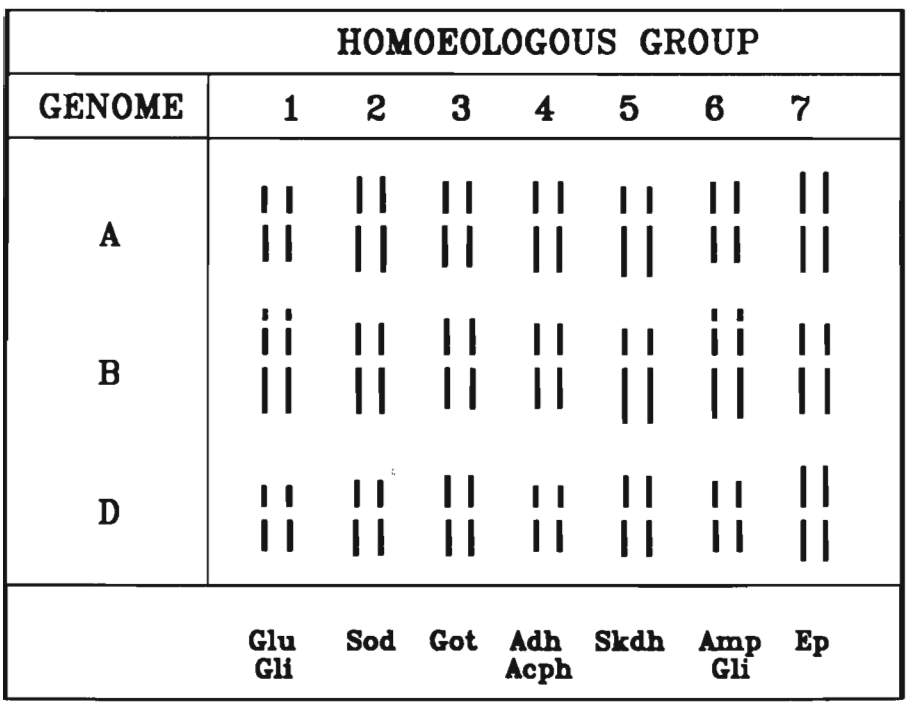


**Figure 2. Different isozyme and protein markers used in our laboratory to mark the seven homoeologous group chromosomes of wheat. For further information on these markers, see Hart (1987).**

used for determining genetic relationships of transferred alien chromatin with the genome of wheat. Once agronomically useful alien chromatin transfers have been identified, available methods akin to dot blots can be used for assaying the alien segments in breeding populations on a large scale. These and other aspects of germplasm enhancement technology can be adapted to a variety of laboratory situations and should be widely used in the management and utilization of genetic resources in wheat improvement programs throughout the world.

## ACKNOWLEDGMENTS

The author thanks W.J. Raupp for expert technical assistance. Research supported in part by USDA-CRGO grant 85-CRCR-1-1557. Contribution no. 89-496-A from the Wheat Genetics Resource Center and Kansas Agricultural Experiment Station, Kansas State University, Manhattan, Kansas.

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**RESUMEN**

A nivel de cromosomas, existe una necesidad urgente de vigilar la cantidad y localización de la cromatina extraña en las cruzas amplias efectuadas mediante la hibridización sexual y de células somáticas. En las hibridizaciones amplias, la planta tolera sólo una pequeña cantidad de cromatina extraña; con cantidades mayores se producen efectos adversos en el comportamiento agronómico. La regulación de la cantidad de dicha cromatina y su localización ahora se puede llevar a cabo con un grado de sensibilidad y eficiencia que era difícil de imaginar hace pocos años. Las nuevas técnicas son indispensables para la ingeniería genética que se usa en la genotecnia. Este estudio describe los trabajos realizados en el laboratorio de la Universidad Estatal de Kansas sobre el uso del bandeado de cromosomas, la hibridización *in situ* y los marcadores moleculares para vigilar la introducción de genes extraños como parte del fitomejoramiento.

# Evaluation and utilization of wild germplasm of wheat

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The germplasm of wild wheats and related genera continues to be an important source of useful variability for improvement of cultivated wheat. At the Punjab Agricultural University, the wheat germplasm collection includes nearly 11,000 lines of cultivated bread wheat, durum wheat, and triticale as well as wild wheats (*Triticum boeoticum*, *T. dicoccoides*, etc.), related genera (species of *Aegilops*, *Agropyron*, *Secale*, etc.), and alien addition and substitution lines collected from various international sources. The germplasm has been screened for resistance to the rusts, powdery mildew, Karnal bunt, and leaf spot diseases of wheat over different years and locations under field and artificial inoculation conditions. The useful variability for resistance to various diseases and abiotic stresses has been identified in a number of species. The resistance of *T. monococcum* to isoproturon herbicide, conditioned by a single dominant gene, has been transferred to the susceptible *T. durum*. The rust resistance of *T. araraticum* has been transferred into *T. aestivum*. The resistance of *T. monococcum*, *T. boeoticum*, and *Aegilops squarrosa* to *Tilletia indica* (Karnal bunt) was maintained in their amphiploids with *T. durum* cultivars. This paper deals with the evaluation of a comprehensive collection of wild germplasm of wheat and related species for identifying sources of resistance to various diseases and abiotic stresses and their utilization in wheat improvement programs.

With the development and adoption of high yielding wheat varieties with a narrow genetic base over large growing areas, wheat crops have become more vulnerable to biotic and abiotic stresses and the tremendous variability existing in the indigenous landraces and wild germplasm has eroded and faces extinction. The eroding genetic base of the cultivated wheats has led workers to investigate the possibility of utilizing

the genetic variation present in wild relatives (Feldman and Sears 1981). In wheat, diseases such as the rusts, Karnal bunt, powdery mildew, and leaf spot/blight cause frequent crop losses and yield instability. To keep pace with global wheat demand, we have to break the existing yield plateau, stabilize production at each level of the production ladder, extend wheat cultivation to problem and marginal areas, and breed wheat varieties with specific industrial and nutritional requirements. This can be accomplished by exploiting the vast genetic variability scattered in the germplasm of wild wheats and alien species of *Aegilops*, *Agropyron*, and other related genera that have been reported to be excellent sources of resistance to various diseases (Dhaliwal and Gill 1982, Gill *et al.* 1983, Tomerlin *et al.* 1984, Krupinsky and Berdahl 1984, Moseman *et al.* 1985, Gill *et al.* 1985; and Warham *et al.* 1986).

## COLLECTION AND MAINTENANCE

At the Punjab Agricultural University (PAU), Ludhiana, we have collected nearly 11,000 accessions (Table 1) comprising *Triticum aestivum*, *T. durum*, *X Triticosecale* (triticale), wild wheats (*T. boeoticum*, *T. dicoccoides*, etc.), and alien species of related genera (*Aegilops*, *Agropyron*, *Elymus*, *Secale*, *Hordeum*, and *Haynaldia*) from various national and international organizations of USSR, Greece, Japan, USA, Israel, Hungary, Mexico, etc. In addition, we have cytogenetic stocks, such as aneuploid series in

**Table 1. Wheat germplasm collection at Punjab Agricultural University, Ludhiana, India, through July 1987.**

| Species/genera | No. of accessions |
|---|---|
| *Triticum aestivum* | 5896 |
| *T. durum* | 1518 |
| *T. urartu* | 772 |
| *Aegilops* spp. | 602 |
| *X Triticosecale* | 526 |
| *T. dicoccoides* | 460 |
| *T. boeoticum* | 438 |
| *T. araraticum* | 116 |
| *Secale cereale* | 67 |
| *Agropyron* spp. | 49 |
| *Hordeum vulgare* | 31 |
| *T. turgidum* | 26 |
| *T. dicoccum* | 22 |
| *T. monococcum* | 16 |
| *Hordeum bulbosum* | 10 |
| *T. spelta* | 4 |
| *Elymus* spp. | 3 |
| *Hordeum sponataneum* | 2 |
| *Haynaldia* spp. | 1 |
| Total No. of accessions | 10559 |

aestivum and durum backgrounds, amphiploid derivatives, and alien addition and substitution lines received from various renown scientists. The germplasm collection is maintained and evaluated at PAU, Ludhiana/Gurdaspur and at Keylong in the Himalayan Valley of Lahaul and Spiti (Himachal Pradesh, India). Earlier studies (Dhaliwal *et al.* 1985) had shown Keylong to be ideally suited for long-term storage of germplasm on account of low temperatures, very low humidity in summer, and negligible incidence of stored-grain pests. To utilize this natural facility, all of the germplasm is stored at Keylong. Germplasm not being actively screened and under long-term storage is periodically rejuvenated.

## SCREENING OF GERMPLASM

### Rusts and powdery mildew

A set of 736 wild wheats and 311 *Aegilops* spp. were screened for resistance against stripe (yellow) rust (*Puccinia striiformis*) and leaf (brown) rust (*P. recondita* f.sp. *tritici*), powdery mildew (*Erysiphe graminis* f.sp. *tritici*), Karnal bunt (*Tilletia indica*), and leaf spot diseases (Dhaliwal *et al.* 1986). Inoculum containing mixtures of various races of stripe rust (14, 19, 20, 20A, 38 and K) and leaf rust (12A, 12B, 77, 77A, 104 and 104B) was used for creating artificial rust epiphytotics. The same set of germplasm was screened at Keylong under natural epiphytotic conditions for stripe rust and powdery mildew. With the exception of some lines of *T. dicoccoides*, all the wild wheats and *Aegilops* spp. were resistant to leaf rust. The incidence of powdery mildew and stripe rust showed that a majority of accessions of *T. araraticum* and *T. boeoticum* were resistant to both diseases. However, in *T. dicoccoides* and *T. urartu*, susceptible lines outnumbered resistant lines. A good number of accessions of *Ae. squarrosa*, *Ae. triuncialis*, *Ae. triaristata*, *Ae. ovata*, *Ae. lorentii*, and *Ae. speltoides* exhibited resistance to both stripe rust and powdery mildew (Singh *et al.* 1987, 1988).

### Screening against specific races

Eighty-four accessions of three wild wheats and nine *Aegilops* spp., which had shown resistance to stripe rust under artificial inoculation in the field for three crop seasons at Gurdaspur, were evaluated further against I (38S 102) and K (47S 102) races of stripe rust and 77 group, 104 group, 12-1 (5R37), and 12-2 (IR5) of leaf rust at IARI, Regional Research Station, Flowerdale, Shimla. None of the wild wheats tested were resistant to either K or both I and K races of stripe rust. Six of ten accessions of *Ae. speltoides* were completely resistant to all four races of leaf rust, indicating that this species is an important source of resistance against the most virulent races of stripe and leaf rusts. In addition to *Ae. speltoides*, one accession each of *Ae. ovata*, *Ae. triuncialis*, and *T. araraticum* also exhibited resistance against all the four races of leaf rust.

Several accessions of *T. araraticum*, *T. boeoticum*, and *Aegilops* spp. tested against a monoconidial isolate of *E. graminis* f.sp. *tritici* had either none or only traces of powdery mildew infection (Dhaliwal *et al.* 1987). No line of *T. dicoccoides* was resistant. All plants of *Agropyron*, *Aegilops*, *Elymus*, and *Hordeum* spp. were free from powdery mildew (Table 2).

### Karnal bunt

A limited number of lines of various species were screened for resistance to Karnal bunt under artificial inoculation conditions following the boot inoculations technique developed by Aujla *et al.* (1982). All 20 accessions of *T. urartu* screened were resistant to Karnal bunt, while other wild wheats had both resistant as well as susceptible lines. A majority of *Aegilops* spp. screened were resistant to Karnal bunt. A large proportion of *Ae. squarrosa* accessions (22 of 31) were free from Karnal bunt. Of 20 accessions of *Secale cereale* screened, only three accessions were susceptible to Karnal bunt. These *Secale* lines were also resistant to other diseases. The wheat-rye and wheat-barley addition lines used to localize the genes for resistance to Karnal bunt indicated that the chromosomes 4R and 6R of 'Imperial' rye and 1H and 4H of 'Betzes' barley carry the genes conferring resistance to Karnal bunt (Gill and Aujla 1986, Dhaliwal *et al.* 1987). The backcross derivatives of *T. aestivum* and *Agropyron* spp., substitution lines of *Elymus giganteus*, primary hexaploid triticales, and several complete/partial

**Table 2. Response of *Triticum* and *Aegilops* species to *Erysiphe graminis* f. sp. *tritici* when potted plants were inoculated by the "touch" method in the laboratory.**

| Species | No. of lines | | |
|---|---|---|---|
| | Tested | Infection | |
| | | None | Traces[a] |
| *Triticum araraticum* | 54 | 19 | 8 |
| *T. boeoticum* | 102 | 9 | 4 |
| *T. dicoccoides* | 132 | 0 | 0 |
| *Aegilops bicornis* | 7 | 2 | 0 |
| *Ae. caudata* | 2 | 0 | 0 |
| *Ae. comosa* | 3 | 0 | 0 |
| *Ae. columnaris* | 4 | 2 | 0 |
| *Ae. crassa* | 8 | 1 | 0 |
| *Ae. cylindrica* | 29 | 1 | 1 |
| *Ae. juvenalis* | 2 | 0 | 0 |
| *Ae. kotschyi* | 2 | 0 | 1 |
| *Ae. longissima* | 7 | 1 | 1 |
| *Ae. lorentii* | 17 | 3 | 1 |
| *Ae. ovata* | 14 | 2 | 1 |
| *Ae. peregrina* | 7 | 0 | 0 |
| *Ae. searsii* | 1 | 0 | 0 |
| *Ae. speltoides* | 43 | 7 | 3 |
| *Ae. squarrosa* | 43 | 11 | 1 |
| *Ae. sharonensis* | 5 | 0 | 0 |
| *Ae. triaristata* | 22 | 8 | 1 |
| *Ae. triuncialis* | 42 | 10 | 7 |
| *Ae. umbellulata* | 4 | 1 | 0 |
| *Ae. ventricosa* | 13 | 0 | 0 |

[a] Few restricted colonies per plant.

synthetic amphiploids along with their parents were also screened for resistance to Karnal bunt. The results revealed that only two plant progeny of backcross derivatives, one substitution line (2n=42), six complete/partial synthetic amphiploids and 15 primary hexaploid triticales were free from Karnal bunt.

**Leaf spot**

There is very little resistance to leaf spot disease caused by *Helminthosporium sativum* in the germplasm of wild wheats and *Aegilops* spp. None of the *T. urartu* accessions was resistant to leaf spot disease. The other wild wheats had only a few resistant lines. *Ae. squarrosa* and other D genome species and some of the S genome *Aegilops* spp. except *Ae. speltoides* possessed resistance to leaf spot disease (Dhaliwal *et al.* 1986).

**Screening for tolerance to saline-sodic soil**

In all, 172 accessions—66 of *T. aestivum* obtained from Brazil; 5 each of four wild wheats; 39 different species of *Aegilops*; 19 of *Secale cereale*; 18 released varieties (PAU) of bread wheat, durum wheat, and triticale; and 10 accessions of *Agropyron caninum*—were evaluated in a patch of saline sodic soil. The soil was a sandy loam with a pH of 10.0-10.3 and electrical conductivity of 1.5-1.8 mmhos. Only 4 of 66 accessions of *T. aestivum* from Brazil were tolerant to saline sodic soil. None of the accessions of *Aegilops* spp., *Secale cereale*, *Agropyron caninum*, wild wheats, or released PAU varieties were resistant. However, 27 accessions of *T. aestivum* (Brazil), 1 of *T. araraticum*, 5 of *Secale cereale*, 1 each of *Ae. squarrosa*, *Ae. triaristata*, *Ae. columnaris*, *Ae. cylindrica*, and 10 released varieties of PAU showed moderate resistance.

**Germplasm utilization**

*T. dicoccoides* is the wild progenitor of *T. durum*, one of the parents of *T. aestivum* and easily crossable with both the cultivated species. The resistant lines of *T. dicoccoides* should, therefore, be used as new sources of rust resistance for transfer into cultivated wheats. The wild diploid wheats *T. boeticum* and *T. urartu* and the wild tetraploid wheat *T. araraticum* possessing resistance to the rusts and powdery mildew are also crossable with *T. durum* as well as with *T. aestivum*. These species constitute the most accessible gene pool next only to that of *T. dicoccoides* for transferring rust and powdery mildew resistance to cultivated wheats. Powdery mildew resistance, rare in cultivated bread and durum wheats, is common in *Aegilops* spp. with S, D, C, M, and U genomes. It should be easier to transfer resistance from S and D genome species, whereas, transfer from other genomes requires certain specialized cytogenetical techniques.

**Transfer of herbicide resistance**

Durum wheat cultivars are highly sensitive to the most commonly used herbicide, Isoproturon, whereas a *T. monococcum* line was identified to carry a high level of resistance to Isoproturon. To transfer this resistance, the *T. monococcum* line was crossed with three durum wheat cultivars. A total of 171 plants was selected on the basis of various characteristics from the $BC_1F_3$ population of the *T. durum*/*T. monococcum*//*T. durum* crosses and screened for herbicide resistance along with durum and

monococcum parents. Arelon (Isoproturon) was sprayed at the rate of 3.0 kg/ha, whereas the recommended dose for bread wheat is 1.250 kg (75 WP)/ha. Six plant progenies showing uniformly resistant reactions to Isoproturon were selected and three of them were found to breed true the following year. Chromosome number, pairing, and fertility of these plant progenies indicate that the gene(s) controlling herbicide resistance in *T. monococcum* have been transferred in *T. durum* cultivars (Gill *et al.* 1986).

To determine the genetics of traits, crosses were attempted between three true breeding resistant progeny, i.e. 27-3, 41-1, and 74-2 with susceptible durum wheat parents (PBW 34, PBW 117, DWL 5023). $F_1$, $F_2$, and $F_3$ generations were raised and screened for herbicide reaction along with their parents. The $F_1$ generation of all three crosses (27-3/PBW 34, 41-1/PBW 117, and 74-2/DWL 5023) behaved like the tolerant parents, indicating that tolerance to Isoproturon was dominant. In the $F_2$ generation, a 3:1 segregation ratio of tolerant to susceptible plants was recorded (Table 3). A goodness fit of the $X^2$ values revealed that the tolerance of 27-3, 41-1, and 74-2 lines to Isoproturon appeared to be under the control of a single dominant gene. In the $F_3$ plant progenies, a segregation ratio of 2:1 for segregating and tolerant (nonsegregating) further confirmed that the resistance of *T. monococcum* to Isoproturon is conditioned by a single dominant gene.

**Table 3. Frequency distribution of durum wheat plants tolerant and susceptible to the application of Isoproturon (Arelon) in different generations of selected herbicide resistant plant/normal susceptible parent crosses.**

| Cross/ generation | No. of plants | | | No. of progenies | | | Genetic ratio (T:S) | $X^2$ value |
|---|---|---|---|---|---|---|---|---|
| | Tolerant (T) | Suscept. (S) | Total | Segregating | Non-segregating | Total | | |
| **27-3/PBW 34** | | | | | | | | |
| 27-3 | 197 | 1 | 198 | | | | | |
| PBW 34 | 0 | 37 | 37 | | | | | |
| $F_1$ | 13 | 0 | 13 | | | | | |
| $F_2$ | 184 | 52 | 236 | | | | 3T:1S | 1.106 |
| $F_3$ progenies | | | | 26 | 16 | 42 | 2Sg:1Nonsg | 0.430 |
| **41-1/PBW 117** | | | | | | | | |
| 41-1 | 143 | 2 | 145 | | | | | |
| PBW 117 | 0 | 49 | 49 | | | | | |
| $F_1$ | 45 | 0 | 45 | | | | | |
| $F_2$ | 286 | 78 | 364 | | | | 3T:1S | 2.476 |
| $F_3$ progenies | | | | 39 | 15 | 54 | 2Sg:1Nonsg | 0.750 |
| **74-2/DWL 5023** | | | | | | | | |
| 74-2 | 158 | 4 | 162 | | | | | |
| DWL 5023 | 0 | 28 | 28 | | | | | |
| $F_1$ | 22 | 1 | 23 | | | | | |
| $F_2$ | 237 | 77 | 314 | | | | 3T:1S | 0.001 |

**Transfer of rust resistance of *T. araraticum* into *T. aestivum***

Different accessions of *T. araraticum* (AAGG) resistant to stripe rust over 3 years were crossed with susceptible bread wheats as the female parents. Meiotic analysis of the pentaploid $F_1$ plants (AAGBD) showed 7-18I, 7-13II, 0-2III, and 0-2IV; this indicated homoeology of the A genomes as well as of the B and G genomes of wheat and *T. araraticum*, respectively.

The $F_1$ plants were backcrossed to their respective recurrent parents and selfed. Backcross seedlings died and the viable $F_2$ seedlings were screened against stripe rust under epiphytotic conditions. Selfed progenies of only resistant plants were carried forward during the 1987-88 crop season. Cytological studies of randomly fixed plants in $F_3$ generation showed that the chromosome number in $F_3$ plants was variable and ranged from 39 to 42. Cytologically stable plants (2n=42), having adult plant resistance under artificial conditions, have been selected for reconstituting their genetic background by backcrossing with a recipient variety. The transfer of stripe rust resistance from other *T. araraticum* lines into bread wheat varieties is in progress.

**Synthesis and utilization of amphiploids**

Karnal bunt resistant accessions of *T. monococcum* ($A^mA^m$), *T. boeoticum* ($A^bA^b$), *T. urartu* ($A^uA^u$), *Ae. squarrosa* (DD), and *Ae. kotschyi* (UUSS) were used in crossing with 20 different susceptible but otherwise well adapted and high yielding diverse durum wheats of the world. All 20 durum wheat cultivars (Guil Mexi 75, Flamingo'S'/Booby'S' (Fg'S'/BO'S'), Flamingo'S'/Dommel'S', Frigate'S' (Frig'S'), Boyeros'S', Mallard, Rokel, Cinclus'S', PMI-D-57, Nagaro, Mexicali 75, Cit C71, Albellus'S', Produra, Ovi 65, PG-GGO, PBW 117, PBW 34, DWL 5023, and DWL 5031) used in combination with seven accessions of *T. boeoticum*, five of *T. urartu*, one of *T. monococcum*, four of *Ae. squarrosa*, and two of *Ae. koschyi* showed cross compatibility. Seed set varied from cross to cross and species to species. The viable $F_1$ seedlings in all the combinations were treated with 0.25% colchicine solution (in 5% DMSO) for 3-1/2 hours and amphiploidized heads or sectors were selected. So far, nine amphiploids involving combinations of *T. durum/T. monococcum*, 12 involving *T. durum/T. boeoticum*, eight involving *T. durum/T. urartu*, four involving *T. durum/Ae. squarrosa* and two involving *T. durum/Ae. kotschyi* have been synthesized.

The synthetic amphiploids *T. monococcum*, *T. boeoticum*, and *T. urartu* showed a high degree of preferential pairing of chromosomes of the A genome of diploid and tetraploid wheats. The amphiploids were meiotically stable and fully fertile. Superiority of four *T. durum/T. monococcum* amphiploids for tiller number per plant, 1000-grain weight, protein content, and resistance to Karnal bunt (Gill *et al.* 1989) demonstrated that these could either be commercially exploited as such after overcoming certain inherent defects or used to introgress desirable genes into durum and bread wheat cultivars.

To introgress genes conferring Karnal bunt resistance into bread wheat cultivars, crosses have been attempted between a *T. durum/T. monococcum* amphiploid and eight bread wheat cultivars. The $F_1$ and $F_2$ generations of these crosses were raised without selection. The $F_3$ selfed progenies of each cross were screened for Karnal bunt resistance following artificial inoculation conditions. Segregation among the progenies of these crosses for resistance to Karnal bunt has been observed. Resistant plants

in $F_3$ progenies have been selected and backcrossed to the recipient wheat parent to reconstitute the genetic background.

The synthetic amphiploids involving Karnal bunt resistant accessions of *T. monococcum*, *T. boeoticum*, and *Ae. squarrosa* and susceptible but otherwise well adapted and high yielding durum wheat cultivars were evaluated for Karnal bunt resistance under artificial inoculation conditions. The origin of the synthetic amphiploids and their parents is given in Tables 4, 5, and 6. Table 4 shows the incidence of Karnal bunt in nine durum wheat parents, *T. monococcum*, and their amphiploids along with the susceptible control *T. aestivum* cv. WL 711 during 1986-87 at Gurdaspur and

**Table 4. Incidence of Karnal bunt on *T. durum*/*T. monococcum* synthetic amphiploids under artificial inoculation conditions over 2 years.**

| Material | Origin | KB infected/total grains (% infection) 1986-87 | 1987-88 | |
|---|---|---|---|---|
| | | | Gurdaspur | Ludhiana |
| **Parents** | | | | |
| *Triticum monococcum* | Dr. B.L. Johnson, Univ. of California, Riverside | 0/181(0.0) | 0/84(0.0) | 0/78(0.0) |
| *T. durum* cv. | | | | |
| PCD 57 | CIMMYT, Mexico | 3/247(1.2) | 0/169(0.0) | 5/93(5.4) |
| DWL 5031 | PAU, Ludhiana | 37/381(9.7) | 3/102(2.8) | 11/163(6.7) |
| WC 6001 | CIMMYT, Mexico | 4/223(1.8) | 5/231(2.1) | 2/104(1.9) |
| Boy'S' | CIMMYT, Mexico | 16/297(5.1) | 1/235(0.4) | 13/228(5.8) |
| Mexicali 75 | CIMMYT, Mexico | 14/305(4.6) | 6/268(2.2) | 13/116(11.2) |
| Produra | Univ. of California | 11/219(5.0) | 1/184(0.5) | 0/165(0.0) |
| PBW 34 | PAU, Ludhiana | 59/365(13.4) | 2/188(1.0) | 3/183(1.6) |
| PBW 117 | PAU, Ludhiana | 1/147(0.7) | 3/512(0.6) | 8/399(2.0) |
| Yavaros 79 | CIMMYT, Mexico | 23/283(8.1) | 2/212(0.9) | 0/129(0.0) |
| **Amphiploids** | | | | |
| PCD 57/*T. mono* Gill *et al.* 1986 | | 0/310(0.0) | 0/81(0.0 | 0/159(0.0) |
| DWL 5031/*T. mono* Gill *et al.* 1986 | | 4/475(0.9) | 0/229(0.0) | 0/190(0.0) |
| WC6001/*T. mono* Gill *et al.* 1986 | | 0/196(0.0) | 0/98(0.0) | 0/93(0.0) |
| Boy'S'/*T. mono* Gill *et al.* 1986 | | 0/473(0.0) | 0/255(0.0) | 0/298(0.0) |
| Mexicali/*T. mono* Gill *et al.* 1986 | | 0/276(0.0) | 0/184(0.0) | 0/394(0.0) |
| Produra/*T. mono* Gill *et al.* 1986 | | 0/341(0.0) | 0/178(0.0) | 0/339(0.0) |
| PBW 34/*T. mono* Gill *et al.* 1986 | | 0/189(0.0) | 0/128(0.0) | 2/180(1.1) |
| PBW 117/*T. mono* Gill *et al.* 1986 | | 0/849(0.0) | 0/352(0.0) | 0/182(0.0) |
| Yavaros/*T. mono* Gill *et al.* 1986 | | 0/427(0.0) | 0/373(0.0) | 0/255(0.0) |
| *T. aestivum* cv. WL711 | PAU, Ludhiana | 119/405(29.5) | – | 116/536(21.6) |

1987-88 at Ludhiana and Gurdaspur. All the durum wheat parents showed high incidence of Karnal bunt, whereas *T. monococcum* and all the synthetic amphiploids except DWL 5031/*T. monococcum* amphiploids during 1986-87 and PBW 34/*T.*

**Table 5. Incidence of Karnal bunt on *T. durum*/*T. boeoticum* synthetic amphiploids under artificial inoculation conditions over two locations during 1987-88.**

| Material | Origin | No. of infected/total grains (% infection) | |
|---|---|---|---|
| | | Gurdaspur | Ludhiana |
| **Parents** | | | |
| *Triticum boeoticum* (Acc. No. 5112) | Dr. B.L. Johnson, Univ. of California, Riverside | – | 0/74(0.0) |
| *T. boeoticum* (Acc. No. 211) | " | – | 0/58(0.0) |
| *T. boeoticum* (Acc. No. 238) | " | – | 0/92(0.0) |
| *T. durum* cv. | | | |
| DWL 5023 | PAU, Ludhiana | 1/418(0.2) | 2/623(0.3) |
| Cit C 71 | CIMMYT, Mexico | 0/438(0.0) | 2/228(0.9) |
| Fg'S'/Bo'S' | " | 32/200(16.0) | 37/349(10.6) |
| Frig'S' | " | 0/606(0.0) | 0/173(0.0) |
| Mexicali 75 | " | 6/268(2.2) | 13/116(11.2) |
| Nagaro | " | 22/276(7.9) | 15/99(15.0) |
| Produra | Univ. of California | 1/184(0.5) | 0/165(0.0) |
| PBW 117 | PAU, Ludhiana | 3/512(0.6) | 8/399(2.0) |
| **Amphiploids** | | | |
| DWL 5023/ *T. boeoticum* | Recently synthesized at PAU, RRS, Gurdaspur | 0/324(0.0) | 0/323(0.0) |
| Cit C 71/ " | " | 0/50(0.0) | 0/25(0.0) |
| Produra/ " | " | 0/95(0.0) | 0/31(0.0) |
| Fg'S'/Bo'S'//" | " | 0/54(0.0) | 0/52(0.0) |
| Frig'S'/ "(211) | " | 0/59(0.0) | 0/51(0.0) |
| PBW 117/ " | " | 0/54(0.0) | 0/28(0.0) |
| Nagaro/ " | " | 0/89(0.0) | 0/117(0.0) |
| Frig'S'/ "(238) | " | 0/124(0.0) | 0/52(0.0) |
| **Check** | | | |
| *T. aestivum* cv. WL 711 | PAU, Ludhiana | 119/405(22.5) | 116/536(21.6) |

*monococcum* during 1987-88 were free from Karnal bunt infection after 2 years and two locations. Karnal bunt incidence in the progeny of some of the amphiploids might be due to the substitution of the critical *T. monococcum* chromosome carrying the gene for Karnal bunt resistance by the corresponding chromosome of the A genome of *T. durum* following quadrivalent formation (Gill *et al.* 1989).

Table 5 shows the incidence of Karnal bunt in the *T. durum*/*T. boeoticum* ($A^bA^b$ AABB) amphiploids along with their parents. The durum wheat parents of these amphiploids were also susceptible to Karnal bunt at both locations and the percentage of infection ranged from 0.0 to 16.0—the maximum being in Fg'S'/BO'S' and minimum in Frig'S' compared with 21.6% in the check. The parental lines of *T. boeoticum* and all their synthetic amphiploids were free from Karnal bunt. Similarly, all the accessions of *Ae. squarrosa* and their synthetic amphiploids except DWL 5023/

**Table 6. Incidence of Karnal bunt on *T. durum*/*Ae. squarrosa* synthetic amphiploids under artificial inoculation conditions over 2 years.**

| Material | Origin | KB infected/total grains (% infection) 1986-87 | 1987-88 |
|---|---|---|---|
| **Parents** | | | |
| *Aegilops squarrosa* (Acc. No. 3745) | Dr. J.P. Gustafson, Univ. of Missouri, Columbia, USA | 0/28(0.0) | 0/92(0.0) |
| *Ae. squarrosa* (Acc. No. 3754) | " | 0/32(0.0) | 0/74(0.0) |
| *Ae. squarrosa* (Acc. No. 3741) | " | 0/45(0.0) | |
| *Triticum durum* cv. | | | |
| Ruff'S' | CIMMYT, Mexico | 15/427(3.5) | 0/275(0.0) |
| PBW 114 | PAU, Ludhiana | 38/355(10.7) | 0/251(0.0) |
| DWL 5023 | " | 13/354(3.7) | 1/418(0.2) |
| **Amphiloids** | | | |
| Ruff'S'/*Ae. squarrosa* (3745) | Synthesized at PAU, RRS, Gurdaspur | 0/204(0.0) | 0/267(0.0) |
| PBW 114/*Ae. squarrosa* (3754) | " | 0/358(0.0) | 0/1505(0.0) |
| DWL 5023/*Ae. squarrosa* (3754) | " | | 0/279(0.0) |
| DWL 5023/*Ae. squarrosa* (3741) | " | | 2/296(0.7) |
| **Check** | | | |
| *T. aestivum* cv. WL-711 | PAU, Ludhiana | 119/405(29.5) | 116/536(21.6) |

*Ae. squarrosa* (Acc. No. 3741) were free from Karnal bunt incidence over 2 years of testing as compared to their durum wheat parents in which the percentage of infection varied from 3.5 to 10.7% compared with 29.5% in the check (Table 6).

Eight of nine *T. durum/T. monococcum* and all the *T. durum/T. boeoticum* and *T. durum/Ae. squarrosa* amphiploids were free from Karnal bunt infection, indicating that the resistance of *T. monococcum*, *T. boeoticum*, and *Ae. squarrosa* to Karnal bunt is dominant and expressed in the presence of the durum wheat genomes. Contrary to the present results, Kerber and Dyck (1977) and Trottet *et al.* (1982) reported the inhibitions of the expression of genes for resistance to stem rust and glume blotch from *Ae. squarrosa* in the amphiploids, respectively.

The diploid species, therefore, offer a unique source of genetic resistance to Karnal bunt that can be effectively transferred to *T. aestivum*. *T. monococcum* and *T. boeoticum* are readily crossable with *T. durum*, whereas embryo culture is necessary for crossing these with bread wheat. The synthetic amphiploids can, however, be crossed with bread wheat easily and can be used for transferring Karnal bunt resistance from wild species to bread wheat varieties. The synthetic amphiploids (AABBDD) between *T. durum* and *Ae. squarrosa*, however, will be of immense importance as it is easier to utilize them for breeding or resynthesizing Karnal bunt-resistant *T. aestivum*. It will be desirable to screen more lines of *Ae. squarrosa* for resistance to Karnal bunt.

## CONCLUSIONS

- Wheat breeders are not exploiting the useful variability from the wild germplasm because of crossability barriers, sterility, lack of chromosome pairing, and transfer of associated undesirable traits.
- Prebreeding centers should be established to transfer useful variability into well adapted high yielding backgrounds for use by the breeders.
- Depending upon the available facilities and breeding objectives, priorities for the sources of required variation should be drawn. Based on the ease of transference, the following order of preference of various related sources should be adopted:
  - Winter wheat
  - Primitive and synthetic hexaploid wheat
  - Durum and other cultivated emmer tetraploids
  - *T. dicoccoides*
  - *Ae. squarrosa*
  - Cultivated and wild diploid wheats
  - *Timopheevi* wheats
  - *Aegilops* section sitopsis
  - Other *Aegilops* species
  - *Secale cereale*
  - *Agropyron*
  - *Hordeum*, *Elymus*, *Haynaldia*

  For durability of transferred variability, the order of the above list should be reversed.
- Only a limited number of accessions of wild species collected for academic studies is circulating among laboratories. An expedition should be planned for a huge collection of germplasm of the related wild species from the centers of diversity.

- Wild wheat germplasm is being maintained by selected centers, but is not being screened exhaustively at 'hot spots' and for most virulent races of various diseases.
- Genetic analysis of newly identified sources should be done at the species level before any attempt is made to obtain effective utilization.
- The aneuploid series, crossability genes, *ph*-mutant, etc., should be made available in the high yielding and well adapted cultivars which, in turn, should be used in development of new addition and substitution lines in the process of transferring useful variability.
- Possibilities of valuable sources of genetic variation for wider adaptation, nutritional attributes, physiological efficiency and yield in the wild germplasm need to be explored.

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## RESUMEN

El germoplasma de los trigos silvestres y de géneros relacionados, tales como *Aegilops*, *Agropyron* y *Secale*, sigue siendo una fuente importante de variabilidad en el mejoramiento de trigo cultivado. En la Universidad Agrícola del Punjab, la colección de germoplasma de trigo incluye más de 10,000 líneas de trigo harinero, trigo duro, triticale, trigos silvestres (*Triticum boeoticum*, *T. dicoccoides*, etc.), especies de *Aegilops*, *Agropyron* y *Secale* y líneas extrañas de acumulación y sustitución recolectadas en diversas fuentes internacionales. El germoplasma se evaluó para determinar si posee resistencia a las royas, mildiú polvoriento, carbón parcial y enfermedades del trigo que producen mancha foliar en diferentes años y localidades y en condiciones de campo y de inoculación artificial. En diversas especies se identificó variabilidad útil para la obtención de resistencia a varias enfermedades y condiciones abióticas adversas. La resistencia de *T. monococcum* al herbicida isoproturón, que está condicionada por un solo gene dominante, se transfirió a *T. durum* susceptible. La resistencia a la roya de *T. araraticum* se transfirió a *T. aestivum*. La resistencia de *T. monococcum*, *T. boeoticum* y *Aegilops squarrosa* a *Tilletia indica* (carbón parcial) se conservó en sus anfidiploides con variedades de *T. durum*.

# Collection, conservation, and potential use of the wild relatives of rice in Asia and Australia

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Rice (*Oryza sativa* L.) occurs in a subfamily of the Poaceae distinct from other major cereals. The potential usefulness to rice improvement of the genera related to *Oryza* needs to be explored. Maximum diversity of the wild relatives of rice occurs in Asia and Australia. Eleven of the 22 species in the genus *Oryza* occur in this region. Living collections of most of the species have been collected and conserved over the last 30 years. The unprecedented recent demand, primarily from biotechnologists, for germplasm of these wild species and the undiminished destruction of wild habitats have led the International Rice Research Institute (IRRI) to embark on collaborative efforts to gather samples of these wild species from diverse locations across the region. Recent collections have increased our knowledge of in situ conservation of *Oryza* spp. Forest reserves and wildlife parks have been identified that are a refuge for the wild relatives of rice. The protection of habitats, conservation of germplasm, and advances in biotechnology are complementary. Evaluation has revealed that the chance of finding resistance to insect pests of rice is about 50 times greater in wild *Oryza* spp. than the cultigen. Populations of wild *Oryza* spp. have already contributed substantially to increasing rice production in Asia. The further potential of these wild relatives of rice is discussed.

In *The Origin of Species*, Darwin (1971) states a general principle, "...The greatest amount of life can be supported by great diversification of structure." Diversity in an agroecosystem has been shown to be more productive than monocultures (Trenbath 1974).

Genetically diverse germplasm, the fuel of current advances in biotechnology and agricultural research, being collected and conserved today is a small portion of what has not already been lost through habitat destruction due to urban expansion, pollution, and man-made or natural calamities. The new Sukarno-Hatta International Airport outside Jakarta has covered populations of *Oryza rufipogon*. Kalimantan is an area rich in diversity of *Oryza* spp., among many others, but in early 1983 a forest fire raged for 3 months consuming an estimated 2 million hectares, which compounded the tremendous destruction of habitats there by forest clearing (Brown and Wolf 1986). During collection expeditions for wild *Oryza* spp., the author has found populations of less than five plants. Two populations of the species *O. granulata* have been found in Thailand and Indonesia consisting of a single plant each.

This paper discusses the genetically diverse germplasm of the wild relatives of rice in the Asian and Australian realm, its collection, its *in situ* and *ex situ* conservation, and its potential use in rice improvement.

## THE GENUS *ORYZA* AND RELATED GENERA

The genus *Oryza* is closely allied to the bamboos, both occurring in the Bambusoideae subfamily but in separate supertribes, the Oryzanae and Bambusanae (Watson *et al.* 1985). Below the rank of supertribe, *Oryza* is placed in the tribe Oryzeae.

The species from Asia and Australia in the tribe Oryzeae are shown in Table 1. A numerical taxonomic analysis of the Poaceae (Watson *et al.* 1985) shows that *Oryza* as a genus is isolated from all the major genera that contain important cereal crops. For genetic improvement of rice, particularly for useful quantitative characteristics, consideration of the evolutionary relationships between rice and other grasses can be of value to biotechnologists and plant breeders in gauging the expected success of intergeneric hybridization. In a recent review of wide crosses among cereals, Brar and Khush (1986) reported no successful crosses between genera in different supertribes or superfamilies as delimited by Watson *et al.* (1985). In the future, intergeneric hybridization with rice might be most successful if researchers concentrate on genera that are evolutionarily related to rice—the wider gene pool of rice.

The taxonomic and evolutionary relationships between genera of the tribe Oryzeae have not been closely studied. With new living collections of several species from genera related to *Oryza*, it should be possible to come up with a much clearer picture of intergeneric relationships. The germplasm collections, though primarily used by agricultural scientists, are a valuable resource for botanists and taxonomists too.

### The genus *Oryza*

Table 2 presents information on the species of *Oryza* in Asia and Australia. Of these species, *O. schlechteri* may be extinct since it has not been collected since the beginning of this century. Populations of *O. ridleyi* and *O. longiglumis*, which usually occur in forest areas, are threatened by habitat destruction. *O. longiglumis* has a restricted distribution and spikelets of both these species have a thin palea and lemma compared to other species in the genus. Across Asia, truly wild habitats are diminishing in area and thus populations of many wild *Oryza* spp. are confined. Some species that

have adapted to man-made habitats, such as *O. granulata* and *O. meyeriana*, which can be found growing in teak plantations, have a relatively stable habitat. However, populations of cosmopolitan species such as *O. nivara* and *O. rufipogon* are vulnerable to destruction as a consequence of modern irrigation schemes and land drainage projects. Weedy hybrids derived from crosses between rice and its close wild relatives are still common in some deepwater rice fields in Thailand, Bangladesh, and Indochina. However, as cultural practices improve, such populations can rapidly disappear.

**Table 1. Genera and species related to *Oryza* in the tribe Oryzeae in Asia and Australia.**

| Genus | No. of species worldwide | Species occurring in Asia and Australia and their distribution | |
|---|---|---|---|
| *Chikusichloa* | 3 | *C. aquatica*[a] | Honshu, Kyushu, Japan; China |
| | | *C. brachyanthera* | Okinawa, Japan |
| | | *C. mutica* | Nanning, Kwangsi, China |
| *Hygroryza* | 1 | *H. aristata*[a] | From Taiwan Province, China, to Pakistan |
| *Leersia* | 17 | *L. hexandra*[a] | Widely distributed in tropics and subtropics |
| | | *L. japonica* | Japan, China, Korean Peninsula |
| | | *L. oryzoides* | Widely distributed in temperate region |
| | | *L. stipitata* | Chantaburi, Thailand |
| *Porteresia* | 1 | *P. coarctata*[a] | Delta region of Indus, Ganges and Irrawady rivers |
| *Potamophila* | 1 | *P. parviflora*[a] | Australia |
| *Zizania* | 3 | *Z. latifolia* | N. China, Korea, Japan, NE India, Burma, Indochina |

[a] Samples in the International Rice Germplasm Center.

**Table 2. Distribution, chromosome number, genome group, life cycle, normal habitat, and collection priority of widely accepted species in the genus *Oryza* from Asia and Australia.**

| Species | Distribution | Chromosome number (Genome group) | Life[a] cycle | Natural habitats[b] | Urgency of collection based on utility and threat to habitats |
|---|---|---|---|---|---|
| *O. schlechteri* | Papua New Guinea | ?(?) | ? | Dry, open habitats | No living collection exists (P1)[c] |
| *O. longiglumis* | Irian Jaya, Indonesia | 48(?) | p | Grows in or near water, usually in shade or partial shade | Restricted distribution (P1) |
| *O. ridleyi* | SE Asia | 48(?) | p | Grows in or near water, usually in shade or partial shade | Wide distribution but uncommon (P1) |
| *O. granulata* | S & SE Asia | 24(?) | p | Grows in well-drained soils, in partial shade forest floor | Common in teak plantations (P3) |
| *O. meyeriana* | SE Asia | 24(??) | p | Grows in well-drained soils, in partial shade forest floor | Common in teak plantations (P3) |
| *O. officinalis* | Tropical Asia | 24(CC) | p | Grows in full sun or partial shade, wet or seasonally dry habitats | Useful source of resistance genes, more collections needed from Burma, Indochina and S. Asia (P2) |
| *O. minuta* | Philippines | 48(BBCC) | p | Usually in or beside water, in partial shade | Well represented in germplasm collections (P3) |
| *O. eichingeri* | Sri Lanka, E. Africa | 24(CC) | p | Seasonally dry habitat, in sun or partial shade of forest scrub | Good collections of this species exit (P3) |

**Table 2. (continued)**

| Species | Distribution | Chromosome number (Genome group) | Life[a] cycle | Natural habitats[b] | Urgency of collection based on utility and threat to habitats |
|---|---|---|---|---|---|
| *O. australiensis* | Australia | 24(EE) | p | Habitat dry in full sun | Habitat relatively stable (P3) |
| *O. sativa* | Worldwide | 24(AA) | a | Adapted to a wide range of hydrological conditions, full sun | Collections from remote areas essential (P2) |
| *O. nivara* | Tropical Asia | 24(AA) | a | Seasonally dry habitats, full sun | Habitats threatened across Asia (P1) |
| *O. rufipogon* | Troical Asia | 24(AA) | p | Deepwater habitats, full sun | Habitat threatened by irrigation, drainage schemes and aggressive weeds (P1) |
| *O. meridionalis* | Tropical Australia | 24(AA) | a/p | Seasonally dry habitats, usually full sun | Habitat relatively stable (P2) |

[a] Life cycle: a = tendency to have one main flush of panicles; p = tendency to have repeated flushes of panicles over several seasons.

[b] Based on field collection passport data, unpublished trip reports and Oka (1980).

[c] P1,P2, P3 = first, second, and third priority for conservation, respectively.

## COLLECTION

Collection of the wild relatives of rice began in the late 1950s. Teams from Japan visited many countries in South and Southeast Asia (Oka 1988). In India national collection efforts gathered a range of germplasm from areas of great ecogeographic diversity (Govindaswamy *et al.* 1966, Sharma *et al.* 1971). In several countries where there is a diversity of wild *Oryza* species—among them Bangladesh, large areas of Burma, and the countries of Indochina—collections were not made. Although the early collections resulted in the range of species in the genus being collected, more recent collections have focused on species with the AA genome—the close relatives of rice.

In the 1970s and 1980s, the massive collection efforts for traditional varieties of rice resulted in only the incidental collection of wild *Oryza* species. Specific collections of wild *Oryza* species have been made comprehensively in China (Kwangtung Agricultural and Forestry College 1975). In Australia, Australian and foreign workers have made several trips to collect the two wild species, *O. australiensis* and *O. meridionalis*, which occur in that country's tropical zone (Marshall and Broué 1981, Oka 1978).

The objectives of the present collaborative collection efforts for wild *Oryza* species are to:

- Visit areas where the relatives of rice have not previously been collected and areas threatened by habitat destruction. Coverage includes species from all complexes in the genus *Oryza* and species in related genera. Thus, comprehensive collections have been made in South India, with the Indian National Bureau of Plant Genetic Resources, to gather samples of rare tetraploid populations of *O. officinalis*. Collaboration with Indonesian workers in West Java has rescued populations threatened by urban expansion and resulted in discovery of a new center of diversity of *O. officinalis* along the west coast of Java. More than 20% of recent collections have been of species not having the AA genome. Specific germplasm, such as *Hygroryza aristata*, which was not previously in the world's genebanks, has been collected and is being requested for use by scientists.
- Visit nationally conserved areas to identify areas of *in situ* conservation. Finding populations of *Oryza* in protected areas allows their presence to be brought to the attention of conservation officers who can monitor these resources.
- Build up a more complete data base on the distribution, habitats, and characteristics of populations of wild *Oryza* species. Information gathered in the field can be a first step in the appropriate evaluation of species. This data base includes information available from herbaria that have sizable collections of the rice relatives.

Collections over the last year have increased the germplasm of wild species in the world collection by about 10%. Thus, the concerns about the representation of wild species in germplasm collections are now being aggressively addressed for rice (Frankel and Soulé 1981).

## CONSERVATION

Before we can know how to adequately conserve wild species, it is necessary to have an understanding of their population structure. Nothing is known about the population structure of most species in the genus *Oryza*. However, in recent years, techniques,

such as isozyme analysis as well as repeated field visits to the same populations, have helped to define more clearly the population structure of species closely related to the two rice cultigens *O. sativa* and *O. glaberrima*.

The results of population studies of wild rice in Thailand indicate that population structure is very complex. Within a single population, one can find plants with a wide range of characteristics, a consequence of genetic dynamism that reflects the micro-ecology of the habitat (Morishima *et al.* 1984, National Institute of Genetics 1987). The wild relatives of rice vary greatly in traits that affect population structure, such as breeding system (from predominantly outcrossing to inbreeding) and life cycle (annual to perennial). Genetic conservation of some wild *Oryza* species with good seed set can be handled quite well *ex situ*. Other species that produce few seeds are better conserved in their natural habitat, *in situ*.

### *In situ* conservation

Recent collectors have found *Oryza* spp. growing *in situ* in forest reserves, wildlife parks, and botanic gardens (Table 3). In forest reserves and wildlife parks, wild *Oryza* spp. are relatively safe unless the forest is cut down for timber or the population of wild animals becomes too large and destroys the habitat.

**Table 3. *In situ* conservation of wild relatives of rice in selected countries of Asia.**

| Country | Conserved area | Species |
|---|---|---|
| **India** | Bhoothathankettu Forest Reserve, Kerala | *Oryza rufipogon* |
| | Parambikulam Game Reserve, Kerala | *O. officinalis* |
| | | *O. granulata* |
| | Karulai Range Teak Plantation/ Forest Reserve, Kerala | *O. nivara* |
| | | *O. granulata* |
| | | *O. officinalis* (tetraploid form) |
| **Thailand** | Pukai Botanic Garden; Mae Sai Valley | *O. granulata* |
| | Forest Reserve and Botanic Garden near Chiang Mai | *O. ridleyi* |
| | Tong Nga Chang Park near Haadyai | *O. ridleyi* |
| **Sri Lanka** | Ritigala Strict Natural Reserve | *O. eichingeri* |
| | Wilapattu National Park | *O. eichingeri* |
| | Yala National Park | *O. nivara* |
| | | *O. eichingeri* |
| | Ruhuna Wildlife Sanctuary | *O. nivara* |
| | | *O. eichingeri* |
| | | *Hygroryza aristata* |
| | Polonnaruwa, ruins of ancient capital | *O. nivara* |
| | | *H. aristata* |
| **Indonesia** | Ujung Kulon National Park | *O. officinalis* |

On the other hand, botanic gardens usually have many visitors as well as gardeners who can trample or disturb herbaceous plants. The Pukai Botanic Garden run by the Royal Thai Forestry Department was an appropriate habitat for *O. granulata*. However, only a single plant of this species was found in the protective base of a large specimen of *Dipterocarpus alatus* Roxb. In this garden, cattle are allowed to roam to keep the grass short and gardeners clear brush and cut vegetation. In another botanic garden in the Mae Rim Valley in northern Thailand, a large population of *O. granulata* was found under 17-year old trees on a steep slope. This slope was soon to be planted with a soil binding grass to prevent soil erosion. This certainly would have destroyed the *O. granulata*, a species that does not tolerate weedy conditions.

Thus, wild *Oryza* spp. in botanic gardens are best conserved consciously, otherwise they may be unwittingly eliminated. Botanic gardens, unless well maintained, suffer considerable attrition. *O. officinalis*, which grew in the nursery of the Singapore Botanic Garden in the 1930s, is no longer there. Estimates of the loss of species diversity from botanic gardens in certain Asian countries are very high (A.J.G.H. Kostermans 1988, personal communication—Herbarium Bogoriense). Natural calamities, such as storms (e.g., the 1987 hurricane that hit the Royal Botanic Gardens, Kew, England) and droughts, can also have sudden dramatic effects.

*O. nivara* is a cosmopolitan species often found near rice fields, village ponds, and roadside ditches. Populations of this species are probably as vulnerable to invasion by other plant species as they are to disturbance of their habitats by man. *O. nivara* is not aggressive, so if aggressive weeds such as water hyacinth (*Eichhornia crassipes* (Mart.) Solms) or sedges (*Carex* spp.) invade the area, *O. nivara* can be easily eliminated. The diversity of the wild species complex of *O. nivara*, *O. spontanea*, and *O. rufipogon* in the Orissa state of India is less than it was, in part, because of the spread of water hyacinth (IBPGR-IRRI Rice Advisory Committee 1982; S.D. Sharma 1987, personal communication—Central Rice Research Institute, India). To achieve appropriate *in situ* conservation of these species, the entire ecosystem, not just one part, must be taken into account.

Farmers in some areas play a positive role in *in situ* conservation. For example, they purposely harvest wild *Oryza* spp. for grain or animal feed, such as in southern Thailand where the perennial wild rice, *O. rufipogon*, is deliberately protected to provide feed for cattle.

### *Ex situ* conservation

*Ex situ* conservation begins during field collection when the population is sampled for seeds (or vegetative parts) and information about the population is recorded. The remaining steps in *ex situ* conservation are essentially the same as for a cultigen, however, the process is more complex and time-consuming because of:

- Strong seed dormancy which requires hand dehusking
- High seed shattering which requires that panicles be placed in bags
- Low or no seed set
- Required growth of plants in pots which need to be watered continuously
- Various ecological requirements of the different species

- Expertise needed for species identification
- More complex taxonomic and morpho-agronomic characteristics.

Further discussion of this topic can be found in Chang (1976, 1985).

## POTENTIAL USE IN RICE IMPROVEMENT

Six Asian and Australian species in the tribe Oryzae are recorded as food for man and/or forage for cattle. *O. meridionalis*, *O. nivara*, *O. rufipogon*, and *Hygroryza aristata* have been or are harvested for their grain (Bancroft 1984, Oka and Morishima 1971, Vaughan and Muralidharan 1988, Bor 1960). *Leersia hexandra* (swamp rice grass), a common companion species of wild *Oryza* in South and Southeast Asia, has been introduced as a forage in some countries (Dhalgren *et al.* 1985). The swollen and fleshy culm bases of *Zizania latifolia* infected by *Ustilago esculenta* are a prized vegetable in Asia; buds and seeds of this species are also eaten (Tanaka 1976).

The genera related to—and wild species of—*Oryza* offer a number of traits of interest to rice scientists:

- The genera *Potamophila* and *Zizania* consist of species with unisexual spikelets. *Z. latifolia* has pistillate spikelets on branches at the apex of the panicle while staminate spikelets are on branches at the base of the panicle. These monoecious species may offer an approach to hybrid rice production in the tropics.
- *Z. latifolia* is reported to grow in polluted water and to be resistant to insecticides and fungicides (Hsu 1978). Similarly, *O. rufipogon* in Indonesia has been found in very polluted areas. Thus, the wild relatives of rice might furnish useful sources of tolerance to adverse conditions.
- The seed physiology of species in the genera *Porteresia* and *Zizania* is quite different from rice; both have recalcitrant seeds, whereas rice has orthodox seeds. Comparative studies of seed physiology in *Oryza* and its relatives are needed since this could extend the range of areas where rice could be productive such as permanent swamps. In addition, germinating seeds and seedlings of genera from temperate regions might have a degree of cold tolerance not found in the genus *Oryza*.
- *Hygroryza aristata* of South and Southeast Asia has inflated leaf sheaths that allow it to float. The morphology, anatomy, and potential of this little known species should be explored.
- Two species in the tribe Oryzeae, *Porteresia coarctata* and *Leersia oryzoides*, grow in brackish water. The former species from marshes around the Bay of Bengal is receiving attention as a potential source of salt tolerance for rice (IRRI 1987). Only one ecotype of *L. oryzoides* (var. *oryzoides* f. *glabra*) is reported to grow in salt water (Pyrah 1969). The salt tolerance mechanism in this ecotype should be investigated for its potential value in breeding rice for saline soils.
- Extensive evaluation of varieties of rice and wild *Oryza* spp. has shown that, in general, the chances of finding insect pest resistance in populations of wild species is about 50 times that for rice varieties (Heinrichs *et al.* 1985). Wild *Oryza* spp. are also a valuable source of resistance to viruses (Khush and Ling 1974, Aguiero *et al.* 1984). Evaluation of the wild rice gene pool at IRRI will hopefully identify genes that will cope with perennial as well as emerging pest and disease problems.

- *O. sativa* f. *spontanea* has been an important source of cytoplasmic male sterility (cms) for hybrid rice production in China (Lin and Yuan 1980). Evaluation of the wild rice gene pool for further sources of cms are underway (Khush *et al.* 1988). *O. rufipogon* outcrosses have long anthers that provide greater pollen dispersal than rice (Oka 1988). These traits could be valuable in hybrid rice production (IRRI 1987) and in improving fertility where sterility is a problem, such as areas where rice is subject to cold temperatures.
- A range of shade tolerance exists in wild *Oryza* spp. (Table 2). The lanceolate leaves of *O. granulata*, which grows in shaded habitats, are much darker green and more rigidly horizontal than species found in full sun, such as *O. australiensis*. Consideration of the plant type and physiology of the *Oryza* spp. occurring in shaded habitats might give useful insights on how to increase rice productivity under shaded or partially shaded conditions. Perenniality (ratooning ability) also varies among *Oryza* spp. (Table 2). This is a potentially valuable trait to incorporate into rice varieties where soil tillage is undesirable.
- Species of the *O. officinalis* complex exhibit different ploidy levels in the same species or closely related species. The mechanism that controls pairing in this complex should be studied since there is already evidence that a mechanism similar to that of *Triticum* exists in this complex (Nowick 1986). It has recently been discovered that two species of the *O. officinalis* complex exist in Sri Lanka—*O. eichingeri*, found in moist habitats such as the Ritigala Strict Natural Reserve, and *O. rhizomatis*, a new species found in Walapattu National Park and Rahuna Wildlife Sanctuary. *O. rhizomatis* occurs in seasonally dry habitats in the sun or partial shade and can probably be found all across Sri Lanka's dry zone. *O. eichingeri* appears to be the less common of the two species in Sri Lanka.

The lag time between collection and utilization of germplasm can be very long. For example, collections of *O. officinalis* made outside Bangkok in 1957 were evaluated (Oka 1980) and found to have multiple pest resistance (Heinrichs *et al.* 1985) and were used in wide crosses in 1984 (Jena and Khush 1986). Now after 30 years of conservation, genes from the original population are in advanced breeding lines.

The seeds and information on the world's rice germplasm at the International Rice Germplasm Center are available to all rice workers. Further gains in stable and sustainable rice production depend on the diversity of germplasm and its appropriate, careful conservation.

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**Resumen**

El arroz (*Oryza sativa* L.) pertenece a una subfamilia de las Poaceae, distintas de otros cereales importantes. Es preciso estudiar la posible utilidad de los géneros afines a *Oryza* en el mejoramiento del arroz. La mayor diversidad de parientes silvestres del arroz se encuentra en Asia y Australia, donde se dan 11 de las 22 especies del género *Oryza*. Durante los últimos 30 años se han reunido y conservado colecciones vivas de casi todas esas especies. No obstante, la demanda reciente sin precedentes, sobre todo por parte de los biotecnólogos, de germoplasma de estas especies silvestres y la destrucción constante de los hábitats silvestres han hecho que el Instituto Internacional de Investigación de Arroz (IRRI) haya iniciado la tarea de recolectar muestras de estas especies silvestres en distintas localidades de la región. Las recientes recolecciones han incrementado nuestros conocimientos sobre la conservación in situ de las especies *Oryza*. Se identificaron reservas forestales y parques nacionales que constituyen un refugio para los parientes silvestres del arroz; algunas actividades complementarias, llevadas a cabo por el Instituto, son la protección de hábitats, la conservación de germoplasma y los adelantos en el campo de la biotecnología. La evaluación demostró que la posibilidad de encontrar resistencia a las plagas de insectos que afectan al arroz es 50 veces mayor en las especies silvestres de *Oryza* que en las cultivadas. Las poblaciones de especies silvestres de *Oryza* ya han contribuido en gran manera a incrementar la producción de arroz en Asia. En este trabajo se analiza también el potencial ulterior de esos parientes silvestres del arroz.

# Artificial embryogenesis and plant regeneration in citrus

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The goal of this research was to explore ways to improve methods of citrus breeding using tissue culture. Polyembryonic seeds of two citrus species were germinated on Murashige and Skoog medium. Nucellar embryonic plantlets were isolated from zygotic embryos and cultured. It was found that plumule explants differentiated new roots while hypocotyl and radicle explants formed calluses on the same medium. The calluses regenerated plantlets. We also used bud proliferation to propagate plumules.

Polyembryonic seeds of two citrus species were germinated on medium after which nucellar embryonic plantlets were isolated from zygotic embryos and cultured. Plumule explants differentiated new roots while hypocotyl and radicle explants formed calluses on the same medium. The calluses regenerated plantlets. Bud proliferation was another method of plumule propagation. Seedling explants of monoembryonic *C. grandis* also regenerated.

## MATERIALS AND METHODS

Polyembryonic seeds of *Citrus grandis* var. *tahungpow* Hort. and *C. sinensis* (L.) Osbeck were sterilized and germinated on MS (Murashige and Skoog 1962) medium under aseptic conditions. The seedlings were separated from the seed coat with forceps. Among them, one seedling had closed heart-like embryonic cotyledons and lacked roots; the others had open heart-like embryonic cotyledons and well developed roots. Seedling heights were similar. It is believed that similar seedlings can come from the asexual embryo, but that individual dissimilar seedlings are sexual plantlets in polyembryonic seeds of citrus (Middle Agriculture School 1979). It can be speculated that the former seedling was a zygotic embryo from the seed and that the latter seedlings developed from nucellar embryonic tissue. The embryo-derived seedlings were subcultured on MS medium to allow further plantlet development.

Growth regulators used in the experiment included: 2,4-Dichlorophenoxy acetic acid (2,4-D); Naphthalene acetic acid (NAA); 6-Benzylaminopurine (BAP); and Indole-3-butyric acid (IBA).

The compositions of the three media used were as follows:

- Medium 1 (Middle Agriculture School 1979)—MS + 2,4-D (0.5 mg/L) + BAP (1.0 mg/L) + NAA (0.25 mg/L) + sucrose (3%).
- Medium 2 (Shao 1985)—MS + BAP (1.0 mg/L) + NAA (0.1 mg/L) + sucrose (3%).
- Medium 3 (Wu 1986)—1/2-strength MS +IBA (1.0 mg/L) + NAA (0.5 mg/L) + sucrose (1.5%).

Explants derived from cotyledons, hypocotyl, or roots of the nucellar embryonic seedlings were inoculated onto medium 1 for callus initiation. After this, the callus was transferred to medium 2, where shoots formed and proliferated constantly via subculturing. Buds (3 to 4 cm high) were cut and transferred onto medium 3 for root formation. All media were adjusted to pH 5.6 to 5.8 and plant materials were cultured in the chamber at 25 °C and 60-70% relative humidity and a light intensity of 1000-3000 Lux, 10-14 hours a day. All plantlets arising from the culture were transplanted into pots.

## RESULTS AND DISCUSSION

The use of nucellar embryonic culture to obtain virus-free seedlings has been applied in citrus production since 1986. The plantlets can come either directly from nucellar embryos or as regenerants from callus-derived embryoids. In addition to these approaches, plantlets were obtained from the proliferation of plumule explants that readily differentiated roots on medium 1.

Under similar conditions, the nucellar embryonic plantlets, which regenerated from the plumule explants, possessed obvious superiority; they also grew stronger, branched more, and ripened earlier than those coming directly from uncultured seedlings.

Successful plantlet regeneration through the formation of multiple shoots from the plumule explant enabled rapid multiplication of virus-free citrus varieties. These plantlets were stable and closely resembled the original parent. It was possible to multiply eight generations in 1 year. Each bud formed 8 to 10 shoots per generation. This approach can shorten the time needed to multiply a citrus variety as well as save manual labor and field space. It also seems a viable approach to the production of virus-free plants.

Hypocotyl and root explants were induced to form callus on medium 1 and formed shoots on medium 2; the frequency of differentiation was as high as 80 and 100%, respectively. The buds were transferred onto medium 3 where root primordia formed. After about 20 days, plantlets were easily transferred onto 1/2-strength MS medium.

Different varieties displayed similar levels of efficiency on these media, which simplifies the procedures needed for such work. These methods will enable the rapid multiplication of virus-free material and hence speed up bud sport breeding, polyploid breeding, somatic cell screening, somatic hybridization, and germplasm preservation.

Differentiating the zygote embryo from nucellar embryos is a difficult technical problem in crossbreeding. It appears the author has found a simple method to resolve this problem. It will be confirmed by biochemical analysis.

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## RESUMEN

El objetivo de esta investigación era mejorar y explorar nuevos métodos de mejoramiento de cítricos mediante el cultivo de tejidos. Se germinaron semillas poliembriónicas de cítricos en medios de Murashige y Skoog (MS). Las plántulas nucelares embrionarias se aislaron de los embriones cigóticos y se cultivaron. Se encontró que los explantes de plúmulas diferenciaban raíces nuevas, en tanto que los explantes de hipócotilos y radículas formaban callos en el mismo medio, y éstos regeneraban plántulas. Otra forma de propagación de las plúmulas se hizo a través de la proliferación de yemas. También se regeneraron los explantes de plántulas monoembrionarias de *Citrus grandis*.

# Induction and cryopreservation of somaclonal variation in wheat and rice

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The dual problem of the depletion of genetic resources and the loss of native germplasm due to cultivation of few improved cultivars over large areas has led to the disappearance of the genetic diversity in many crops. Thus, biotechnological approaches are being evaluated for the induction of genetic variability and the conservation of germplasm. In this regard, studies on *in vitro* culture combined with cryopreservation have yielded some valuable information for their use in wheat and rice improvement programs. *In vitro* cultures have been observed to exhibit somaclonal and gametoclonal variation and the regenerants show heritable changes. The callus-derived plants showed a wide range of morphological traits. Wheat plants with larger spikes, better yield, and gliadin variation have been obtained. Likewise, rice variants with higher protein content, resistance to *Xanthomonas oryzae*, and tolerance to salt have been reported. The regeneration of complete plants from frozen (-196 °C) zygotic and pollen embryos of wheat and rice establish the use of cryopreservation for the conservation of somaclonal and gametoclonal variants. It is highly desirable to incorporate the in vitro-generated variability in wheat and rice improvement programs. That may enable the early release of improved cultivars. Cryopreservation would further help to conserve the variant cell lines and tissues.

Wheat and rice are the two most important cereals. Though they have been tremendously improved through conventional breeding, they lack the genetic variability to bring about any further significant improvement. Depletion of the germplasm pool and shrinkage of naturally occurring genetic resources are causing great concern. The introduction of new cultivars and exotic germplasm has resulted in the loss of native genetic stocks. In fact, there is a dual problem of induction and conservation of genetic variability, thus there is an urgent need to incorporate *in vitro* technology in breeding programs. The success of a crop improvement program depends on selection of desirable plants, and is possible only if wide variation is present

in the base population. The progress made in biotechnology during the last decade has helped to generate variation in a number of plant species, including wheat and rice (Bajaj 1986a). This paper summarizes work on the induction of genetic variability/ somaclonal variation in callus, and the freeze-preservation of *in vitro* cultures of wheat and rice.

## *IN-VITRO* INDUCTION OF GENETIC VARIABILITY

The natural occurrence of different types of variation in callus cultures has been known for a long time (D'Amato 1977). The callus tissues on prolonged culturing undergo endomitosis, chromosome loss, polyploidy, aneuploidy, mutations, and other genetic changes. Though most of these changes may not be of much significance, there may yet be some which can be selected and utilized as has been demonstrated in other agricultural crops (Bajaj 1989). Larkin and Scowcroft (1981) speculated that tissue culture may generate an environment for enhancing chromosome breakage and reunion events, and thus a tissue culture cycle of the hybrid material may provide the means for obtaining the genetic exchange needed between the genomes in the interspecific hybrid. The hybrid callus may enhance the frequency of requisite exchange. Hybrid callus, which is a rich source of variation (Bajaj and Gill 1985), needs to be exploited for crop improvement.

### Wheat

Wheat has proved to be excellent material for the induction of somaclonal variation and a number of publications have appeared on this aspect during the last 6 years, such as Sears and Deckard, (1982), Davies *et al.* (1986), Metakovsky *et al.* (1987). The callus derived from pollen/anthers yields plants varying in chromosomal constitution (Hu 1986, Kudirka *et al.* 1986). A number of doubled haploid lines have been released as cultivars in China (Hu 1986) and an anther culture-derived cultivar 'Florin' was released in France (de Buyser *et al.* 1987).

Our work on the induction of somaclonal variation through the culture of immature embryos was described in detail by Bajaj (1986b) and is summarized below.

The immature embryos cultured on MS (Murashige and Skoog 1962) + 2,4-Dichlorophenoxy acetic acid (2,4-D) started to swell and increased considerably in size within 1 week; a mass of callus was formed in 3 to 4 weeks. In the older embryos, in addition to the callus formation, germination was also observed. The presence of 2,4-D in the medium, however, inhibited further development of the root and shoot unless the plants were transferred to a new medium. The callus cultures varied a great deal in their morphology, and, in general, four patterns were observed, 1) cream, loose, fluffy, and highly friable callus; 2) callus mixed with numerous root hairs; 3) light brown callus; and 4) callus with white, round and compact organized masses and green patches. The last type of callus was highly embryogenic. The callus could be subcultured and maintained on MS + 2,4-D (1-2 mg/L) medium.

The callus transferred to a medium without 2,4-D, or to a medium containing indole acetic acid (IAA) and cytokinin underwent regeneration. In some cases, as many as 8-10 plantlets appeared on one callus. These studies produced a wide range of morphological variation, especially in the height of the plants, size and shape of the

leaves, length of the awns, fertility of the spikes (Bajaj 1986b), and the size, shape, and color of the seeds. In a few instances, two or three spikes were formed on one culm.

In addition to morphological changes, wheat somaclonal variants have also shown changes in various enzymes and proteins (Maddock *et al.* 1985, Davies *et al.* 1986, Ryan and Scowcroft 1987, Metakovsky *et al.* 1987). Although field assessment of somaclonal variants (Maddock and Semple 1986) and heritability studies, especially for gliadin protein (Cooper *et al.* 1986) have been made, the practical utility of these somaclones has yet to be realized.

**Rice**

*Embryo and seedling-derived callus and plants.* Bajaj and Bidani (1980) demonstrated callus proliferation from excised embryos and segments of root, mesocotyl and shoot of various cultivars after 4-5 weeks of culture on MS + 2,4-D (2 mg/L) medium. The nature of the callus, its growth rate, texture, and color varied between cultivars. The mesocotyl was the best segment for raising callus, followed by shoot and root segments. In most of the cases, the callus underwent rhizogenesis after 5 to 15 days, but no shoot formation was observed. The excised embryos in cultures enlarged considerably and proliferated. After transfer to MS + 4 mg/L of IAA + 2 mg/L of Kinetin (KT), the callus regenerated shoots and plantlets in 3-5% of the culture. These plants continued to grow when transferred to the soil. The production of callus from the excised segments and embryos, and the induction of organogenesis has been reported by a number of workers (Henke *et al.* 1978, Nakano and Maeda 1979, Bajaj 1982a). However, the major problem was the reproducible induction of differentiation in the subcultured callus. Using this technique, shoots are obtained from the callus passed through several subcultures. There is a genotypic variation for plant regeneration as shown by Abe and Futsuhara (1986). The callus-derived plants varied in number ranging from 11 to 60 in embryo-derived calluses and 24 to 105 in endosperm divided calluses. Aneuploids were the most common (Bajaj *et al.* 1980).

Cell suspensions were raised on liquid medium of MS + 2,4-D (2 mg/L) + 1 mg/L of naphthalene acetic acid (NAA) + 0.5 mg/L of KT. When 14 days old, cultures were used as inoculum, the cells started growing immediately. However, in older cultures (24 days), a lag period of 1 week was noticed before growth was resumed. Cell suspensions were subcultured periodically. IAA and KT, when added to the medium in place of 2,4-D, promoted root formation. The microscopic examination of the cell suspension showed various stages of growth from a single cell to a clump of cells. Single cells of various shapes were observed.

Sun *et al.* (1983) studied inheritance and variation in more than 2000 somatic cell-derived plants and reported that the phenotypic variation observed in the second generation was heritable. Subsequently mutants (Oono 1985) and plants resistant to *Xanthomonas oryzae* (Sun *et al.* 1986), and sodium chloride (Wong *et al.* 1986) were selected from among somaclonal derivatives.

*Endosperm culture and the regeneration of triploid plants (Bajaj et al. 1980).* The immature and mature endosperm of various cultivars showed striking differences in their growth response (Bajaj and Bidani 1986). The immature endosperm underwent two modes of differentiation, i.e. the direct regeneration of plants without the

intervening callus phase and indirect through the differentiation of the callus. The immature endosperm gave a higher response than the mature endosperm. On MS + 2,4-D (2 mg/L) the segments of the mature endosperm proliferated to form a mass of callus in 3 to 4 weeks, but there was no shoot formation. On transferring to MS + IAA (4 mg/L) + KT (2 mg/L), the callus differentiated to form shoots and complete plants in 4 to 6 weeks. These plants continued their growth when transferred to the soil.

The proliferation of immature endosperm and the occasional formation of shoots has been showed to occur in a medium fortified with yeast extract. A combination of IAA and KT induced and enhanced shoot formation, whereas yeast extract did not induce shoot growth (Bajaj and Bidani 1986).

The triploid plants produced from the endosperm showed broader leaves, a faster rate of growth, and greater tillering than the embryo-derived plants. The triploid and hexaploid plants are more vigorous than diploids (Morinaga and Fukushima 1935) and their foremost use is in hybridization programs, especially for the augmentation of reservoirs of germplasm. Hybrid selection can then be made for the desirable agronomic traits.

The study of the endosperm callus showed wide differences in chromosome number. Though the cells were predominantly triploid (3n=36), cells with the chromosome number varying from 24 (2n) to 105 (8n=96) were also observed (Bajaj and Bidani 1980).

*Anther and pollen-derived plants (Bajaj and Bidani 1986).* The anthers containing pollen at an early uninucleate stage gave the maximum growth response. There were two modes of development: 1) the direct formation of haploid pollen-embryos, and 2) the callusing of the pollen and anthers (Bajaj 1980b, Bajaj *et al.* 1986).

After a week of culture, the microspores enlarged and underwent mitosis to form either two similar looking nuclei, or a generative and a vegetative cell. The continued mitosis resulted in the formation of multinucleate pollen or a multicellular globular embryo. In some cultures, callus was formed from pollen and the anthers that later differentiated into plants. Thus, plants originating from both sources in the same anther were obviously mixoploid. Extensive cytological studies have been done (Chu *et al.* 1985) and variants showing improved protein and yield have been selected (Schaeffer *et al.* 1984).

During the last decade, much literature on the production of haploid and homozygous plants through anther and pollen culture has accumulated (Bajaj 1983d, Hu 1986). In rice, starting with the pioneering work of Niizeki and Oono (1968), a number of investigations on various aspects of androgenesis have been done. Although an optimum yield of haploids has been obtained from the young anthers cultured at a uninucleate pollen stage, on $N_6$ medium with 9% sucrose, the frequency of androgenesis has been rather low. However, with persistent efforts a number of rice cultivars have been released including 'Hua Yu 1' and 'Hua Yu II' (see Loo and Xu 1986).

## CRYOPRESERVATION OF CELLS, TISSUES, AND ORGANS

Wheat and rice seeds present no problem for long-term storage; however, due to an enthusiasm for obtaining novel plants through various biotechnological approaches,

such as the induction of somaclones and transformation, the number of cell lines is becoming so large that it is not only difficult, but at times impossible, to maintain them. Moreover, with periodical subculturing, they undergo genetic erosion and the original cell lines/somaclones are lost.

At present, no method is known by which genetic stability of cultures is ensured. The freeze-preservation of cells in liquid nitrogen (-196 °C) is an optimistic approach. It involves bringing the cells to a zero level of metabolism. During the last decade, considerable work has been done on the cryopreservation of cells, tissues, zygotic and pollen-embryos of wheat and rice (Bajaj and Bidani 1986), and complete plants have regenerated from retrieved cultures. The freeze-preservation method is useful because: 1) it avoids the need for periodic transfer and subculturing, 2) it avoids genetic instability, 3) it retains morphogenetic potentials, and 4) the cryopreservation of pollen would solve some of the problems encountered with incompatibility and pollen longevity. Work on the cryopreservation of *in vitro* cultures of rice and wheat is reviewed here.

**Cryopreservation of zygotic embryos**

There are a number of reports on the freeze-preservation of embryos (Bajaj 1985). The mature zygotic embryos of wheat (Bajaj 1980a) and rice (Bajaj 1981) frozen in liquid nitrogen regenerated entire plants. In all these cases, quick freezing followed by thawing at 35-40 °C was employed. However, the viability varied considerably (Bajaj 1980a). The survival of immature embryos varied from 42 to 55%. In the controls, the embryos elongated and started to proliferate within 3 days; the frozen embryos underwent a lag period of about 2 weeks before they proliferated and formed shoots. Plantlets transferred to the field matured and the seeds obtained germinated to form normal plants (Bajaj 1986c).

**Cryopreservation of endosperm cultures**

The segments of mature endosperm of rice subjected to sudden freezing in liquid nitrogen and cultured on MS + 2,4-D (2 mg/L) were quiescent for about 4 weeks, after which 11% proliferated to form callus (Bajaj 1981). Upon transfer to MS + IAA (4 mg/L) + KT (2 mg/L), the callus differentiated to form shoots and plantlets.

**Cryopreservation of anthers and pollen-embryos**

Anthers of rice cultivar 'Basmati-370' subjected to quick freezing became soft and spongy, turned brown, and appeared dead. Of 927 anthers subjected to freezing, only five showed visual signs of growth (Bajaj 1980b), i.e. localized proliferation to form callus. The callus was compact and slow growing, and showed various modes of differentiation and regenerated albino plants. Growth of the retrieved anthers was considerably delayed.

The segments of androgenic wheat anthers (4- to 6-week old cultures) freeze-stored for 1 year resumed growth (Bajaj 1983a, 1984). The retrieved cultures underwent a lag phase of 4 to 6 weeks, after which 5% of the cultures proliferated, and the callus occasionally differentiated shoots (Bajaj 1984). The survival of segments of wheat was observed in both methods of freezing, i.e. quick freezing in a solvent, as well

as by the dry method in which the segments are wrapped in aluminium foil. However, revival was better in the latter, as the anthers that were frozen in a solvent tended to become spongy.

The results obtained with wheat and rice are comparable (Bajaj 1984). The segments of the androgenic anthers showed post-freezing viability of 5-6%, whereas 19-21% of the pollen-embryos survived freezing. The low viability, long lag phase, and the regeneration of malformed shoots are evidence of the extent of cryodamage caused during various steps of the cryogenic protocol. Thus to obtain increased survival, a thorough study of various factors affecting cryoability should be undertaken.

### Cryopreservation of cells

The callus tissue and cell suspensions are able to withstand freezing and regenerate entire plants (Bajaj 1976). The highest degree of viability has been obtained with young and actively growing cells that are highly cytoplasmic, small, thin-walled, and nonvacuolated. The callus cells of wheat (Bajaj 1980a) and rice (Sala *et al.* 1979) yielded a viability of 26 and 65%, respectively.

### Cryopreservation of somatic hybrid protoplasts

Fusion of protoplasts is being employed to induce genetic variability by synthesizing new combinations of germplasm. The genetic variability thus induced by various *in vitro* means leads to a number of cell lines that need to be periodically subcultured. During this process, there is an erosion of the stocks which can hopefully be circumvented by cryopreservation. The following summarizes our published results (Bajaj 1983b).

The mesophyll protoplasts of pea (*Pisum sativum* L.) and callus cell-derived protoplasts of wheat, rice, and sorghum fused by PEG and frozen in liquid nitrogen showed various degrees of survival (Bajaj 1983c). In the control (unfrozen) cultures, the hybrid protoplasts showed mixing of the cytoplasm, yellowing and redistribution of chloroplasts, and an overall increase in size in 5 to 7 days. The retrieved, fused protoplasts, however, were quiescent for the first 2 weeks in culture. Thereafter, changes in their morphology were observed, such as mixing of the cytoplasm and visible cyclosis in the cytoplasm. With the passage of time, the chloroplasts became scanty and yellowish. The results in the three systems were comparable, with survival in wheat x pea being higher (12.2-17.9%) than rice x pea (10.8-13.9%). Budding of the protoplasts was a common phenomenon. The heterokaryon showed two nuclei of different sizes, and occasionally fusion of the nuclei was also observed in the somatic hybrid cells. Only in one instance, the retrieved hybrid cell underwent repeated division. The survival of hybrid protoplasts frozen in liquid nitrogen is a significant step toward the induction and conservation of genetic variability.

### Germplasm banks

Storage of seeds in banks is the traditional method for germplasm conservation. Seed banks have been established at various centers so that genetic diversity can be made available for plant improvement and for developing new plants that are high yielding and resistant to disease and pests, and tolerant to harsh climatic and soil conditions.

However, with the recent advances in the area of plant tissue culture, it has become increasingly evident that various *in vitro* methods can be used for the conservation and international exchange of germplasm as well as the induction of genetic variability and the synthesis of novel plants (Bajaj 1979a,b, 1983e, 1986c). Based on these observations, the establishment of germplasm banks of cells, tissue, and organ culture is recommended. Likewise, storage of pollen would circumvent some of the seasonal, geographical, and physiological limitations experienced in wide hybridization. These banks (Bajaj 1982b, 1987) would be somewhat like 'semen banks' for animals. Institutes such as the International Maize and Wheat Improvement Center (CIMMYT) in Mexico and the International Rice Research Institute (IRRI) in the Philippines may be entrusted with the task of using tissue culture and cryopreservation for the conservation, maintenance, distribution, and international exchange of germplasm of elite, desirable, and rare plants of wheat, rice and wild relatives.

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**RESUMEN**

El doble problema del agotamiento de los recursos genéticos y la pérdida del germoplasma nativo como resultado del cultivo de unas pocas variedades mejoradas en zonas extensas, ha llevado a la desaparición de la diversidad genética de numerosos cultivos. En consecuencia, se evalúan los métodos biotecnológicos para determinar su capacidad de inducir variabilidad genética y conservar el germoplasma. En este sentido, los estudios en medios de cultivo *in vitro* combinados con la criopreservación han producido información valiosa para los programas de mejoramiento de trigo y arroz. Se ha observado que los cultivos *in vitro* poseen variación somaclonal y gametoclonal y que las plantas regeneradas muestran cambios heredables. Las plantas derivadas de los callos exhibieron una gran diversidad de características morfológicas. Se han obtenido plantas de trigo con espigas más grandes, mejor rendimiento y variación gliadina. De la misma forma, se han registrado variantes de arroz con un mayor contenido de proteína, resistencia a *Xanthomonas orysae* y tolerancia a la sal. La regeneración de plantas completas a partir de embriones cigotas o de polen de trigo y arroz que han sido congelados (-196 °C) establecen el uso de la criopreservación como medio para conservar las variantes somaclonales y gametoclonales. Es muy importante que se incorpore la variabilidad generada *in vitro* en los programas de mejoramiento de trigo y arroz, pues podría permitir el rápido lanzamiento de variedades mejoradas. La criopreservación podría ayudar también a conservar las líneas y tejidos de células variantes.

# High frequency embryogenic callus induction and its regeneration in three wheat cultivars

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In this study, mature seeds were used for callus induction in three wheat cultivars, 'Pavon-76', 'Lyallpur-73', and 'Pak-81'. Cultures were incubated in both dark and light. High frequency embryogenic callus induction and its regeneration were studied by using different combinations of 2,4-Dichlorophenoxy acetic acid (2,4-D). Callus induction was observed after 1 week of culture in all the cultivars tested. Lower concentrations of 2,4-D (0.5 mg/L) failed to induce callus. It was further observed that callus formation was cultivar-specific, when higher concentrations of 2,4-D (2-4 mg/L) were used. The percentage of callus induction was highest in 'Pavon-76' followed by 'Lyallpur-73' and 'Pak-81'. The effect of light is also cultivar-specific. It was also possible to distinguish embryogenic calluses (E callus) from nonembryogenic calluses (NE callus) by visual observation. The NE calluses are loose, dirty white, whereas the E calluses are compact and green. Maximum E calluses were produced in 'Pak-81'. Both E and NE calluses were transferred onto regeneration medium supplemented with Benzylaminopurine (BAP) and Indole acetic acid (IAA) after four passages. Only E calluses turned organogenetic. 'Pavon-76' has the best potential for regeneration.

Wheat is the most important of the cereal crops in terms of area and production and is a staple food for more than one third of the world's population (Reitz 1967). Wheat contributes more calories and protein to the world's diet than any other food crop (Hanson *et al.* 1982). To a great extent in Pakistan, low wheat production means failure to achieve self-sufficiency in food.

It is imperative that nonconventional breeding methods be integrated to amplify or accelerate varietal improvement in wheat. Early maturing wheat cultivars are

essential to the cropping systems in Pakistan, which are based on rice/wheat, cotton/wheat, or sugarcane/wheat.

Diseases are a major cause for low wheat yields. Adding disease-resistant cultivars to the existing gene pool can not be overemphasized. Salinity is also a major problem that can drastically reduce wheat production and underlines the importance of salt- and drought-tolerant cultivars.

Plant tissue culture has often been acknowledged as one of the more promising avenues to plant improvement. This is usually seen in terms of the ability to apply cellular selection for recovering useful genetic variants, anther culture to expedite the attainment of homozygosity, somatic hybridization for recombining genomes of sexually incompatible species, and, more recently, the possibility of specific gene addition or modification by recombinant DNA techniques (Scowcroft 1977, Nitzche and Wenzel 1977, Thomas *et al.* 1979, Kado and Kleinhofts 1980). A tissue culture cycle involves the establishment of a more or less differentiated cell or tissue culture under defined culture conditions, proliferation for a number of cell generations, and the subsequent regeneration of plants (Larkin and Scowcroft 1981). Cereal tissue culture systems need to be ameliorated as the property of cereal cells to form somatic embryos which seem to be lost early in the development of the explant source. Mature seeds are a more convenient source. Although plant regeneration has been achieved previously from callus cultures derived from mature and immature embryos of wheat and sorghum, results were inconsistent from these short-term cultures (Mackinnon *et al.* 1987).

Regeneration from wheat (*Triticum aestivum* L.) tissue culture has been demonstrated for many cultivars. However, callus cultures of some wheat cultivars produce few shoots (Maddock *et al.* 1983). Improvements in wheat tissue culture should result from an increased understanding of the hormonal and nutritional requirements of various morphogenetic phenomena, thereby allowing the continuous production of plantlets in long-term cultures.

This paper describes attempts to develop high-frequency embryogenic callus induction, long-term maintenance, and subsequent regeneration in three selected wheat cultivars. The study is further supported with a cytological examination of embryogenic cultures to determine the tissues that take part in callus proliferation and embryo formation.

## MATERIALS AND METHODS

Material for the study was provided by the National Coordinator, Wheat Program. Three wheat cultivars ('Lyallpur-73', 'Pavon-76', and 'Pak-81') were tested. Explants selected were dry seeds that were washed thoroughly with running tap water and then dipped in a commercial detergent for a few minutes. Excess detergent was removed by thoroughly washing in tap water followed by several rinses in distilled water. The material was then sterilized by first quickly washing in 70% ethanol, followed by a 20-minute sterilization with chlorox bleach containing 5.25% sodium hypochlorite. After sterilization, excessive bleach was washed off with sterile distilled water under aseptic conditions at least five times successively for 15 minutes each and cultured directly on the medium. One seed was cultured per test tube containing 6 ml of medium.

The cultures were incubated under both dark and lighted conditions (16 hours day/8 hours night). The temperature was maintained at 22 °C±3 °C. The MS (Murashige and Skoog 1962) culture medium was supplemented with 3.0% sucrose, 0.8% agar, and various concentrations of growth regulators. The medium pH was adjusted to 5.8 prior to autoclaving at 15 psi for 20 minutes.

### Maintenance and proliferation

Both embryogenic (E) and nonembryogenic (NE) calluses were distinguished by their external appearance. The E calluses were compact, either green or greenish white, depending on the cultivar. The NE calluses were loose, dirty white crystalline masses in all three cultivars. Calluses were subcultured after approximately 4 weeks of incubation on the maintenance medium. In the second and subsequent passages, E calluses were separated from NE calluses. After four passages, fresh weight of the calluses was taken, following which they were transferred onto a regeneration medium. The regenerated plants were transferred to pots in a glasshouse.

### Protocol for histological studies

Calluses of the three cultivars were fixed in formalin acetic acid (FAA) and then dehydrated in a series of ethanol solutions before being embedded in paraffin. Sections of 10 μ were prepared with a hand microtome and mounted on glass slides stained with 0.5% safranin and 0.1% fast green, separately and in combination.

## RESULTS AND DISCUSSION

### Callus induction

Using the three cultivars, the effect of various concentrations (0.5 to 4.0 mg/L) of 2,4-dichlorophenoxy acetic acid (2,4-D) on callus induction was tested both in the dark and the light. Callus initiation was observed after 1 week of culture in all cultivars, irrespective of the light or dark period.

A lower concentration of 2,4-D (0.5 mg/L) failed to induce callus. Callus formation at higher concentrations of 2,4-D was cultivar-specific. The amount and size of callus varied from cultivar to cultivar. In 'Pavon-76', the highest percentage of callus induction was observed on medium containing 2 mg/L of 2,4-D maintained in the dark. High concentrations of 2,4-D (4 mg/L) had an adverse effect. Callus induction frequency in light was less than in the dark in all media (Fig. 1). In 'Lyallpur-73', 3 mg/

**Table 1. Percentage of embryogenic callus formation in relation to fresh weight after four passages.**

| Variety | Percentage of embryogenic callus | Average fresh weight of calluses (g/culture) with standard error |
|---|---|---|
| Pavon-76 | 67.76 | 1.048 ± 0.300 |
| Lyallpur-73 | 59.64 | 0.67 ± 0.132 |
| Pak-81 | 75.00 | 0.843 ± 0.030 |

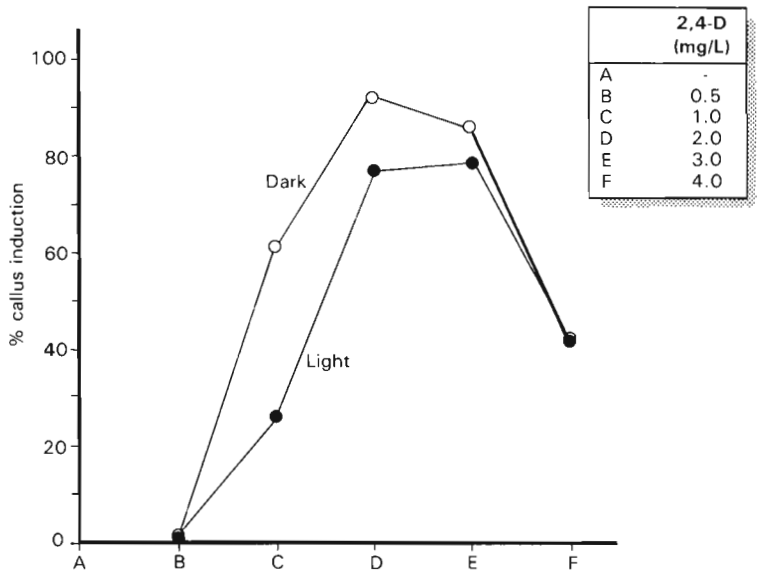


**Figure 1. Callus induction frequency in 'Pavon-76'.**
Concentrations of 2,4-dicholorophenoxy acetic acid (2,4-D)

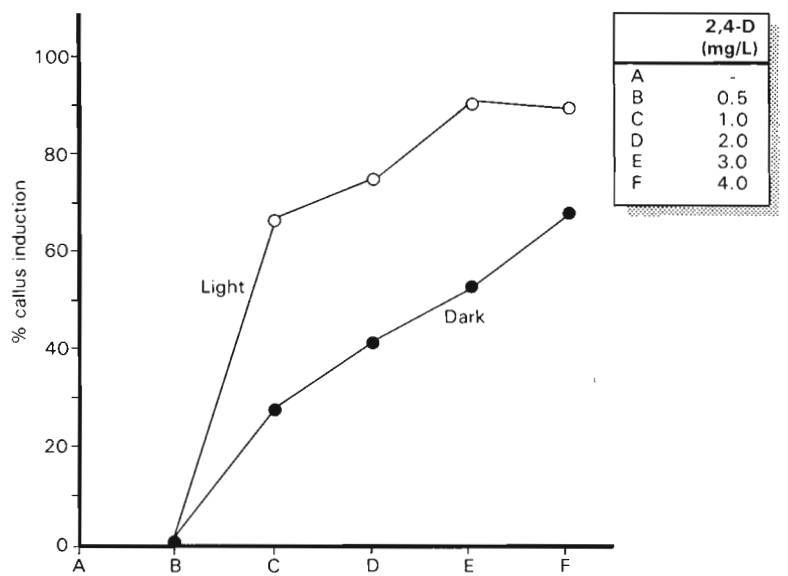


**Figure 2. Callus induction frequency in 'Lyallpur-73'.**
Concentrations of 2,4-dicholorophenoxy acetic acid (2,4-D)

L of 2,4-D produced good callus induction in light, with little difference in the callus induction frequency between 3 and 4 mg/L of 2,4-D. In the dark, there was a significant decrease in callus induction in all media (Fig. 2). In 'Pak-81', callus induction frequency was high at 4 mg/L of 2,4-D in the dark. At the rates of 2 and 3 mg/L of 2,4-D, a very slight difference in callus induction frequency was observed between light and dark (Fig. 3).

The frequency of good callus induction was noted to be highest in 'Pavon-76' followed by 'Lyallpur-73'. The frequency of embryogenic callus was less during the first passage, however, during subsequent passages, its frequency increased substantially. This result is in accordance with the work reported by Mackinnon *et al.* (1987).

**Callus consistency**

Two distinct callus types were recognized during the first culture passage, i.e. the NE callus that remained smooth and crystalline during several subsequent culture passages (Fig. 4), and the E callus recognized by greenish papillae on the callus surface (Fig. 5). The E callus was separated to maintain its identity over several passages. The frequency of callus formation, both E and NE, varied from cultivar to cultivar, and even within the same cultivar depending on the concentration of 2,4-D in the culture medium.

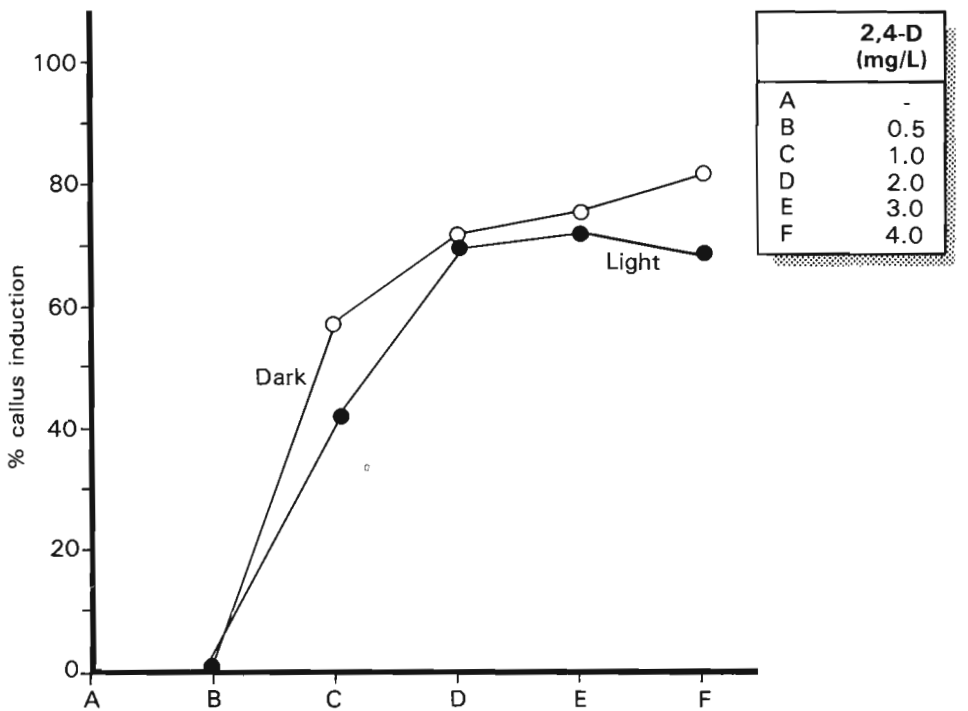


**Figure 3. Callus induction frequency in 'Pak-81'.**
Concentrations of 2,4-dicholorophenoxy acetic acid (2,4-D)

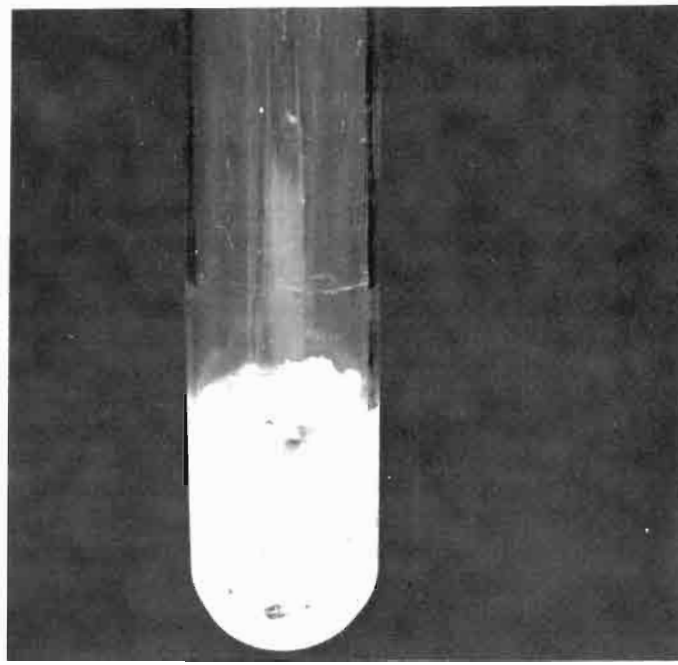

Figure 4. Nonembryogenic callus of 'Pak-81' after five passages.

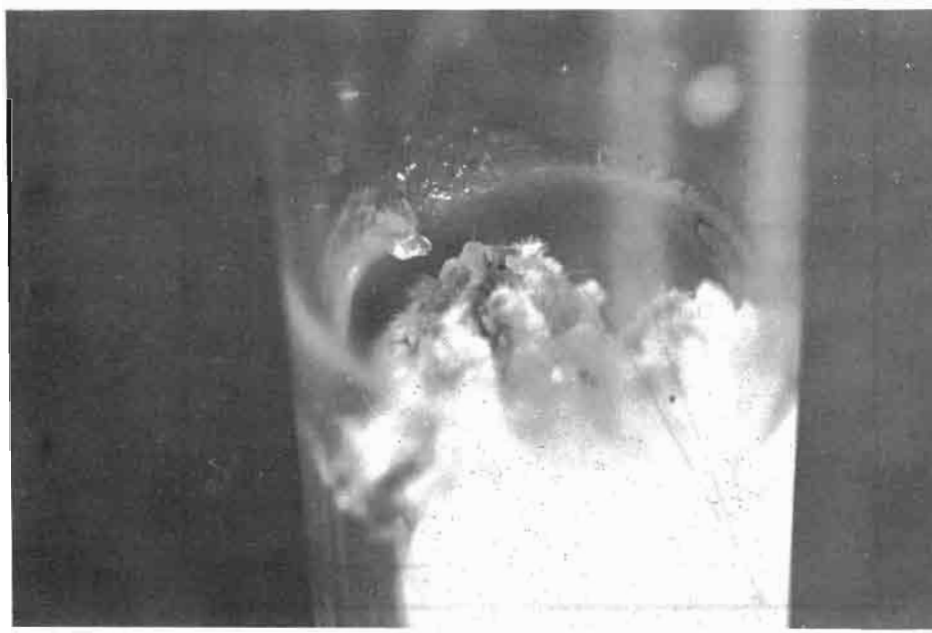

Figure 5. Embryogenic callus of 'Pavon-76' after three passages.

Callus texture and color varied from cultivar to cultivar. In 'Pavon-76', the callus was compact and greenish white; in 'Lyallpur-73', it appeared to be a loose crystalline mass and dirty white; in Pak-81, the callus was compact and yellowish white.

**Callus maintenance**

Callus performance after subculturing was also studied using different medium combinations. When the calluses on 'Pavon-76' were subcultured on a medium with 2 mg/L of 2,4-D, good proliferation of tissues was noted (Fig. 6). When the 2,4-D concentration of was lowered from 3 to 0.5 mg/L, proliferation was profuse. At elevated levels of 2,4-D (4 mg/L), proliferation decreased but embryo-like structures were clearly visible, indicating that such levels could initiate differentiation. For 'Lyallpur-73', the optimum 2,4-D concentration for good callus formation was 3 mg/L (Fig. 7). 'Pak-81' could be maintained very well on a medium supplemented with 1 mg/L of 2,4-D. The calluses appeared to be regenerative with a good proliferation.

Under the conditions specified above, calluses are being maintained for the sixth passage. The cultures were 10 months old as of this writing.

The average percentages of E callus formation were 67.76% for 'Pavon-76', 59.64% for 'Lyallpur-73', and 75.00% for 'Pak-81'. The fresh callus weight in g/culture was less in both 'Lyallpur-73' (0.67 g) and 'Pak-81' (0.843 g) than in 'Pavon-76' (1.048 g). The fresh weight of NE calluses noted after the fourth passage showed no significant difference (0.183 to 0.251 g) irrespective of the media combination tested.

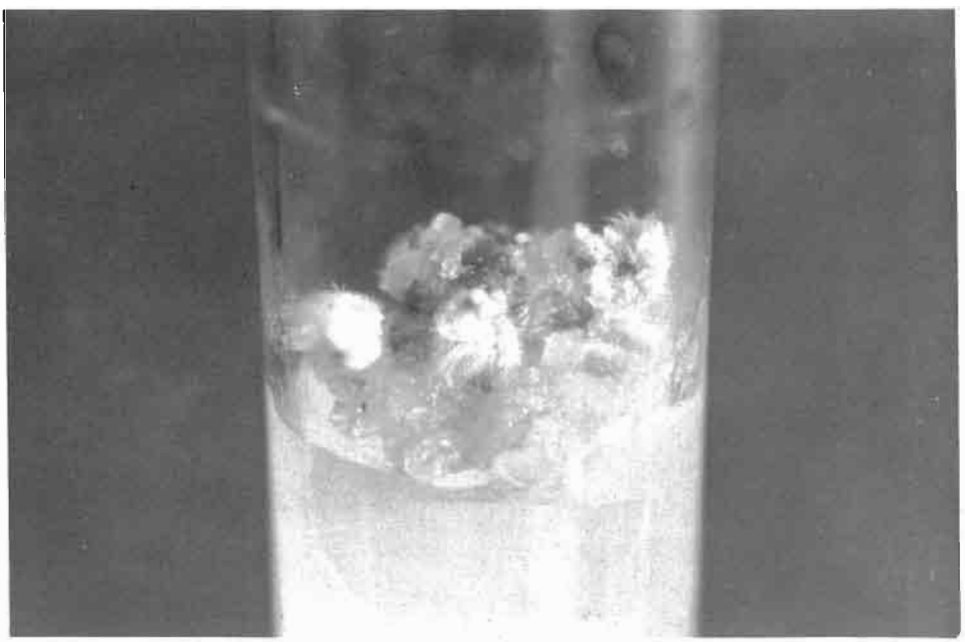

**Figure 6. Callus proliferation in 'Pavon-76' on 2,4-D medium of 2 mg/L.**

### Callus histology

The histological examination showed various E stages in the three wheat cultivars. In 'Lyallpur-73', embryoids were observed that originated under the epidermal tissues and pushed the peripheral cells outward, forming a meristematic region. An angular or spiral thickening in certain cells was observed giving rise to tracheids. The callus of 'Pak-81' showed the first regular division leading to the suspensor. In some sections, well developed root structures were observed arising from the meristematic tissues of the subepidermal layer. Well spaced cells with large vacuoles and deeply stained nuclei were observed in the callus from 'Pavon-76'.

### Regeneration

Nabors *et al.* (1983) reported that NE callus was not regenerable. In this study, the NE callus also failed to regenerate when transferred to the regeneration MS medium supplemented with 0.5 to 1.0 mg/L of Benzylaminopurine (BAP) and 0.1 mg/L of Indole acetic acid (IAA). On the other hand, when E callus was transferred to the regeneration medium, root initiation and callus differentiation was observed within 1 week of incubation. Visible shoot formation was noted 4 weeks later. The presence of IAA in the regeneration medium directed morphogenesis toward excessive root formation in all three cultivars. It was for this reason that in further studies IAA was replaced with NAA at the rate of 1 mg/L, which ultimately gave better results. The shoots were transferred to pots under glasshouse conditions; some grew to maturity (Figs. 8 and 9). Mackinnon *et al.* (1987) also reported that E callus established both from mature and immature embryos readily regenerated shoots.

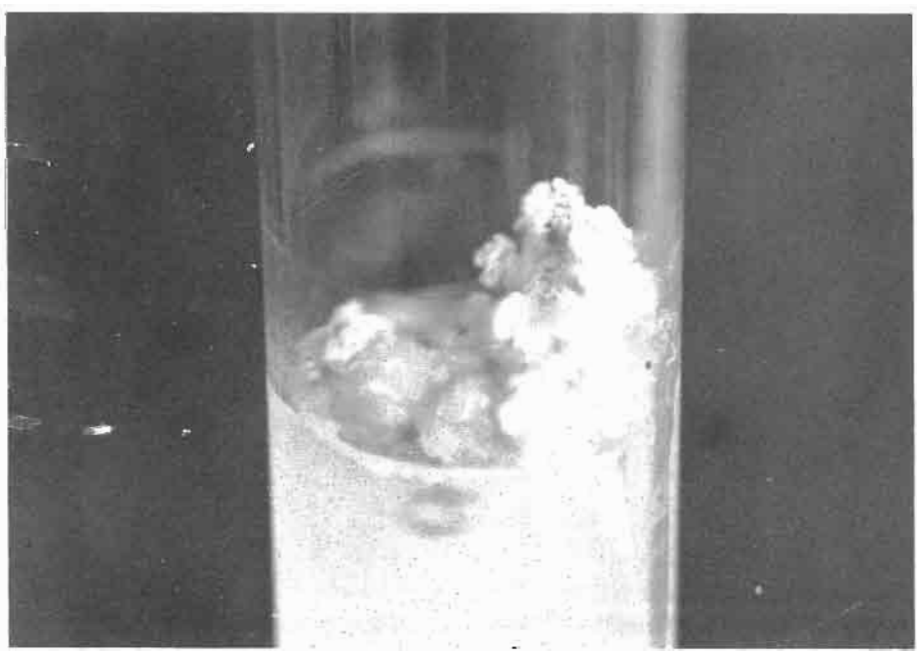

**Figure 7. Callus proliferation in Lyallpur-73 on 2,4-D medium of 3 mg/L.**

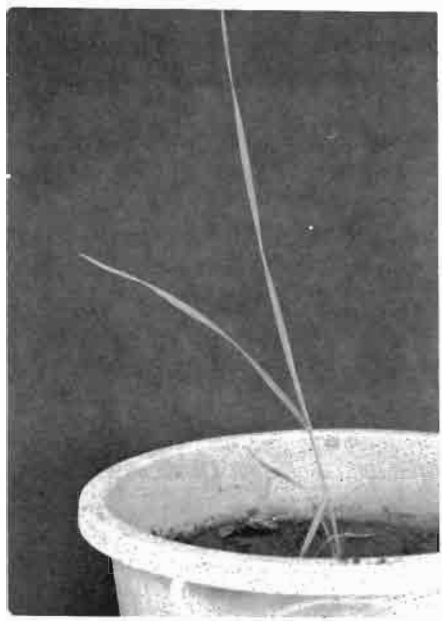

**Figure 8. Wheat regenerants ('Lyallpur-73').**

**Figure 9. Earing stage ('Pavon-76').**

## CONCLUSION

It can be inferred from this study that it is possible to induce a high frequency of E callus in 'Pavon-76', 'Lyallpur-73', and 'Pak-81'. Regeneration can be achieved from E calluses in long-term cultures.

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## RESUMEN

En este estudio se emplearon semillas maduras para la inducción de callos en tres variedades de trigo: 'Pavón-76', 'Lyallpur-73' y 'Pak-81'. Los cultivos se incubaron en condiciones de luz y de oscuridad. Se estudiaron la inducción de alta frecuencia de callos embrionarios y su regeneración usando distintas combinaciones de ácido 2,4-diclorofenoxiacético (2,4-D). En todas las variedades sometidas a prueba se observó la inducción de callos después de una semana de cultivo. No fue posible inducir los callos con concentraciones más bajas de 2,4-D (0.5 mg/L). Por otra parte, se observó que la formación de callos era específica de la variedad cuando se empleaban concentraciones más altas de 2,4-D (2-4 mg/L). El porcentaje de la frecuencia de inducción de los callos fue mayor en 'Pavón-76', seguida por 'Lyallpur-73' y 'Pak-81'. El efecto producido por la luz también es específico de la variedad. Asímismo, fue posible distinguir entre los callos embrionarios (callos E) y los no embrionarios (callos NE) mediante la observación visual. Los callos NE están diseminados y son de un color blanco sucio, en tanto que los callos E son compactos y de color verde. La mayor cantidad de callos E se produjo en la variedad 'Pak-81'. Los callos E y los NE se transfirieron a un medio regenerativo, complementando con benciloaminopurina (BAP) y ácido indolácetico (AIA) después de cuatro pasajes. Sólo los callos E se volvieron organogenéticos. La variedad 'Pavón-76' presentó el mayor potencial de regeneración.

# Genetics of plant regeneration in anther culture (AC) of rice (*Oryza sativa* L.)

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The genetics of plant regeneration from anther-derived callus was investigated using the four rice cultivars 'Lemont', 'Short Tetep','IR36', and 'Gui Chow'; the $F_1$, $F_2$, and $F_3$ generations from a diallel cross of these four cultivars; and 24 $BCF_1$s. Green plant regeneration rates and albino plantlet production rates were calculated as the number of green and albino plants regenerated from 100 calluses following transfer to a differentiation medium under uniform culture conditions. 'Lemont' regenerated plants at a rate of three plants per 100 calluses (3%), while 'IR36' and 'Gui Chow' regenerated no plants. The cultivar 'Short Tetep' possessed a high regeneration rate for both green plants (63%) and albinos (316%). The rates for 'IR36' and 'Gui Chow' were significantly different from the rate for 'Lemont', which was statistically different from the rate for 'Short Tetep'. The mean green plant and albino regeneration rates among the 12 $F_1$s showed overdominance and recessiveness in some crosses. A significant reciprocal difference was found in the cross involving 'Lemont' and 'Short Tetep'. Evaluation of the 24 $BCF_1$s showed that mean plant regeneration rates generally increased upon backcrossing to high culturability parents and decreased upon backcrossing to low culturability parents. Generation mean analysis indicates that dominance (d), additive x dominance (ad), and dominance x dominance (dd) are major contributors to the variation of generation means, although additive gene effects (a) are important in some crosses. From a practical standpoint for rice anther culture (AC), higher regeneration rates can be obtained from crosses involving a high and low parent by utilizing the $F_2$ rather than the $F_1$ generation for AC.

Genotypic differences in plant regeneration were first reported by Niizeki and Oono (1968). Among the 10 japonica rices they tested, only two cultivars produced plantlets. Since then, similar findings have been reported by various research groups (Mukherjee 1973, Chen *et al.* 1974, Lin *et al.* 1974, Oono 1975, Chen and Lin 1976, Yin *et al.* 1976,

Chen 1978, Woo *et al.* 1978, Woo and Huang 1980, Cornejo-Martin and Primo-Millo 1981, Abe and Sasahara 1982, Sheng *et al.* 1982, Wakasa 1982, Zhang and Chu 1984, Croughan *et al.* 1984, Chu *et al.* 1984, 1985, Croughan *et al.* 1985, Miah *et al.* 1985, Boyajiev and Kuong 1986, Chu *et al.* 1986, Croughan *et al.* 1986, Davoyan 1987). These reports indicated that plant regeneration not only differed among various wild species and subspecies of rice such as japonica, indica, and javanica, but also between cultivars of cultivated rice.

Xui and Liou (1984) analyzed the genetic aspects of culturability by diallel analysis. They found significant differences in general combining ability (GCA) and specific combining ability (SCA) effects, indicating that additive and dominance gene effects were important aspects of culturability. Zhang and Chu (1985) and Chu (1986) made a 5x5 diallel cross to study the genetics of plant regeneration. The results indicated that maternal, additive, and slightly high overdominance effects contributed to the observed genotypic differences.

This study was conducted to systematically evaluate the genetics underlying plant regeneration in rice anther culture (AC). Specifically, the objectives were to clarify the role of maternal effects in plant regeneration by comparing $F_1$ hybrids and their reciprocals; to analyze the genetic aspects of the trait through analysis of the $P_1$, $P_2$, $F_1$, $F_2$, $F_3$, $P_1F_1$, and $P_2F_1$ generations; to investigate the contribution of various gene effects by generation mean analysis; and to calculate the heritability of the character by parent-offspring regression analysis.

## MATERIALS AND METHODS

### Establishment of experimental material

The cultivars 'Lemont', 'IR36', 'Short Tetep', and 'Gui Chow' were selected from a screening of 10 cultivars to represent high and low culturability parents for use in a 4x4 diallel cross. The details of the hybridization method were previously described (Chu and Croughan 1988a). Twelve $F_1$s were backcrossed to their corresponding parents to produce $P_1F_1$ and $P_2F_1$ populations. $F_2$ and $F_3$ populations were established by selfing.

### Plant regeneration

Rice stems in the booting stage were collected on an individual plant basis when the flag leaf collar was 2 to 5 cm above the penultimate leaf collar. Following leaf blade removal, the stems were sealed in plastic bags and given a cold pretreatment of 5 °C for 5 to 7 days. Before inoculation, the rice boots were sterilized in a 50% bleach solution (v/v) for 30 minutes. Panicles were aseptically dissected from the stems and rinsed with distilled water three times. Anthers of parents, 12 $F_1$s, 24 $BCF_1$s, 12 $F_2$s, and 12 $F_3$s were cultured on N6 medium to induce callus formation (Chu and Croughan 1988b). Upon reaching 2 m in diameter, calluses were transferred to MS (Murashige and Skoog 1962) regeneration medium supplemented with 0.5 mg/L naphthalene acetic acid (NAA) and 2.0 mg/L Kinetin (KT). Cultures were maintained at 25 °C with 16:8 hours of fluorescent light. Upon reaching the three-leaf stage, regenerated plantlets were subcultured to fresh dishes of the same medium to promote continued development.

### Statistical analysis

The plant regeneration rate was expressed as a percentage calculated as the number of green plantlets obtained divided by the number of calluses transferred. Generation mean analysis (Gamble 1962) was used to estimate genetic components of means for green plant and albino plant regeneration rates. To evaluate the contribution of various gene effects to the total variation among generation means, a six-parameter model was used to calculate estimates of genetic effects. The general model for the observed mean of any generation, Y, is

$$Y = m + \alpha + \beta d + \alpha^2 aa + 2\alpha\beta ad + \beta^2 dd,$$

where m represents the mean of a reference population ($F_2$); a and d represent pooled additive and pooled dominance effects, respectively; and aa, ad, and dd are the pooled digenic interaction effects of additive x additive, additive x dominance, and dominance x dominance gene effects, respectively.

## RESULTS

### Green and albino plant regeneration rates for parents and $F_1$s

Green and albino plant regeneration rates are presented in Table 1. The cultivar 'Short Tetep' had the highest value for green plant regeneration (64%). 'Lemont' had an intermediate plant regeneration rate (3%), while 'IR36' and 'Gui Chow' produced no plantlets. Significant differences in plant regeneration rates were found in mean comparisons between 'Short Tetep' and 'Lemont', and between 'Lemont' and 'IR36', and between 'Gui Chow' and 'Lemont'. The mean plant regeneration rates in the 12 $F_1$s ranged from 0 to 200% and significant differences were found among $F_1$s and their

**Table 1. Plant regeneration in four parents and their 12 $F_1$s.**

| Parents and $F_1$s | No. calluses transferred | No. green plantlets produced | No. albino plantlets produced | Mean green regen. rate (%) | Mean albino regen. rate (%) |
|---|---|---|---|---|---|
| 'Lemont' | 362 | 11 | 53 | 3.0 | 14.6 |
| Lemont/IR36 | 19 | 16 | 5 | 84.2 | 26.3 |
| Lemont/ST | 41 | 5 | 42 | 12.2 | 102.4 |
| Lemont/GC | 2 | 0 | 0 | 0.0 | 0.0 |
| 'IR36' | 1 | 0 | 1 | 0.0 | 100.0 |
| IR36/Lemont | 40 | 11 | 14 | 27.5 | 35.0 |
| IR36/ST | 14 | 0 | 3 | 0.0 | 21.4 |
| 'Short Tetep' | 44 | 28 | 139 | 63.6 | 315.9 |
| ST/Lemont | 414 | 830 | 419 | 200.4 | 101.2 |
| ST/IR36 | 5 | 0 | 18 | 0.0 | 360.0 |
| Gui Chow | 13 | 0 | 8 | 0.0 | 61.5 |
| GC/Lemont | 3 | 3 | 3 | 100.0 | 100.0 |

reciprocals. This indicates that maternal effects may play a role in plant regeneration. All crosses having 'Lemont' as one parent produced green plants except Lemont/Gui Chow. The mean plant regeneration rates in the crosses Lemont/IR36, IR36/Lemont, Short Tetep/Lemont, and Gui Chow/Lemont exceeded both parents, indicating overdominance. Four crosses, IR36/Gui Chow, Short Tetep/Gui Chow, Gui Chow/IR36, and Gui Chow/Short Tetep, failed to produce callus and, therefore, regenerated no plants. Regarding albino plant regeneration rates, 'Short Tetep' was the highest among the four cultivars. Significant differences existed among cultivars and their $F_1$s for this trait.

### Green and albino plant regeneration rates in $F_2$ and $F_3$ populations

A total of 3207 calluses from 12 $F_2$ populations were transferred to a differentiation medium to evaluate plant regeneration rates (Table 2). No plants were regenerated from the cross IR36/Gui Chow and its reciprocal, reflecting the poor regeneration rates of both parents. The other 10 crosses varied in their mean regeneration rates, ranging from 2.5% for Short Tetep/IR36 to 121% for Short Tetep/Lemont. Genotypic differences were significant among $F_2$s. All $F_2$s produced albinos, ranging from 13% for Lemont/IR36 to 360% for Gui Chow/Lemont.

Green plant and albino regeneration rates for the 12 $F_3$ populations are shown in Table 3. The mean green plant regeneration rate varied from 0% for IR36/Gui Chow to 335% for Gui Chow/IR36. The albino regeneration rates of the 12 $F_3$s ranged from 0% to 311%.

**Table 2. Green and albino plant regeneration rates in the $F_2$s.**

| Cross | No. calluses transferred | No. green plantlets produced | No. albino plantlets produced | Mean green regen. rate (%) | Mean albino regen. rate (%) |
|---|---|---|---|---|---|
| Lemont/IR36 | 241 | 37 | 32 | 15.3 | 13.2 |
| Lemont/ST | 630 | 624 | 543 | 99.0 | 86.1 |
| Lemont/GC | 613 | 576 | 303 | 93.9 | 9.4 |
| IR36/Lemont | 225 | 24 | 92 | 10.6 | 40.8 |
| IR36/ST | 77 | 53 | 186 | 68.8 | 241.5 |
| IR36/GC | 28 | 0 | 17 | 0.0 | 60.7 |
| ST/Lemont | 981 | 1191 | 1393 | 121.4 | 142.0 |
| ST/IR36 | 79 | 2 | 137 | 2.5 | 173.4 |
| ST/GC | 92 | 29 | 235 | 31.5 | 255.4 |
| GC/Lemont | 92 | 75 | 331 | 81.5 | 359.7 |
| GC/IR36 | 43 | 0 | 115 | 0.0 | 267.4 |
| GC/ST | 106 | 31 | 339 | 29.2 | 319.8 |

### Green plant and albino regeneration rates in 24 $BCF_1$s

The mean green plant and albino regeneration rates of 24 $BCF_1$s are presented in Table 4. The mean green plant regeneration rates of $BCF_1$s increased, in general, when the $F_1$ was backcrossed to the high parent, and decreased when it was backcrossed to the low. An example can be found in the $BCF_1$ of Lemont/IR36//Lemont (9.8%) and in Lemont/IR36//IR36 (0%). However, $BCF_1$s of Lemont/Gui Chow//Lemont, Lemont/Gui Chow//Gui Chow, Gui Chow/Short Tetep//Gui Chow, and Gui Chow/Short Tetep//Short Tetep did not follow this general trend, possibly due to some unique genetic aspect of the 'Gui Chow' genotype.

Albino rates in $BCF_1$s were dependent on the individual cross. Most crosses that involved 'Short Tetep' as a parent produced high rates of albinos. The trait of producing albino regenerates appears highly heritable, since 'Short Tetep' had the highest albino production rate (316%) among the four parents.

### Genetic estimates of green plant and albino regeneration rates

Mean plant regeneration rates are shown in Table 5. Based on these data, the genetic estimates of additive (a), dominance (d), additive x additive (aa), additive x dominance (ad), and dominance x dominance (dd) were calculated (Table 6). Additive (a) gene effects affected plant regeneration rates in most crosses except Lemont/Gui Chow, IR36/Gui Chow, and Gui Chow/IR36. In the case of IR36/Gui Chow and its reciprocal cross, both parents produced no regenerants and genetic estimates could not be made. However, the relative magnitude of additive gene effects to the mean effects (m) is small compared to dominance effects (d). Therefore, it contributes less to the total variation of the generation means. In contrast, dominance (d), additive x additive (aa), and dominance x dominance (dd) gene effects showed highly significant values, indicating that these factors are significant contributors to the variation of the means.

**Table 3. Plant regeneration in the $F_3$ generation.**

| Cross | No. calluses transferred | No. green plantlets produced | No. albino plantlets produced | Mean green regen. rate (%) | Mean albino regen. rate (%) |
|---|---|---|---|---|---|
| Lemont/IR36 | 27 | 0 | 0 | 0.0 | 0.0 |
| Lemont/ST | 120 | 147 | 229 | 122.5 | 190.8 |
| Lemont/GC | 685 | 252 | 311 | 36.7 | 45.4 |
| IR36/Lemont | 362 | 75 | 78 | 20.7 | 21.5 |
| IR36/ST | 22 | 39 | 13 | 177.2 | 59.0 |
| IR36/GC | 10 | 0 | 0 | 0.0 | 0.0 |
| ST/Lemont | 500 | 414 | 350 | 82.8 | 70.0 |
| ST/IR36 | 7 | 16 | 9 | 228.5 | 128.5 |
| ST/GC | 222 | 72 | 691 | 32.4 | 311.2 |
| GC/Lemont | 15 | 42 | 18 | 280.0 | 128.5 |
| GC/1R36 | 17 | 57 | 16 | 335.2 | 94.1 |
| GC/ST | 69 | 5 | 127 | 7.2 | 184.0 |

The mean albino plant regeneration rates are shown in Table 7. The genetic estimates calculated by generation mean analysis are presented in Table 8. Additive gene effects (a) appear to be significant in 8 of 12 crosses with relatively small magnitudes for the mean effects (m). However, dominance (d), additive x additive (aa), and dominance x dominance (dd) are significant with relatively large magnitudes to their mean effects. These gene effects play an important role in the total variation of the generation means.

## DISCUSSION

The mean regeneration rates of the four parents and their $F_1$s in this study showed overdominance and maternal effects in some crosses. However, high plant regeneration rates appeared recessive in the crosses Lemont/Short Tetep, Lemont/Gui Chow, IR36/Short Tetep, IR36/Gui Chow, Short Tetep/IR36, Short Tetep/Gui Chow, Gui

**Table 4. Regeneration rates of 24 $BCF_1$s.**

| Cross | No. calluses transferred | No. green plantlets produced | No. albino plantlets produced | Mean green regen. rate (%) | Mean albino regen. rate (%) |
|---|---|---|---|---|---|
| Lemont/IR36//Lemont | 235 | 23 | 18 | 9.7 | 7.6 |
| Lemont/IR36//IR36 | 15 | 0 | 0 | 0.0 | 0.0 |
| Lemont/ST//Lemont | 1241 | 732 | 940 | 58.9 | 75.7 |
| Lemont/ST//ST | 35 | 117 | 99 | 334.2 | 282.8 |
| Lemont/GC//Lemont | 361 | 84 | 143 | 23.2 | 39.6 |
| Lemont/GC//GC | 137 | 32 | 91 | 23.3 | 66.4 |
| IR36/Lemont//IR36 | 53 | 2 | 4 | 3.7 | 7.5 |
| IR36/Lemont//Lemont | 275 | 17 | 36 | 6.1 | 13.0 |
| IR36/ST//IR36 | 7 | 0 | 6 | 0.0 | 85.7 |
| IR36/ST//ST | 44 | 8 | 94 | 18.1 | 213.6 |
| IR36/GC//IR36 | 11 | 0 | 4 | 0.0 | 36.3 |
| IR36/GC/GC | 21 | 0 | 14 | 0.0 | 66.6 |
| ST/Lemont//ST | 1571 | 1149 | 1132 | 73.1 | 72.0 |
| ST/Lemont//Lemont | 414 | 458 | 538 | 110.6 | 129.9 |
| ST/IR36//ST | 38 | 28 | 42 | 73.6 | 110.5 |
| ST/IR36//IR36 | 24 | 17 | 15 | 70.8 | 62.5 |
| ST/GC//ST | 59 | 47 | 138 | 79.6 | 233.9 |
| ST/GC//GC | 41 | 15 | 112 | 36.5 | 273.1 |
| GC/Lemont//GC | 7 | 0 | 14 | 0.0 | 200.0 |
| GC/Lemont//Lemont | 10 | 11 | 22 | 110.0 | 220.0 |
| GC/IR36//GC | 6 | 0 | 0 | 0.0 | 0.0 |
| GC/IR36//IR36 | 3 | 0 | 0 | 0.0 | 0.0 |
| GC/ST//GC | 15 | 63 | 13 | 420.0 | 86.6 |
| GC/ST//ST | 130 | 24 | 257 | 18.4 | 197.6 |

**Table 5. Mean plant regeneration rates (%) for parents and progeny.**

| Cross | $P_1$ | $P_2$ | $F_1$ | $F_2$ | $F_2$ | $P_1F_1$ | $P_2F_1$ |
|---|---|---|---|---|---|---|---|
| Lemont/IR36 | 3.0 | 0.0 | 84.2 | 15.3 | 0.0 | 9.8 | 0.0 |
| Lemont/ST | 3.0 | 63.6 | 12.2 | 99.0 | 122.5 | 58.9 | 334.2 |
| Lemont/GC | 3.0 | 0.0 | 0.0 | 93.9 | 36.7 | 23.2 | 23.3 |
| IR36/Lemont | 0.0 | 3.0 | 27.5 | 10.6 | 20.7 | 3.7 | 6.1 |
| IR36/ST | 0.0 | 63.6 | 0.0 | 68.8 | 177.2 | 0.0 | 18.1 |
| IR36/GC | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| ST/Lemont | 63.6 | 3.0 | 200.4 | 121.4 | 82.8 | 73.1 | 110.6 |
| ST/IR36 | 63.6 | 0.0 | 0.0 | 2.5 | 228.5 | 73.6 | 70.8 |
| ST/GC | 63.6 | 0.0 | 0.0 | 31.5 | 32.4 | 79.6 | 36.5 |
| GC/Lemont | 0.0 | 3.0 | 100.0 | 81.5 | 280.0 | 0.0 | 110.0 |
| GC/IR36 | 0.0 | 0.0 | 0.0 | 0.0 | 335.2 | 0.0 | 0.0 |
| GC/ST | 0.0 | 63.6 | 0.0 | 29.2 | 7.2 | 420.0 | 18.4 |

$P_1$ = female parent in cross.
$P_2$ = male parent in cross.
$F_1 = P_1/P_2$.
$F_2$ = selfed seeds from $F_1$.
$F_3$ = selfed seeds from $F_2$.
$P_1F_1$ = $F_1$ backcrossed to female parent.
$P_2F_1$ = $F_1$ backcrossed to male parent.

**Table 6. Genetic components of plant regeneration rates (%) of parents and progeny.**

| Cross | m | a | d | aa | ad | dd |
|---|---|---|---|---|---|---|
| Lemont/IR36 | 15.3 | 9.8* | 41.0** | -41.6** | 8.3* | 193.3** |
| Lemont/ST | 99.0 | -275.3** | 401.0** | 390.3** | -245.01** | -1,085.8** |
| Lemont/Gui Chow | 93.9 | -0.1 | -284.1** | -282.5** | -1.6 | 192.3** |
| IR36/Lemont | 10.6 | -2.4* | 3.2* | -22.7** | -0.9 | 60.8** |
| IR36/ST | 68.8 | -18.1* | -270.7** | -238.9** | 13.6* | -266.2** |
| IR36/Gui Chow | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| ST/Lemont | 121.4 | -37.4* | 49.0* | -118.1** | -67.7* | 218.2** |
| ST/IR36 | 2.5 | 2.8* | 247.0** | 278.9** | -28.9** | -504.2** |
| ST/Gui Chow | 31.5 | 43.0** | 74.6** | 106.4** | 11.2* | -275.2** |
| Gui Chow/Lemont | 81.5 | -110.0** | -7.6 | -106.0** | -108.4** | 89.1** |
| Gui Chow/IR36 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Gui Chow/ST | 29.2 | 401.3** | 728.1** | 759.9** | 443.3** | -1,573.2** |

m, mean of $F_2$ generation; a and d, pooled additive and dominance effects, respectively; aa, ad, and dd, pooled additive x additive, additive x dominance, and dominance x dominance effects, respectively.
Estimates of m were always highly significant.
*, ** Significant at 0.05 and 0.01 probability levels, respectively.

**Table 7. Mean albino plant regeneration rates (%) for parents and progeny.**

| Cross | $P_1$ | $P_2$ | $F_1$ | $F_2$ | $F_3$ | $P_1F_1$ | $P_2F_1$ |
|---|---|---|---|---|---|---|---|
| Lemont/IR36 | 14.6 | 100.0 | 26.3 | 3.2 | 0.0 | 7.6 | 0.0 |
| Lemont/ST | 14.6 | 315.9 | 102.4 | 86.1 | 190.8 | 75.7 | 282.8 |
| Lemont/GC | 14.6 | 0.0 | 0.0 | 49.4 | 45.4 | 39.6 | 66.4 |
| IR36/Lemont | 100.0 | 14.6 | 35.0 | 40.8 | 21.5 | 7.5 | 13.0 |
| IR36/ST | 100.0 | 315.9 | 21.4 | 241.5 | 59.0 | 85.7 | 213.6 |
| IR36/GC | 100.0 | 0.0 | 0.0 | 60.7 | 0.0 | 36.3 | 66.6 |
| ST/Lemont | 315.9 | 14.6 | 124.3 | 142.0 | 70.0 | 72.0 | 129.9 |
| ST/IR36 | 315.9 | 100.0 | 360.0 | 173.4 | 128.5 | 110.5 | 62.5 |
| ST/GC | 315.9 | 0.0 | 0.0 | 255.4 | 311.2 | 233.9 | 273.1 |
| GC/Lemont | 0.0 | 14.0 | 100.0 | 359.7 | 120.0 | 200.0 | 220.0 |
| GC/IR36 | 0.0 | 100.0 | 0.0 | 267.4 | 94.1 | 0.0 | 0.0 |
| GC/ST | 0.0 | 315.9 | 0.0 | 319.8 | 184.0 | 86.6 | 197.6 |

$P_1$ = female parent in cross.
$P_2$ = male parent in cross.
$F_1 = P_1/P_2$.
$F_2$ = selfed seeds from $F_1$.
$F_3$ = selfed seeds from $F_2$.
$P_1F_1 = F_1$ backcrossed to female parent.
$P_2F_1 = F_1$ backcrossed to male parent.

**Table 8. Genetic components of albino regeneration rates (%).**

| Cross | m | a | d | aa | ad | dd |
|---|---|---|---|---|---|---|
| Lemont/IR36 | 13.2 | 7.6 | -68.7** | -37.7** | 50.3** | 189.7** |
| Lemont/ST | 86.1 | -207.1** | 309.6** | 372.4** | -56.4** | -554.2** |
| Lemont/Gui Chow | 49.4 | -26.8* | 7.0 | 14.3 | -34.1* | -211.7** |
| IR36/Lemont | 40.8 | -5.5 | -144.6** | -122.2** | -48.2* | 256.6** |
| IR36/ST | 241.5 | -127.9** | -554.0** | -367.5** | -19.9 | 227.6** |
| IR36/Gui Chow | 60.7 | -30.3** | -86.7** | -36.7** | -80.3** | -69.2** |
| ST/Lemont | 142.0 | -57.8* | -204.9** | -163.9** | -20.8 | 339.1** |
| ST/IR36 | 173.4 | 40.8* | -159.5** | -347.6** | -59.9* | 1,137.4** |
| ST/Gui Chow | 255.4 | 39.2 | -165.5** | -7.5 | -197.2** | -690.6** |
| Gui Chow/Lemont | 267.4 | 0.0 | -1,119.7** | -1,069.7** | 50.0 | 1,169.7** |
| Gui Chow/IR36 | 267.4 | 0.0 | -1,119.7** | -1,069.7** | 50.0 | 1,169.7** |
| Gui Chow/ST | 319.8 | -112.0* | -868.4** | -710.5** | 46.9 | 457.7** |

m, mean of $F_2$ generation; a and d, pooled additive and dominance effects, respectively; aa, ad, and dd, pooled additive x additive, additive x dominance, and dominance x dominance effects, respectively. Estimates of m were always highly significant.

* ** Significant at 0.05 and 0.01 probability levels, respectively.

Chow/Short Tetep, and Gui Chow/IR36. The low plant regeneration rates in these $F_1$ crosses may be attributable to the parents Gui Chow and IR36 possessing a dominant trait for low regeneration capabilities.

Green plant and albino regeneration rates in the $F_2$ and $F_3$ generations followed a continuous segregation pattern. However, generation means of the traits shifted for individual crosses. The genetic factors involved in regeneration from AC therefore appear more complex than those involved in immature panicle culture (Chu and Croughan 1988a) and the formation of callus from anthers (Chu and Croughan 1988b).

This study on green and albino plant regeneration revealed a complicated genetic background controlling these traits. Overdominance, recessiveness, and maternal effects were observed, suggesting that expression of high regeneration rates may involve an interaction of cytoplasmic factors and nuclear genes. Values for dominance gene effects (d) and epistatic effects (aa, ad, and dd) suggest that they contribute more to the variance of generation means than additive effects (a).

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**RESUMEN**

Se investigó la genética de la regeneración de plantas a partir de callos derivados mediante el cultivo de anteras empleando cuatro variedades de arroz ('Lemont', 'Short Tetep', 'IR36' y 'Gui Chow'), las generaciones $F_1$, $F_2$ y $F_3$ de un cruzamiento dialélico de estas cuatro variedades, y 24 $BCF_1$ (líneas retrocruzadas $F_1$). Se calcularon los índices de regeneración de plantas verdes y de plántulas albinas como el número de plantas verdes y albinas regeneradas a partir de 100 callos después de la transferencia a un medio de diferenciación en condiciones de cultivo uniformes. En la variedad Lemont, se regeneraron plantas a razón de tres plantas por cada 100 callos (3%), en tanto que en las variedades 'IR36' y 'Gui Chow' no se regeneró ninguna planta. La variedad 'Short Tetep' presentó un elevado índice de regeneración de plantas verdes (63%) y de plantas albinas (316%). Los índices correspondientes a 'IR36' y a 'Gui Chow' fueron muy diferentes del índice de Lemont, que fue estadísticamente diferente del índice de 'Short Tetep'. Los índices medios de regeneración de plantas verdes y albinas en las 12 generaciones $F_1$ presentaron dominio excesivo y recesividad en algunos cruzamientos. Se encontró una diferencia recíproca importante en la cruza de 'Lemont' y 'Short Tetep'. La evaluación de las 24 $BCF_1$ indicó que los índices medios de regeneración de plantas suelen aumentar mediante el retrocruzamiento con progenitores de alta fecundidad y disminuir con el retrocruzamiento con progenitores de baja fecundidad. El análisis de la media generacional indica que la dominación (d), la acumulación x dominación (ad) y la dominación x dominación (dd) constituyen los principales factores de variación de las medias generacionales, aunque en ciertos cruzamientos es importante el efecto de los genes acumulativos (a). Desde un punto de vista práctico para el cultivo de anteras del arroz, pueden obtenerse índices más elevados de regeneración a partir de cruzamientos que incluyan un progenitor de alta fecundidad y uno de baja fecundidad, utilizando para el cultivo de anteras la generación $F_2$ en lugar de la $F_1$.

# Studies of somaclonal variation in *Brassica* spp. and its relevance to improvement of stress tolerance and yield

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Through a combination of biotechnological and conventional approaches, the following gains have been achieved for improvement of oilseed brassicas: 1) genetic variability for productivity traits and stress tolerance has been induced in agronomically acceptable genotypes of *Brassica juncea*, *B. napus*, and *B. carinata*; a high proportion of induced variability is in the positive direction; 2) protocols have been developed to generate somatic embryos, both directly and indirectly, from cultured protoplasts of *B. juncea* and these have been successfully utilized for making *in vitro* selection for resistance to salt and alternaria toxin; 3) male sterile somaclones have been identified in *B. juncea*, *B. napus*, and *B. carinata*; 4) a male sterile alloplasmic line has been developed in *B. campestris* with *B. oxyrrhina* cytoplasm; the somaclonal and alloplasmic male sterility is being developed into a system suited to producing heterotic hybrids; and 5) shatter-resistant *B. napus* has been produced by introgression of this trait from B. juncea by nonhomologous recombination.

Oleiferous brassicas are an important source of edible oil in India. In area and production they rank second only to groundnut. In 1986, brassicas were grown on 3.8 million ha and yielded 2.6 million tons of oilseeds. Despite their antiquity (their seeds have been excavated from sites of the Indus Valley civilization and numerous references exist in Vedic literature dating back to 1500 BC), the productivity of brassica varieties in India is very low. The average Indian yield of 695 kg/ha in 1986, compared with 2693 kg/ha in countries of western Europe, underscores the wide yield

gap. If this gap were narrowed, it would save the Indian economy massive expenditures on edible oil imports (more than 1.35 million tons in 1987). Among *Brassica* spp. grown for oilseeds in India, *B. juncea* accounts for 85% of the area under cultivation. Three ecotypes of *B. campestris* sp. *oleifera* (yellow sarson, brown sarson, and toria) make up the rest of the area under brassicas.

Success in breeding genotypes of *B. juncea* and *B. campestris* with yield stability has been limited by a lack of exploitable genetic variability for productivity traits and sources for resistance to diseases (notably white rust caused by *Albugo candida* and alternaria caused by *Alternaria brassicae*) and pests (aphids) and tolerance to drought and salinity. Utilization of biotechnological approaches such as somaclonal variation, *in vitro* selection, production of alloplasmic lines, and realization of products of wide hybridization, therefore, assume great relevance. Biotechnological procedures can also be exploited for achieving diversification of cultivable brassicas by modifying species such as *B. napus* and *B. carinata* to fit into the Indian cropping system. In this paper, we summarize promising work for the improvement of *B. juncea*, *B. napus*, and *B. carinata* using biotechnological intervention.

## CREATION OF USABLE VARIABILITY FOR PRODUCTIVITY TRAITS

### Somaclonal variation for agronomic characters

Plants regenerated from a tissue culture cycle involving a de-differentiated (callus) phase are known to show variation for a wide range of characteristics. The frequency with which such variation occurs is, in many cases, far greater than that created by conventional mutagenesis. In maize, it can be as high as one mutation in every regenerant (Edallo *et al.* 1981) and in tomato, one mutation among 20 to 25 regenerants (Evans and Sharp 1983). In tomato, somaclonal variants were produced that have not been recorded before in induced mutation experiments. A major breeding advantage of somaclonal variation is that the relatively mild conditions of *in vitro* growth do not drastically alter the genetic background, and therefore the agronomic value of the variant is not adversely affected. Our experiments with *B. juncea* and *B. carinata* have shown that the patterns of variation are different among somaclones and their progenies compared with corresponding mutagenized population. Also, the generated variation is of distinct breeding value.

*Assessment of yield and its components in a replicated trial.* Two hundred and sixty-nine R3 somaclonal lines originating from 30 R1 plants were evaluated for yield and its major components in a randomized block design with four replications. A number of results are summarized in Table 1. The range of variability for plant height is high. Dwarfs have a height as low as 118 cm while the tall plants exceed 3 m. While the mean height of the parental line was 190 cm, 6.6% of the lines were less than 165 cm and 2.9% more than 215 cm in height. Earliness in *B. juncea* is a desirable agronomic trait and 20% of the somaclones were earlier than the control in 50% flowering. The major seed yield components in *Brassica* spp. are the number of primary branches, secondary branches, pods, and seeds per pod. Plant architecture—in terms of number of secondaries on primaries, and the number of pods on the primaries and the secondaries, and the contribution of primaries and secondaries to seed yield—is important for devising selection criteria. Data in Table 1 show that the

generated variability for all yield-contributing traits is high among the somaclones and a sizable proportion of this variability is in the desired direction. For single plant yield, specifically, 57% of the lines showed superior performance. The best plants showed threefold improvement in yield over the control yield. It appears that the high magnitude of variability in the positive direction should lend itself to genetic advance with significant yield improvement.

*Comparative assessment of somaclonal variation with ethyl-methol-sulphanate and gamma ray-induced variability.* The kind of assessment described in the previous section has also been done in a statistically laid out trial to compare the effect of the somaclonal cycle with variation induced by ethyl-methol-sulphanate (EMS) and gamma rays. The statistical analysis of metric traits is not yet complete, but the data on

**Table 1. Range of variability and number of lines superior to control mean for some economically important traits among 269 $R_3$ lines of *B. juncea*.**

| Character | Range | Control (pop. mean) | No. of lines superior to control | % lines superior to control |
|---|---|---|---|---|
| Plant height (cm) | 118-301 | 190 | 18[a]<br>8[b] | 6.6[a]<br>2.9[b] |
| No. of primary branches/plant | 2.6-9 | 6 | 58 | 21.5 |
| No. of pods on primary brances | 40.6-165 | 80 | 105 | 39 |
| No. of secondary branches in primary branches | 2.0-13 | 9 | 22 | 8.1 |
| No. of pods on secondary branches | 17-191.6 | 90 | 92 | 34.2 |
| Yield due to primary branches (g) | 0.15-19.9 | 2.6 | 167 | 62.0 |
| Yield due to secondary branches (g) | 0.02-18.8 | 4.3 | 104 | 38.6 |
| Single plant yield (g) | 0.2-36.5 | 12.5 | 150 | 55.7 |

[a] Less than 165 cm.

[b] Greater than 215 cm.

days to 50% flowering are given in Tables 2 and 3 and show a number of interesting points. The number of progenies flowering in the parental range of 54-58 days is highest among the somaclones (40.8%). A considerably higher number of progenies belonging to gamma ray (59.8%) and EMS (66.1%) flower later than the control. Progenies flowering earlier than the control are higher among somaclones (20.1%) compared with gamma rays (12.6%) and EMS (7.8%) treatments. And it is likely that early flowering variation arises from gene mutations, while late flowering is a reflection of gross alterations in the genetic apparatus. If this is confirmed, variability arising from somaclonal variation will have a clear advantage in that the genetic background is not severely altered.

*Variation in R2 generation of B. carinata.* Scoring of R2 variation has revealed that of the 452 lines, each derived from a different R1 regenerant, 18.8% segregated for pigmentation of stem, petiole or leaf; 11.7% for waxy leaf; and 8.1% for flower color. This is a high frequency of variation. From a more practical viewpoint, the early flowering and early maturing somaclones recovered in *B. carinata* are significant because yield evaluation trials have shown that *B. carinata* has yield advantage over *B. juncea*, but it does not fit into the cropping system under Indian conditions because of its prolonged vegetative growth and late maturity. Under New Delhi conditions, *B. carinata* takes 105 days for 50% flowering. The early flowering somaclones have desirable features such as early flowering (75 days for 50% flowering) and a dwarf, semi-determinate growth habit that gives synchronous development of pods on the plant canopy exposed to sunlight. Thus, it should be possible to introduce *B. carinata* into cultivation in India and take advantage of its yield potential and natural characteristics of disease and pest resistance.

## Gene introgression by wide crosses

*Introgression of resistance to pod shattering into B. napus through nonhomologous recombination.* A range of wild and weedy relatives of crop brassicas grouped under *Brassica coenospecies*, and including *Diplotaxis*, *Erucastrum*, *Eruca*, *Sinapis*, *Sinapi-*

**Table 2. Frequency distribution for days to 50% flowering in $R_3$ and $M_3$ populations of *B. juncea* cv. Varuna.**

| Class interval for days to flower | No. of progenies | | |
|---|---|---|---|
| | Somaclones $R_3$ | radiation-derived $M_3$ | EMS-derived $M_3$ |
| 49-53 | 54 | 34 | 21 |
| 54-58 | 110 | 74 | 70 |
| 59-63 | 68 | 136 | 134 |
| 64-68 | 22 | 15 | 29 |
| 69-73 | 13 | 4 | 7 |
| 74-78 | 2 | 5 | 8 |
| more than 80 | 0 | 1 | 0 |

Control Varuna falls in the second class interval, i.e. 54 to 58 days.

*dendron*, and *Raphanus*, are a reservoir of useful genes. Hybrids between cultivated brassicas and members of the *coenospecies* group are not easily obtained. In addition, several mechanisms hinder gene flow even when wide cross hybrids are obtained. When a character of critical importance is not available within a species, however, interspecific and intergeneric transfers become imperative. Resistance to pod shattering in *B. napus* is one such character not available in the world germplasm. We have successfully introgressed resistance to pod shattering from *B. juncea* to *B. napus* (Prakash and Chopra 1988a).

A synthetic *B. napus*, obtained from the cross between *B. campestris* ssp. *oleifera* var. *brown sarson* and *B. oleracea* var. *botrytis*, was used as the recipient. The donor of shattering resistance was also a synthetic amphiploid of *B. juncea* obtained by chromosome doubling of the cross *B. campestris* ssp. *oleifera* var. *brown sarson* x *B. nigra*. The $F_1$ (2n=37, AABC) was resistant to pod shattering. Pollen mother cells of the hybrid showed 10II + 17I in 90.7% of the cells and 14II + 9I in 9.3% of the cells at metaphase I. The $F_1$ was backcrossed to *B. napus* and the BC2 yielded three types of plants: 1) 9 plants resembling *B. juncea*, 2) 43 plants intermediate in the phenotype between *B. juncea* and *B. napus*, and 3) 17 plants resembling the *B. napus* parent. One of these 17 was resistant to pod shattering and was further backcrossed to *B. napus*. Among the progeny, 69 plants resembling *B. napus* were obtained, but only one was resistant to shattering. This 38-chromosome plant formed regular 19II at metaphase I. The plant had high pollen fertility (84%), but low seed fertility (23%). We attribute the introgression of shattering resistance to nonhomologous transfer of the concerned genetic segment from the B genome of *B. nigra* to the C genome of *B. oleracea* in the $F_1$ hybrid AABC. Cells with 14II + 9I (9.3%) in which four bivalents would result from pairing between the B and C genome chromosomes provide cytological evidence in support of this suggestion.

The low seed fertility and synthetic origin of *B. napus* limit the direct applied use of this plant, but it provides an important genetic stock for *B. napus* breeding for overcoming a problem that has defied solution so far.

*Developing protocols for protoplast culture and regeneration for making somatic hybrids*. One way of producing hybrids between parents, irrespective of their genetic relationship, is to resort to parasexual crosses. Such somatic hybrids can bring together both nuclear and cytoplasmic genomes and have been successfully produced both within the genus *Brassica* and between it and other genera. As a prerequisite to making

**Table 3. Grouping of somaclones $R_3$ and corresponding EMS and gamma radiation, derived $M_3$ for days to 50% flowering in relation to parental class.**

| Category | Somaclones $R_3$ | Gamma ray $M_3$ | EMS $M_3$ |
|---|---|---|---|
| Later than control | 105 (39.0) | 161 (59.8) | 178 (66.1) |
| Parental or control class | 110 (40.8) | 74 (27.5) | 70 (26.0) |
| Earlier than control | 54 (20.1) | 34 (12.6) | 21 (7.8) |

Percent population falling in each category is presented in parentheses.

somatic hybrids for improvement of crop brassicas, we have developed protocols of regenerating plants from cultured hypocotyl protoplasts of *B. juncea* cv. RLM-198 through direct and indirect somatic embryogenesis and shoot organogenesis.

Protoplasts were isolated from etiolated 6-day-old hypocotyls and cultured in a modified V47 medium (Binding 1974). On the 14th day of culture, protoplast-derived cell colonies were plated in agarose. Microcalluses, which were obtained during 4 weeks of culture, developed into calluses on solid medium. About 35% of the plated calluses produced 1 to 18 shoots. These shoots could be grown into intact plants.

Direct and indirect somatic embryogenesis also occurred in protoplast culture. Direct somatic embryogenesis, occurring with a very low frequency (0.0001%), followed a specific pathway. An unequal cell division resulted in a small cell with dense cytoplasm and a large cell with vacuolated cytoplasm. Such unequal cell division leading to direct somatic embryogenesis has been reported in *Foeniculum vulgare* (Miura and Tabata 1986).

Indirect somatic embryogenesis—i.e., embryoids developing on protoplast-derived calluses—occurs more frequently (6-8%) when microcalluses are plated on media containing a reduced amount of 2,4-D and/or another auxin, for example, naphthalene acetic acid. The embryoids proliferated on hormone-free media and produced multiple shoots.

Reasonably high frequency of shoot formation from hypocotyl protoplast-derived calluses provides a suitable system for producing somatic hybrids. Direct somatic embryogenesis offers a pathway for selecting nonchimeral mutants of agronomic importance in mustard.

## HETEROSIS EXPLOITATION

### Isolation of male steriles in somaclonal population

Stably expressing male sterility coupled with fertility restoration is a necessary requirement for producing a commercially exploitable heterotic hybrid. Diversification of cytoplasmic sterility sources is equally important to provide insurance against outbreaks of disease epidemics. Since conventional mutagenic treatments are not very effective for inducing mutations in cytoplasmic genomes and somaclonal variation does not discriminate between the nuclear and cytoplasmic genomes for generating variation, we have screened somaclonal populations of *B. juncea*, *B. napus*, and *B. carinata* for male steriles and have been successful in identifying them. Initially identified by the stainability tests, male sterility has been confirmed by controlled pollination. All male steriles set no seed when selfed, but yield seed on pollination with fertile pollen. The mechanism of sterility, its genetic control, and fertility restoration are under investigation.

*Exploiting nuclear cytoplasmic interactions for inducing male sterility.* Interactions between nuclear and plasmagenes brought about by wide hybridization in *Brassica* have been reported to cause cytoplasmic male sterility. Ogura (1968) discovered a sterility inducing cytoplasm in Japanese wild radish (*Raphanus sativus*), which was transferred to *B. oleracea*, *B. campestris*, and *B. napus*. Similarly, replacement of cytoplasm of *B. campestris* ssp. *pekinensis* with that of *Diplotaxis muralis*

resulted in cytoplasmic male sterility (Hinata and Konno 1979). We have synthesized a *B. campestris* ssp. *oleifera* var. *brown sarson* alloplasmic strain with *B. oxyrrhina* cytoplasm which is male sterile (Prakash and Chopra 1988b).

The male sterile plant closely resembles *B. campestris* in morphology and growth pattern. However, the leaves were mildly chlorotic at earlier stages of growth, but turned normal green later. The anthers were slender, nondehiscent, and contained only sterile pollen. The plant has cytologically normal meiosis and pollen abortion occurs at the tetrad stage. The sterility-inducing cytoplasm is being transferred to *B. juncea*, *B. napus*, and other species. The use of different cytoplasmic donors for producing sterility in alloplasmic lines provides the necessary diversification to guard against the dangers inherent in the use of a single source of male sterility.

## IMPROVING STRESS TOLERANCE

*In vitro* selection for tolerance to abiotic stresses (salinity, acidity, toxic ions) and resistance to diseases causing damage via toxins offers the advantage that large populations can be screened for rare resistant mutants under uniform conditions of restrictive environments. There are three requirements. First, it should be possible to regenerate plants from the selected resistant cells/calluses. Second, the intervening callus phase should be limited, if it cannot be avoided altogether. This becomes necessary to maintain genetic integrity of the material into which resistance is to be incorporated. And third, the selection must be a true genetic change and not merely a physiological adaptation. We have developed an approach for *in vitro* selection for salt tolerance and resistance to the *Alternaria* toxin in *B. juncea*.

### *In vitro* selection for salt resistance

Since somatic embryos generally arise from single cells or very small groups of cells, somatic embryogenesis coupled with *in vitro* selection offers a promising system for mutant selection. A system of effective somatic embryogenesis from hypocotyl explants has been developed for *B. juncea* (Kirti *et al.* 1987, Kirti and Chopra 1989) and has been used in two ways for studies on salt tolerance.

*Producing somatic embryos on salt-containing medium.* Earlier, we described a method by which hypocotyl explants produced somatic embryos within 3 to 4 weeks of total culture (Kirti and Chopra 1989). The method was extended to generating somatic embryos on embryogenic medium supplemented with sodium and potassium chloride salts. It was found that the addition of 0.5% salt reduces the percentage of cultures showing embryogenesis. Increasing the salt content to 1% inhibited even the callusing on the cut ends and further reduced embryogenesis (Table 4). Of a total of 2000 explants inoculated on a medium containing 1% salt in three experiments, only one embryo was produced. In a subsequent experiment, embryos were produced on a medium with 0.5% salt and then transferred for proliferation on a medium with 1% salt. Of 50 such embryos, six survived. From the seven selected embryos, eight plants were raised and all set seed. Prior to testing the salt resistance of the seed progeny, explants from the stems of regenerated plants have been cultured on a MS (Murashige and Skoog 1962) medium containing 0.5 mg/L of 2,4-D + 0.5 mg/L of Benzylaminopurine

(BAP) with 1% salt. While the control explants did not survive on this medium, explants from the resistant regenerants produced profuse calluses. It suggests, therefore, that the selected resistance is not epigenetic.

*Selection for salt resistance among embryos regenerated on a normal medium.* In this approach, embryoids were regenerated on a normal embryogenic medium and selection made for variants resistant to salt in the medium. In an experiment, of 200 embryoids inoculated on a medium containing 1% salt, one proliferated. The advantage of this method is that it allows selection of embryoids capable of germination and development into a plantlet directly. No post selection callusing, is involved and hence there is no chance of loss of genetic integrity of the parental material.

### *In vitro* selection for resistance to *Alternaria brassicae* toxin

Blight, caused by *Alternaria brassicae*, is the most destructive of diseases on *Brassica* spp. in India and causes yield losses up to 40% (Labana 1982). Since *Alternaria* symptoms are mediated through a toxin, the system is ideally suited to making selection for resistance *in vitro*. The crude toxin, produced by the Central Institute for Medicinal and Aromatic Plants from an infective culture, was incorporated into the culture medium according to the protocol essentially similar to that used for salt tolerance. The response to toxin concentration gradient for callusing and somatic embryogenesis is in Table 5. It shows that a toxin concentration exceeding 100 mg/L inhibits callusing and prevents embryogenesis. At a concentration of 100 mg/L toxin, of 750 explants, callus from three explants produced six embryoids. These embryoids, on transfer to a proliferation medium containing 250 mg/L toxin, yielded six plantlets that were grown to maturity.

**Table 4. Generation of somatic embryos on salt-containing medium in *B. juncea* cv. RLM 198.**

| Medium | No. of explants plated | % callusing | No. of explants producing somatic embryos |
|---|---|---|---|
| Embryogenesis Medium[a] without added salt | 279 | 95-100 | 97 |
| Embryogenesis Medium[a] + 0.25% KCl + 0.50% NaCl | 255 | 95-100 | 31 |
| Embryogenesis Medium[a] + 0.50% KCl + 0.50% NaCl | 268 | 0 | 0 |

[a] Embryogenesis medium = MS + 2% Sucrose + 0.25 mg/L 2,4-D + 0.50 mg/L NAA + 0.50 mg/L BAP-riboside.

Thus, it appears that prudent application of biotechnological tools and techniques can be very helpful in rapid advancement towards achieving difficult crop improvement objectives. Ultimate success, however, will depend on identification of a relevant problem, choice of the right approach to solve the problem, and complete integration of biotechnological methods with conventional plant breeding programs.

**Table 5. Somatic embryogenesis in *B. juncea* on medium containing *Alternaria* toxin.**

| Medium | Number of explants | Percentage of callusing | Number of explants giving embryogenesis |
|---|---|---|---|
| Embryogenesis medium[a] | 279 | 95-100 | 97 |
| Embryogenesis medium[a] + 100 mg/L toxin | 750 | 80 | 3 |
| + 250 mg/L toxin | 750 | 27 | 0 |
| + 500 mg/L toxin | 750 | 0 | 0 |

[a] Embryogenesis medium = MS + 2% Sucrose + 0.25 mg/L 2,4-D + 0.50 mg/L NAA + 0.50 mg/L BAP-riboside

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## RESUMEN

Los siguientes adelantos se han logrado en el mejoramiento de la oleaginosa brassicas mediante una combinación de métodos biotecnológicos y convencionales: 1) se creó variabilidad genética para las características de productividad y de la tolerancia al estrés en genotipos agronómicamente aceptables de *Brassica juncea*, *B. napus* y *B. carinata*; una alta proporción de la variabilidad inducida es positiva; 2) se han establecido protocolos para la generación, tanto directa como indirecta, de embriones somáticos a partir de protoplastos cultivados de *B. juncea* y éstos se han utilizado para la selección *in vitro* de la resistencia a la sal y la toxina de alternaria; 3) somaclones androestériles han sido identificados en *B. juncea*, *B. napus* y *B. carinata*; 4) una línea aloplásmica androestéril ha sido desarrollada en *B. campestris* con citoplasma de *B. oxyrrhina*; la androesterilidad somaclonal y aloplásmica se está convirtiendo en un sistema adecuado para la producción de híbridos heteróticos, y 5) *B. napus* resistente al rompimiento se ha producido incorporando esa resistencia de *B. juncea* mediante la recombinación no homóloga.

# *In vitro* tissue culture selection for sodium chloride (NaCl) tolerance in rice and the performance of the regenerants under saline conditions

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Tissue culture procedures for rice have been improved and are currently highly efficient. Plant regeneration from rice cultures derived from mature seeds can be routinely maintained for over a year. One of the goals is to develop long-term cultures. A regenerating cell line from 'IR36' has been identified and maintained for more than 4 years. Other important aspects are to improve embryogenic callus formation and growth and plant regeneration. Embryogenic callus growth and plant regeneration can be increased by the addition of 1% sorbitol or by culturing R1 seed. The core tissue culture research in the Tissue Culture for Crops Project (TCCP) involves *in vitro* stress selection, regeneration of plants from selected cultures, and evaluation of the regenerated plants for tolerance. *In vitro* selection for sodium chloride (NaCl) tolerance began in 1984. Under greenhouse screening of seedlings, 25% of the regenerated lines performed better than the nonregenerant check, 83% of which came from NaCl-selected cultures. Other individuals that survived saline conditions have been identified in two field evaluations. The *in vitro* selection was with NaCl and individuals plants have shown tolerance to NaCl as well as to alkaline soils with mixed salts.

Rice tissue culture has been prominent in the literature since the early 1980s. Plant regeneration has been achieved from roots (Abe and Futsuhara 1984, Toshinori and Futshuhara 1985), young inflorescence (Chen 1985), leaves (Changjing and Qinghua 1983, Yan and Zhao 1982), mature embryos (Heyser *et al.* 1983), suspension cultures (Abe and Futsuhara 1986), and protoplasts (Coulibaly and Demarly 1986, Abdullah *et*

*al.* 1986). The ability to retain long-term plant regeneration more than 1 year in culture has been discussed by Kishor and Reddy (1986), Heyser *et al.* (1983), and Ram and Nabors (1984) and is a primary concern for *in vitro* selection experiments. Wong *et al.* (1986) reported plant regeneration on sodium chloride (NaCl) stressed medium.

At the Tissue Culture for Crops Project (TCCP), research in rice callus tissue culture has been in progress for several years. This paper reports on the project's current status of tissue culture technology. Our primary objective is to demonstrate that useful germplasm can be obtained from tissue culture. A significant feature of this work is to determine what *in vitro* treatment, including concentration of the stressing agent and the period of time in culture, will produce the greatest probability of field-tolerant plants for a specific environment. Three evaluations are discussed as to the effect of the *in vitro* treatments on salinity tolerance.

## *IN VITRO* TECHNIQUES

### Callus culture and plant regeneration

The basal medium for rice tissue culture used is similar to that developed by Linsmaier and Skoog (1965) with the pH adjusted to 5.5 prior to adding 1.1% agar and autoclaving. Unlike many other crops, rice requires a unique growth regulator combination, sucrose concentration, and light regime for each cultivar (Table 1). Culture vessels are either glass screw vials (26 x 70 mm) or jars (40 x 72 mm). The vials contain 10 ml of medium and are used for callus initiation. The jars containing 20 ml of medium are used after embryogenic callus is isolated and for plant regeneration.

For callus induction, the mature seeds are surface-sterilized and placed, one per vial, on the appropriate medium for that cultivar. At the end of 28 days—referred to as a "passage"—the original explant and all of its associated callus is transferred to a fresh medium. After the second passage, embryogenic (E) callus is isolated and transferred again to a fresh medium. E callus is usually yellow or cream colored and dense in appearance. For long-term maintenance, E callus must be carefully selected and all nonembryogenic and dead callus removed at each transfer. The optimum ratio of E callus to medium for E callus production and plant regeneration is 70 mg in vials and 100 mg in jars.

### Long-term culture and plant regeneration

With careful selection of E callus, a cell line is occasionally isolated that can be continued for extremely long periods. Leaf tissue of Mahsuri was initiated in February 1982. After 72 passages in culture, these cultures are still producing E callus and an occasional regenerated plant. Also, a cell line of 'IR36' initiated from mature seed in June 1984 has been producing an average of 20 plants/g of E callus over the last 12 passages. This cell line has been maintained for 47 passages.

### Embryonic callus formation

Genotypic differences for the ability to form E callus have been reported in several plant species and have also been observed in rice.

Typically, out of 'IR36' or 'Giza 159', 64 or 35 cultures, respectively, will be identified as containing E callus by the fourth passage. The number of cultures with E

**Table 1. Sucrose, light, and growth regulator requirements for embryogenic callus formation and plant regeneration for five rice cultivars.**

| Cultivar | Sucrose (%) | Growth Regulators (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Initiation | | | Maintenance | | Regeneration | | |
| | | 2,4-D[a] | KT[a] | Trp[a] | 2,4-D[a] | KT[a] | IAA[a] | BA[a] | TIBA[a] |
| Calrose | 4.0 | 1.0 | 0.2 | 100 (D)[b] | 2.0 | 0.2 (L) | | | 0.5 |
| Giza 159 | 4.0 | 1.0 | 0.3 | (D) | 1.0 | 0.3 (D) | 0.5 | 0.4 | |
| IR36 | 1.5 | 1.0 | 0.5 | 50 (L) | 0.5 | 0.25 (L) | 0.5 | 0.2 | |
| Mahsuri | 4.0 | 0.5 | 0.5 | (L) | 0.5 | 0.5 (L) | 0.5 | 0.4 | |
| Pokkali | 4.0 | 0.5 | 0.2 | 50 (D) | 0.5 | 0.4 (L) | | 0.5 | |

[a] 2,4-D = 2,4-Dichlorophenoxy acetic acid; KT = Kinetin; Trp = tryptophan; IAA =Indole-3-acetic acid; BA = Benzyladenine; TIBA = 2,3,5-triiodo-benzoic acid.

[b] Letter in parentheses indicates either cultured in the light (L) or dark (D). Regeneration was always in the light.

callus continues to moderate so that by the seventh passage there will be 20 cultures of 'IR36' left and 'Giza 159' will have remained relatively constant. This is a selection process that identifies embryogenic cell lines with the ability to perform in tissue culture. In 'Giza 159', these cell lines can be identified by the fourth passage; in 'IR36' it takes seven passages.

One possible way to increase the number of cell lines, as well as the E callus weight, may be to culture seed from regenerated plants (R1 seed). In a small experiment with 'Giza 159', R1 seed from two plants derived from previous salt selection experiments were put back into tissue culture. One line, R1A, came from a nonstressed culture, and the other, R1J, from a culture selected on 9 g/L of NaCl. The percentage of seed forming E callus was similar for both nonregenerated and regenerated lines. However, the mean E callus weight at the end of the third passage increased from 0.37 g/cell line for the nonregenerated parent to 0.49 g/cell line for both regenerated lines combined. The E callus weight distribution (Fig. 1) indicates that, from the regenerated lines, there are cell lines that will produce between 1.0 and 1.4 g of E callus per passage as compared to less than 1.0 g for the parent. Plant regeneration was 6 times higher in both of the regenerated lines than in the parent.

Another factor under investigation to increase E callus growth is the addition of sorbitol to the medium. The addition of 1 or 2% sorbitol to established E callus for 'Giza 159' at the tenth passage significantly enhanced E callus weight by the end of that passage (Fig. 2). Embryogenic callus weights were higher from the sorbitol treatment over the three passages of the study. Cultures were moved to the regeneration medium following the third passage on sorbitol. The E callus from 2% sorbitol, although

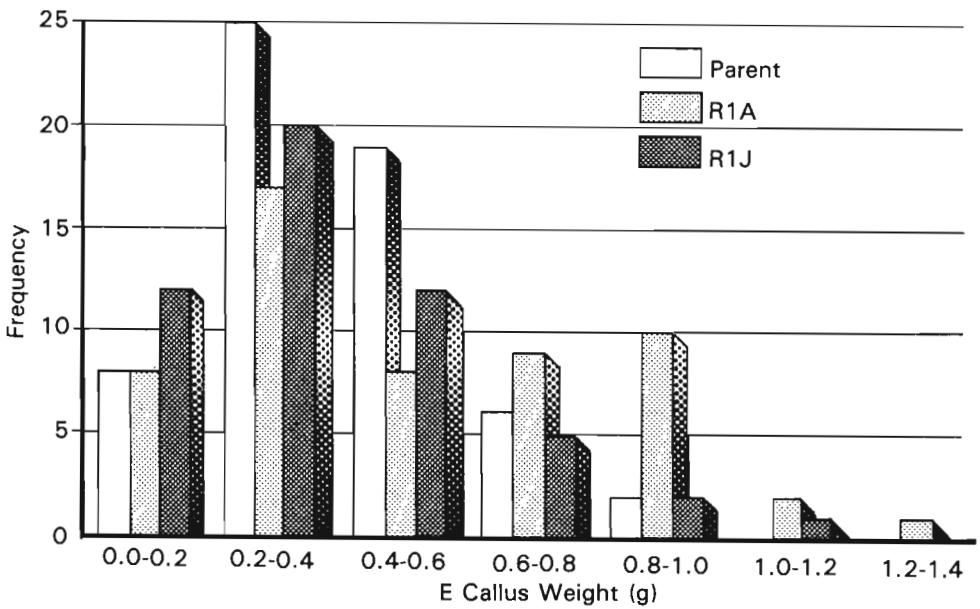


**Figure 1. Frequency distribution of embryogenic callus weights. Distribution of E callus weights showing a near-normal curve for the nonregenerated seed (Parent) and skewed curves for the two regenerated lines.**

significantly more than 0 or 1%, was of poor quality and did not regenerate while the 1% sorbitol-derived E callus regenerated more plants than the control. After finding that sorbitol was a significant factor for E callus maintenance, sorbitol was tested for E callus formation. Embryogenic callus growth is significantly improved in 'Giza 159'. At the end of the third passage, there was an average of 0.44 g/culture with 1% sorbitol and 0.18 g/culture without sorbitol. In 'IR36', E callus weight of the control was 0.06 g/culture and 0.19 g/culture on 1% sorbitol after the second passage. Cultures of 'IR36' require less sucrose than 'Giza 159' but respond equally well to 1% sorbitol, indicating that the sorbitol and sucrose have different functions. The sorbitol effect has been reported by Dr. Nguyen Thanh-Tuyen at the Visayas State College of Agriculture (ViSCA), Philippines (per. comm. 1988) where plant regeneration was enhanced with 3% sorbitol. Kishor and Reddy (1986) also reported a positive effect of 3% sorbitol when added to 6% sucrose. Plant regeneration was maintained for 1400 days with this combination.

These two factors, culturing seed of regenerated material and the addition of sorbitol, have increased E callus growth. Neither technique has been proven to increase the percent of the seeds initiated that will form E callus.

### *In vitro* selection for NaCl tolerance

The first step in our development of an *in vitro* selection technique is to determine the salts to be used and their concentrations. In rice, only NaCl has been used as a selection agent, although other salts or combinations of salts could be used to tailor the *in vitro*

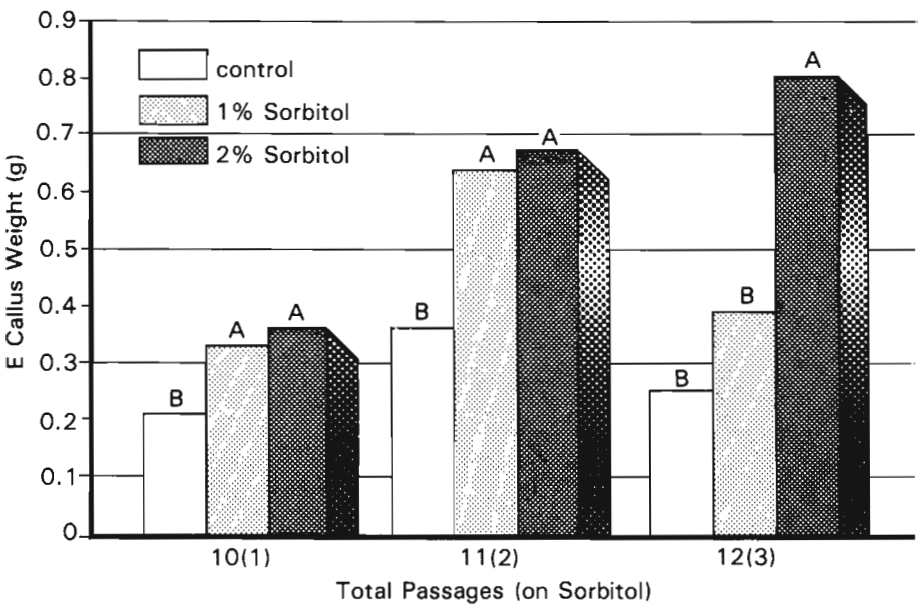


**Figure 2. Effect of sorbitol on maintaining embryogenic callus of Giza 159. Older calluses from ninth passage were moved to two sorbitol treatments and embryogenic callus was weighed after each passage.**

stress to a field environment. Based on preliminary tests, selection levels are chosen so that plant regeneration can be reasonably certain after nine passages of selection. For 'IR36', a more sensitive line than 'Giza 159' to *in vitro* salt, 9 g/L of NaCl is a very high selection level. After determining the selection levels, many selection procedures could be used. The two methods adopted are selection either from callus initiation or after E callus has been identified. When initiating callus under stress, a large number of explants will not form callus. This must be compensated for by plating a larger number of explants. Identification and maintenance of the E callus prior to the *in vitro* selection is an alternate method. With this method, the same cell line can often be placed on all selection levels.

In germplasm development experiments, the number of cultures containing E callus and the plant regeneration rates were recorded. Four treatments were used, two constant levels of NaCl, a stepwise treatment where the stress began at the lower of the two constant levels and then increased to the higher level, and a control. The number of cultures provided a rough estimate of E callus weight since the amount of callus used to initiate the next is controlled at approximately 70 mg. Cell lines were maintained and once the E callus fell below 70 mg the cell line was lost. Often, callus growth was enhanced in the first passage under stress, but rapidly declined afterwards (Fig. 3). The

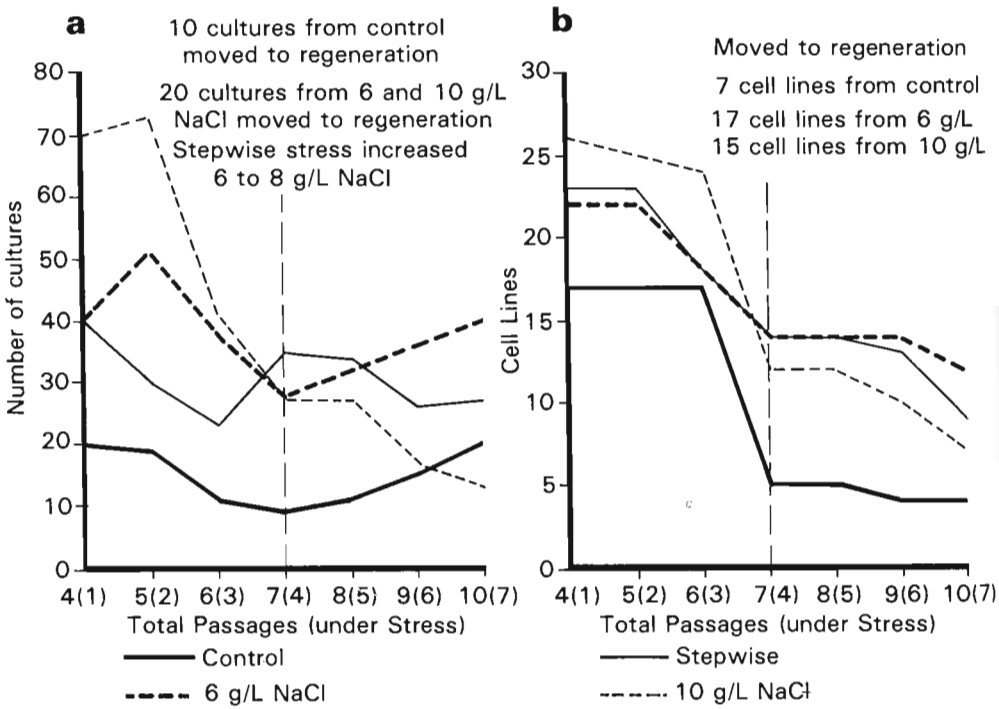


**Figure 3. The effect of NaCl on (a) number of cultures and (b) number of cell lines. NaCl stress began after three passages. After seven passages, some cultures were moved to regeneration media which reduced the number of cultures and cell lines.**

decline was due to a loss of cell lines as well as multiple cultures from the same cell line. The large drop at the seventh passage was partly due to the transfer of callus to regeneration media.

The stepwise treatment began with 6 g/L of NaCl and was expected to perform similarly to the 6 g/L NaCl constant-level selection. This did not occur and reinforces the hypothesis that many cell lines perform differently. There was one cell line, represented only in the 6-g/L constant-selection treatment, that dramatically increased in the first passage on NaCl, thus explaining most of the discrepancy in the number of cultures between the stepwise and 6-g/L constant-selection treatment (Fig. 3a).

In the control, 17 cell lines were represented in 20 cultures at the fourth passage. At the tenth passage, only four cell lines represented 20 cultures, demonstrating more efficient production of E callus. Figure 3 indicates that 6 g/L of NaCl is not a stressful level since the curve for culture number on 6 g/L is very similar to that for the control; however, 10 g/L triggers a strong stress response with a decline in culture number from 70 to 13 at the end of six passages under stress. Plant regeneration rates remained constant for the control from the sixth to ninth passages (Table 2). The 6-g/L treatment is significantly better for plant regeneration than the control after three passages under stress, but little difference is seen after six passages. Plant regeneration from the 10-g/L treatment after three passages was not different from the control, but was better after six passages. The stepwise treatment was moved to the regeneration media only after the sixth passage and the plant regeneration rate was similar to the control.

## EVALUATION OF REGENERATED MATERIAL

### Greenhouse screening

During the first and second weeks of seedling screening in the greenhouse, the plants were grown in 5-cm pots with deionized water only. During the third week a 20-19-18 N-P-K nutrient solution was added. Starting with the fourth week, 3 g/L of NaCl was added to the nutrient solution for the 'IR36' regenerated lines. Chemical analysis showed that the electrical conductivity (EC) increased to 13 mmhos/cm by the seventh week. At the end of the seventh week, the plants were rated for the number of tillers, plant height, and vigor on a 1-to-5 scale, 5 being a healthy plant.

**Table 2. Number of plants regenerated per gram of E callus after third and sixth passages under NaCl stress for Giza 159.**

| Passages | | | |
|---|---|---|---|
| Total | Under stress | Treatment | Plants/g |
| 6 | 3 | Control | 3.4 ± 2.6 |
| | | 6 g/L | 10.5 ± 3.1 |
| | | 10 g/L | 2.1 ± 1.0 |
| 9 | 6 | Control | 2.0 ± 1.9 |
| | | 6 g/L | 3.9 ± 1.5 |
| | | 10 g/L | 11.9 ± 5.7 |
| | | Stepwise | 1.9 ± 6.6 |

A total of 142 'IR36' lines have been evaluated for seedling tolerance to NaCl. Of the checks (nonregenerated seeds), 24% had a vigor rating of at least 4. Of the regenerated lines, 36 of 142 lines had more plants than the check which rated from 4 to 5 for vigor. Of the 36 lines, 30 lines came from salt-selected cultures. Eight lines had at least one plant rated as 5. Of these eight, six came from salt-selected cultures.

The culture conditions including number of passages and NaCl levels were evaluated for their effect on seedling tolerance to NaCl (Fig. 4). Neither number of passages nor *in vitro* NaCl levels were significant for the number of lines that rated better than the check. From evaluation of the data (Fig. 4), most salt tolerance was observed at the seedling stage under short-term, high level salt stress. Future research will clarify this observation.

### Field testing for salt tolerance in the Philippines

In the Philippines, R1 and R2 lines have been increased and field tested for NaCl tolerance. Four cultivars were included in the evaluation: 'Calrose 76', 'Giza 159', 'IR36', and 'Mahsuri'. Approximately 350 R1 lines were planted for a seed increase. Another 63 R2 lines increased in Texas and Louisiana were planted in a saline environment in Pampanga Province where the salinity level increases with the intrusion of sea water during dry periods. At that site, ECs in the paddy water reached as high as 12 mmhos/cm in March 1987. The surviving plants were transplanted in a recovery plot. Number of passages and *in vitro* stress levels were not significant factors for the plant ability to set seed, which was the only variable studied.

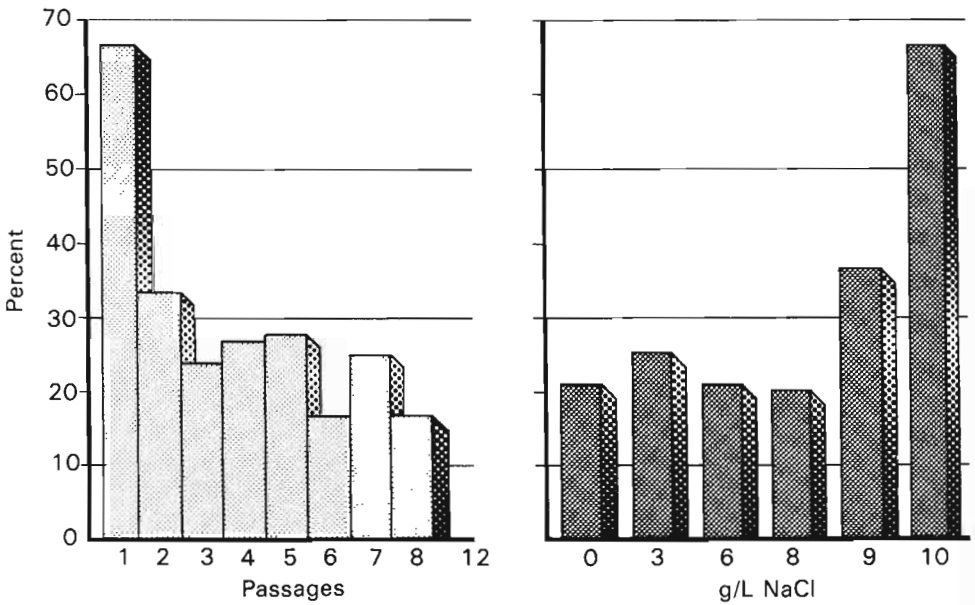


**Figure 4. Effect of number of passages and levels of NaCl *in vitro* on performance during greenhouse screening of seedlings. Percent of lines evaluated within each passage or NaCl level, which had 28% or more of the plants within a line with a vigor rating of 4 or 5.**

### NIAB saline field evaluations

In Pakistan 120 R1 and R2 lines were evaluated on an alkaline soil with high saline content by the Nuclear Institute of Agriculture and Biology (NIAB) at its Biosaline Research Site in Lahore. This environment has a mixture of salts and is different than the *in vitro* single stress of NaCl; however, several lines set seed and performed well. Conclusions from the data were difficult to make concerning the effect of passage and *in vitro* NaCl levels, but the *in vitro* treatments were apparently not significant for field tolerance. Of the lines evaluated, 5% of the R1 lines and 20% of the R2 lines set seed. Sixty-five percent of the lines were from salt-selected cultures; of the lines which set seed, 75% were from salt-selected cultures.

Regenerated lines of Mahsuri and Pokkali did not perform significantly better than their nonregenerated check for seed weight. Regenerated lines of 'IR36' averaged 32 g/plant, while the control yielded 16 g/plant.

One line of 'Pokkali' was significantly shorter than the nonregenerated 'Pokkali'. This short line averaged 47 cm in height as compared to 'Pokkali' (128 cm). Both lines set seed; however, the shorter line had more tillers (35 per plant) than the nonregenerated 'Pokkali' (15 per plant).

## SUMMARY

Formation and growth of embryogenic callus can be significantly improved by the addition of sorbitol to the medium. The sorbitol concentration can range from 1 to 3% depending upon the cultivar. A second factor that may increase tissue culture ability is to culture seed from regenerated plants. In preliminary studies, this has been shown to be true, but requires further research. Evaluation of R1 and R2 seed has yielded individual plants and lines that can survive in a saline environment. Confirmation experiments are underway.

## ACKNOWLEDGMENTS

The authors wish to acknowledge Dr. Thanh-Tuyen, Julie Cotton, and Cathy Rivera for their work in tissue culture; Michael Thompson, Dan Miller, and Reagan Waskon for their work in evaluating plants for saline tolerance; Drs. Javier Hernandez, Paningbatan Malik, and Islah ul Hag for their collaboration in field testing; and Oluf Gamborg and Julie Ketchum for their editorial remarks on this manuscript. The research was supported by Cooperative Agreement #DAN-4137-A-00-4053-00 from the United States Agency for International Development (USAID).

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## RESUMEN

Se mejoraron los procedimientos de cultivo de tejidos del arroz, que son ahora sumamente eficaces. La regeneración de plantas de cultivos de arroz derivados de semillas maduras puede mantenerse durante más de un año. Uno de los objetivos consiste en crear cultivos a largo plazo. Se identificó y mantuvo durante más de cuatro años una línea de células regeneradoras de la variedad 'IR36'. Otro aspecto importante es el mejoramiento de la formación de callos embrionarios, de su crecimiento y de la regeneración de plantas. El crecimiento de los callos embrionarios y la regeneración de plantas puede aumentar si se agrega 1% de sorbitol o si se preparan cultivos de semilla R1. La investigación efectuada por el TCCP sobre el cultivo de tejidos, tiene por objeto el estudio de la selección *in vitro* para obtener resistencia al estrés, la regeneración de plantas a partir de cultivos seleccionados y la evaluación de las plantas regeneradas para determinar su tolerancia. En 1984 se inició la selección *in vitro* para obtener tolerancia al NaCl. En la selección de las plántulas en el invernadero, 25% de las líneas regeneradas se comportaron mejor que el testigo no regenerado; 83% de dichas líneas procedía de cultivos seleccionados por su tolerancia al NaCl. En dos evaluaciones de campo se identificaron otras plantas que sobrevivieron a las condiciones salinas. La selección *in vitro* se efectuó con NaCl y las plantas presentaron tolerancia al NaCl y a suelos alcalinos que contenían diversas sales.

# Assessment of somaclonal variation in *Stylosanthes guianensis*, a tropical forage legume

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One hundred and fourteen *Stylosanthes guianensis* (Aubl.) Sw. plants regenerated from callus culture of leaf or hypocotyl explants originating from a single plant of germplasm accession CIAT 2243 were analyzed for ploidy level. First-generation, selfed lines derived from 76 of these plants were compared with the original check genotype in a replicated field trial at CIAT-Quilichao (Cauca Department, Colombia) for eight agronomic traits. Approximately 20% of regenerated plants were tetraploids. Neither explant source (leaf or hypocotyl) nor subculture affected the rate of generation of tetraploidy. For most agronomic traits, the mean of the diploid lines differed from that of the tetraploid lines and the tetraploid lines differed among themselves. For all traits, except 100-seed weight, at least some of the diploid lines differed from the check genotype, demonstrating the generation of heritable genetic variation through *in vitro* tissue culture. However, in most cases, a majority of these variant lines were inferior (lower stem number, lower internode length, lower maximum plant radius, higher disease rating, and lower seed yield). Thus, somaclonal variation will be useful in *S. guianensis* improvement only where the generation of this variation can be coupled with very efficient selection procedures.

The tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw. is highly amenable to *in vitro* regeneration from undifferentiated callus tissue (Meijer and Broughton 1981). In many species, whole plants recovered from tissue culture have been shown to be

genetically distinct from the original source plant (Larkin 1987). This variation has been termed "somaclonal" variation (Larkin and Scowcroft 1981) and in some cases it has been shown to be heritable over one or more generations of sexual reproduction (Larkin 1987). The generation of genetic variation by *in vitro* culture has two important implications: 1) if the variation is extensive, micro-propagation cannot be relied upon for exact genotype replication as is obtained through conventional methods of vegetative propagation by stem cutting; and 2) the variation arising from *in vitro* culture may be a potential source of novel genetic variants of agronomic value in plant improvement programs.

Three plant breeding programs are currently seeking to improve *S. guianensis* (Cameron *et al.* 1984). One of these programs relies heavily on the evaluation of vegetative replicates of chance outcrosses to select superior *S. guianensis* genotypes and might benefit from the large-scale replication possible using *in vitro* techniques. Any plant breeding program might benefit from novel sources of genetic variability, if these provide genes not easily accessible from conventional sources. Of particular value would be novel genetic attributes arising in otherwise adapted varieties.

The present study sought to assess the importance of somaclonal variation arising from *in vitro* tissue culture in *S. guianensis*.

## MATERIALS AND METHODS

### *In vitro* culture

Leaf or hypocotyl explants (4 or 5 mm, respectively) were taken from a single seedling of the accession CIAT 2243 from the tropical forage germplasm collection held by the International Centre for Tropical Agriculture (CIAT). The plant used as the original source of explants was from a line that had undergone six generations of single-seed descent with selfing and which is, therefore, considered highly inbred and uniform.

Callus arising from the explants was cultured on MS (Murashige and Skoog 1962) medium, supplemented with pyridoxine (1.0 mg/L), nicotinic acid (1.0 mg/L), naphthalene acetic acid (NAA) (2.0 mg/L), and benzilaminopurine (BAP) (8.0 mg/L). Cultures were incubated at 27 °C (±1 °C) and illuminated at 2000 lux on a 12-hour photoperiod.

The original leaf of hypocotyl calluses were subcultured at 30 and 60 days after the original callus cultures were established. Plants were regenerated from the original and from the subcultured calluses in MS medium supplemented with NAA (0.02 mg/L), BAP (0.05 mg/l), and gibberellic acid (GA3, 0.05 mg/L).

Regenerated plants (SC1 using Larkin's (1987) terminology) could be grouped into six classes: by explant source (leaf or hypocotyl) and subculture (original callus or first or second subculture). A total of 114 SC1 plants was obtained (Table 1).

### Glasshouse evaluation, SC1 plants

Regenerated plants were transplanted to soil and grown to maturity in the glasshouse. Seed (resulting from natural self-pollination) was harvested from individual plants. Leaf width and length (mean of 10 leaves per plant), flower width and length (mean of three flowers per plant), internode length (mean of the first three internodes below the first fully expanded leaf on each of three random stems per plant), length of longest leaf

on each of three random stems per plant, length of longest stem, and 100-seed weight were measured on each of the 114 SC1 plants. Somatic chromosome counts were made from root tips fixed in 1 N HCl and stained in lactopropionic orcein.

The effect of explant source or subculture on the proportion of tetraploids recovered was assessed by the Chi-square test (with one or two degrees of freedom, respectively). Comparisons between explant sources, subcultures, or ploidy levels for morphological attributes of SC1 plants were by t-test.

### Field evaluation, SC2 progenies

Seventy-six SC1 plants produced sufficient seed for subsequent evaluation of SC2 progenies. These progenies, along with the selfed progeny of four individual plants of the inbred line of CIAT 2243, were evaluated in a field trial at the CIAT-Quilichao substation in Santander de Quilichao (Cauca) Colombia (3° 06' N lat., 990 masl) on a highly acid, organic clay soil (7.5% organic matter, 74% clay, pH 4.0). Five-week-old seedlings were transplanted to the field in a randomized complete block design with 15 replications on 4 Feb. 1986. Single-plant experimental units were spaced on 1 x 1-m centers. Plants were observed over a period of 12 months and the following morphological traits assessed on a single plant (experimental unit) basis:

- Leaf area: mean of five random leaves per plant, sampled on 20 Oct. 1986, and measured on a LICOR LI-3000 portable area meter with a LI-3050A conveyor assembly (LICOR, Inc., Lincoln, NE, USA).
- Internode length: mean of the first three internodes below the first fully expanded leaf on three random stems per plant on 4 June 1986.
- Anthracnose (caused by *Colletotrichum gloeosporioides*) severity: mean of six ratings taken at monthly intervals between 20 May, and 20 Oct. 1986 on a 0 (= no disease) to 6 (= dead plant) visual scale.
- Length of longest branch, measured on 5 and 6 June 1986.
- Number of basal branches counted on 26 May 1986.
- Seed yield from harvest of mature plants between 9 Jan. and 5 Feb. 1987. Whole plants were cut and dried; seed manually threshed.
- 100-seed weight.
- Proportion dry matter of forage harvested between 9 Jan. and 5 Feb. 1987.

**Table 1. Classification of 114 $SC_1$ *Stylosanthes guianensis* plants regenerated from *in vitro* callus culture by explant source and subculture.**

| Explant source | Subcultures | | | Total |
|---|---|---|---|---|
| | Original | First | Second | |
| Leaf | 44 | 22 | 9 | 75 |
| Hypocotyl | 28 | 4 | 7 | 39 |
| Total | 72 | 26 | 16 | 114 |

The morphological traits were assessed on all 15 replicates except leaf area which was measured on seven replicates only.

Data were analyzed by standard analysis of variance. The 70 degrees of freedom due to "progenies" were partitioned into three orthogonal comparisons: a comparison among the 70 diploid lines (69 df); a comparison among the 10 tetraploid lines (9 df); and a single degree of freedom comparison between diploids and tetraploids. Where differences among diploid lines were detected by F-test ($P < 0.05$), then individual lines were compared with the mean of the four check progenies by t-test, using the pooled error term.

Where comparable variables were measured on parent plant (SC1) and progenies (SC2) (leaf dimensions vs. leaf area; internode length; stem length: 100-seed weight) the regression of progeny mean on parent plant value was calculated. These regressions were calculated separately for diploids (excluding checks) or for tetraploids.

## RESULTS

### *In vitro* culture

Calluses, weighing approximately 40 mg, yielded from five to seven plantlets per callus. No attempt was made to assess differences in regeneration due to explant source or subculture.

### SC1 plants

Approximately 20% tetraploid SC1 plants were recovered from tissue culture. Neither explant source nor subculture affected the proportion of tetraploids recovered (Table 2). Tetraploid SC1 plants differed on average from diploids for all seven traits

**Table 2. Number of diploid or tetraploid *Stylosanthes guianensis* $SC_1$ plants recovered from leaf, hypocotyl explants, original callus culture, or first or second subculture callus.**

| | Ploidy Level | | Total | $X^2$ |
|---|---|---|---|---|
| | Diploid | Tetraploid | | |
| **Explant Source** | | | | |
| Leaf | 58 | 17 | 75 | |
| Hypocotyl | 31 | 8 | 39 | |
| Total | 89 | 25 | 114 | 0.07 (1df) (0.75<P<0.90) |
| **Subculture** | | | | |
| 0 | 59 | 13 | 72 | |
| 1 | 18 | 8 | 26 | |
| 2 | 12 | 4 | 16 | |
| Total | 89 | 25 | 114 | 1.91 (2df) (0.25<P<0.50) |

measured (Table 3). The group of diploid SC1 plants differed from the four (diploid) check plants for four of the seven traits measured (Table 4).

Explant source (leaf or hypocotyl) did not affect any of the seven traits measured on diploid SC1 plants. Leaf width and flower length were slightly greater for tetraploid SC1 plants originating from hypocotyl explants than for those originating from leaf explants (data not shown).

Subculture of callus affected three of seven traits measured on diploid SC1 plants (data not shown). Leaf width and length were greater and maximum stem length less on plants regenerated from second subculture calluses than on plants from original or first subculture calluses. Plants originating from the first subculture differed from those originating from the original callus. Tetraploid SC1 plants did not differ by subculture.

### SC2 progenies

For five of the eight traits measured, diploid and tetraploid lines differed (Table 5). Where comparable traits were measured in the SC1 and the SC2 generation the differences between tetraploids and diploids were in the same direction, i.e. larger

**Table 3. Means for seven traits assessed on diploid or tetraploid $SC_1$ *Stylosanthes guianensis* plants regenerated from tissue culture.**

| Trait | Diploids | Tetraploids |
|---|---|---|
| Leaf width (cm) | 0.51 | 0.67*** |
| Leaf length (cm) | 2.90 | 3.28*** |
| Flower width (cm) | 0.59 | 0.70*** |
| Flower length (cm) | 1.27 | 1.37*** |
| Internode length (cm) | 4.42 | 5.37*** |
| Max. stem length (cm) | 118.8 | 111.2* |
| 100-seed weight (mg) | 259.1 | 308.3*** |

* = P<0.05; *** = P<0.001.

**Table 4. Means for seven traits assessed on diploid $SC_1$ *Stylosanthes guianensis* plants regenerated from tissue culture or on the check accession (CIAT 2243).**

| Trait | Ro Diploids | Check (CIAT 2243) |
|---|---|---|
| Leaf width (cm) | 0.51 | 0.40*** |
| Leaf length (cm) | 2.90 | 1.98*** |
| Flower width (cm) | 0.59 | 0.60ns |
| Flower length (cm) | 1.27 | 1.20* |
| Internode length (cm) | 4.42 | 3.57** |
| Max. stem length (cm) | 118.8 | 108.3ns |
| 100-seed weight (mg) | 172.2 | 262.5ns |

ns = no significant difference; * = P<0.05; ** = P<0.01; *** = P<0.001.

leaves, longer internodes, and shorter stems for tetraploids than for diploids. Seed weight of the tetraploid SC1 plants was greater than for diploid, but did not differ by ploidy in the SC2 lines.

For all traits except seed weight, diploid SC2 progenies differed and check entries were obtained (Table 6). Leaf size tended to be greater for SC2 progenies than for checks (20 lines had larger leaves while only two had smaller leaves), a trend similar to that found in the SC1 generation. However, the direction of induced differences for internode length and maximum stem length detected in the SC2 was reversed relative to that found in the SC1. SC1 plants had greater internode length and maximum stem length than the check plants, while no SC2 line was superior to the checks for these traits and four or 20 lines, respectively, were inferior. Differences were detected among the 10 tetraploid SC2 progenies for four of eight traits measured (data not shown).

No correlation was detected between comparable traits in the SC1 plants and their SC2 progenies (leaf length or width vs. leaf area: maximum stem length or internode length).

## DISCUSSION AND CONCLUSIONS

The induction of heritable variation by *in vitro* culture has been demonstrated in a number of plant species (Larkin 1987). It had not previously been shown for *S. guianensis*. The major effect of *in vitro* callus culture on the *S. guianensis* line used was a relatively high rate of induction of tetraploidy. The fact that tetraploids differed phenotypically from diploids suggests that the induction of tetraploidy might be

**Table 5. Means for eight traits assessed on selfed progeny of diploid or tetraploid *Stylosanthes guianensis* plants regenerated from tissue culture.**

| Trait | Diploids (70) | Tetraploids (10) |
|---|---|---|
| Leaf area ($cm^2$) | 0.818 | 1.142** |
| Basal branch number | 3.3 | 3.3ns |
| Internode length (cm) | 4.16 | 4.32** |
| Length of longest lateral branch (cm) | 97.4 | 75.7*** |
| % dry matter | 50.14 | 54.0*** |
| Anthracnose reaction (score) | 2.7 | 2.5ns |
| Seed yield (g/plant) | 0.317 | 0.09*** |
| 100-seed weight (mg) | 219.4 | 217.8ns |

ns = no significant difference; ** = P<0.01; *** = P<0.001.

exploited in *S. guianensis* breeding. The phenotypic differences between induced tetraploid and diploid genotypes appear to be stable over at least a single sexual generation. However, there was an indication that seed yield may be less on tetraploid than on diploid lines.

SC1 plants differed from checks for most traits measured. However, the differences observed in the SC1 plants were not consistently expressed in the SC2 progenies, and in fact no correlation between SC1 parent phenotype and mean SC2 progeny phenotype was detected suggesting that the SC1 phenotype is not a reliable expression of the genetic change induced by *in vitro* culture. Hence, selection among the induced genetic variants should be delayed at least until the SC2 generation.

**Table 6. Comparison of check progeny (CIAT 2243) with first generation selfed progenies of tissue culture-derived diploid *Stylosanthes guianensis* plants for eight traits.**

| Trait | Check[a] | Range among Diploids | No. of diploid lines <Check[b] | >Check |
|---|---|---|---|---|
| Leaf area ($cm^2$) | 0.762 | 0.636 to 1.080 | 2 | 20 |
| Basal branch number | 3.66 | 2.67 to 3.86 | 18 | 0 |
| Internode length (cm) | 4.18 | 3.57 to 4.49 | 4 | 0 |
| Length of longest lateral branch (cm) | 106.6 | 71.6 to 110.0 | 20 | 0 |
| % dry matter | 50.56 | 46.05 to 54.29 | 4 | 1 |
| Anthracnose reaction (score) | 2.24 | 1.53 to 3.91 | 0 | 16 |
| Seed yield (g/plant) | 0.479 | 0.074 to 0.886 | 24 | 1 |
| 100-seed weight (mg) | 221.8 | 192.0 to 288.6 | 0 | 0 |

[a] Means of 60 single-plant replicates.

[b] Significantly different from check mean ($P<0.05$).

It appears that variation induced by *in vitro* culture is generally detrimental, as might be expected if somaclonal variation represents random alteration of a highly evolved genotype. Induced differences were in the direction of fewer and shorter stems, shorter internodes, lower seed yield, and higher anthracnose score (greater disease susceptibility) than the checks. The number of progenies observed was relatively small (only 76), and larger populations would probably yield genotypes superior to the original. However, an efficient screening procedure will be needed to fully realize the potential of somaclonal variation in the genetic improvement of *S. guianensis*.

Our results clearly demonstrate that *in vitro* regeneration is not a reliable method of faithfully propagating *S. guianensis* genotypes.

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**RESUMEN**

Se analizaron 114 plantas de *Stylosanthes guianensis* (Aubl.) Sw., regeneradas a partir del cultivo de callos de explantes foliares o de hipocótilos de una sola planta perteneciente a la entrada de germoplasma CIAT 2243, con el fin de determinar el nivel de ploidia. Se compararon líneas autofecundadas de primera generación de 76 de estas plantas con el genotipo testigo original en un ensayo de campo con repeticiones realizado en CIAT-Quilichao (Departamento de Cauca, Colombia) en relación con ocho características agronómicas. Cerca de 20% de las plantas regeneradas eran tetraploides. El índice de generación de tetraploidia no se vio afectado ni por la fuente de explante (foliar o de hipocótilo) ni el tipo de subcultivo. En cuanto a la mayor parte de las características agronómicas, la media de las líneas diploides fue diferente de la media de las líneas tetraploides y las líneas tetraploides presentaron diferencias entre sí. En todas las características, excepto en el caso del peso de 100 semillas, algunas de las líneas diploides difirieron del genotipo testigo, hecho que demuestra la generación de variación genética hereditaria mediante el cultivo de tejidos *in vitro*. No obstante, en la mayoría de los casos, casi todas las líneas que presentaron variación fueron inferiores (menor número de vástagos, menor longitud entre nudos, menor radio máximo de la planta, mayor incidencia de enfermedades y menor rendimiento de semilla). En conclusión, la variación somaclonal de *S. guianensis* sólo será de utilidad en las actividades de mejoramiento si la generación de esta variación puede combinarse con procedimientos de selección sumamente eficaces.

# Using biotechnology in international research for development

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This paper deals with scientific organization as it relates to biotechnology derived from the author's recent experiences as chairman of the External Program Reviews of the International Rice Research Institute (IRRI) in 1987 and the International Maize and Wheat Improvement Center (CIMMYT) in 1988. There can be no doubt that when the core work of an International Agricultural Research Center (IARC) is germplasm improvement—in which the central discipline is genetics—biotechnology must have the potential to improve its efficiency. A detailed definition of biotechnology is given as it relates to protoplast manipulation, anther culture, probes, genetic engineering, and engineered commensals. The primary function of the IARCs will be to bring biotechnological research findings and materials into forms that can be used by developing countries. Regarding patent rights, the IARCs must understand the ways in which plant material is afforded protection in developed countries. Forms of protection available are outlined. Limitations to the use of biotechnology due to patent infringement by the IARCs appear slight.

Most of my brief comments today are not about technical methodologies or results but about scientific organization. They have been derived from my recent experiences as chairman of the External Program Reviews (EPRs) of the International Rice Research Institute (IRRI) in 1987 and of the International Maize and Wheat Improvement Center (CIMMYT) in 1988. Both are International Agricultural Research Centers (IARCs) with a commitment to develop research capabilities in biotechnology, which they will use to supplement their many other responsibilities to assist developing countries by the provisions of improved germplasm, other research products, and of training and education.

Clearly all IARCs should use all methodologies relevant to research on the agricultural organisms covered by their mandates that will improve their efficiency. So the

first question is: is biotechnology such a methodology? In my view there can be no doubt that when the core work of a Center is germplasm improvement—in which the central discipline is genetics—biotechnology must have the potentiality to improve its efficiency in a significant fashion.

## WHAT IS BIOTECHNOLOGY?

Perhaps at the outset I should tell you what I think crop-plant biotechnology is (recognizing as I write this before this Symposium that other definitions of biotechnology may have been given before it is my turn to speak). I will use the definition that I wrote for the Report of the 1988 CIMMYT EPR.

The term *biotechnology* is now applied to a wide range of activities from chemical engineering and vaccine production to aspects of food processing. For the purpose of this report, it will not be applied to work carried out using entire plants and the normal processes of hybridization, even in widecrossing involving embryo rescue. It will be used to refer to the manipulation of DNA or cells which have been separated from entire plants or to the use of biological reagents. It may have the following components:

### Protoplast manipulation

Protoplasts are plant cells from which the wall has been digested enzymatically. In this state, they are used for the insertion of new DNA. Also protoplasts of different species may be fused to create hybrids even between species that cannot be hybridized sexually. Protoplasts will synthesize new walls and can regenerate into entire plants, so perpetuating the changes induced. So far, protoplast regeneration in wheat and maize has not been generally successful.

### Anther culture

Anthers or pollen grains may be cultured on artificial medium and some pollen grains will grow into entire plants. Since pollen grains are haploid (bearing the gametic chromosome number) so are many of the plants produced. This enables the rapid return to homozygosity in breeding programs.

### Probes

Nucleic acid probes, antibodies, or enzymes may be applied to plants, or to plant extracts, to determine their genetic constitutions. Consequently, precise information is provided that increases the speed and certainty of selection in conventional plant breeding. Probes will also detect the presence of pathogenic organisms in plants so enabling rigorous selection for disease resistance.

### Genetic engineering

DNA can be introduced into entire plants or into protoplasts using vectors or by direct physical insertion. The sequences to be inserted will usually have been selected in bacterial cells initially. Foreign DNA present in the transformed regenerated plants can be inherited normally, its expression controlled, and it can increase resistance to abiotic or biotic stress or improve performance in other ways. DNA extracted from a species may be modified *in vitro* and returned to the same species. This procedure will be

especially useful in modifying seed storage proteins to improve their nutritional value or suitability for baking or other processing.

### Engineered commensals

Microorganisms that are normally tenants (endophytes) of plant cells without any apparent effect can be transformed to contain genetic information that will be beneficial to the plants they infest. DNA determining the production of a substance toxic to insects has been incorporated in such an organism and is now being tested as a corn borer protection strategy for maize.

## COUNTRIES

It seems clear that it will not be the function of the IARCs to innovate in biotechnology, but to bring biotechnological research findings and materials into forms that can be used by developing countries. The developing countries will have a range of attitudes to biotechnology. Some will be deeply involved in innovative research and will build considerable research capability. This is already happening in Brazil, China, Mexico, and India, to name a few of the leading countries. The relationship of the IARCs to such national programs will be, if needed, to assist developing countries in securing the necessary enzymes, isotopes, reagents and equipment.

Many developing countries will not have the scale of national agricultural programs to make it economical for them to participate directly in original research in biotechnology. Nevertheless they may benefit from the use of biotechnological tools or materials coming from the work of others. Such items may be genetically engineered parental plants having novel genetic characteristics derived from an alien source. Alternatively, they may be marker probes—isozyme or other protein markers, or RFLPs—that are tightly linked to genes that are useful in plant breeding, but whose presence cannot easily be detected in selection programs. Probes may also be useful in diagnosing pathogens and so on. IARCs may be construed as having a responsibility to provide such materials to developing countries or to provide guidance in their use.

## IARCS AS TRANSMISSION AGENCIES FOR BIOTECHNOLOGY

All of this presupposes that the IARCs will have the competence to service the national programs in the ways just described. A necessary condition for this is that they are staffed with biotechnologists of considerable competence whose capabilities are honed by participation in research. On this proposal, the IARCs would act as a transfer agency for biotechnology in a mode essentially similar to that traditionally employed in germplasm improvement, crop management research, or in the many other forms of scientific assistance with which they help the national programs.

When an IARC acts as a transmission agency, it should have the following responsibilities:

- Disseminating to appropriate countries or regions new genotypes created by genetic engineering at a Center or elsewhere.
- Communicating to in-house biotechnologists or others knowledge of characteristics potentially achievable by genetic engineering that are needed in crops in developing countries or regions.

developing countries and whether there would be alternative solutions to the problem. If there are no alternatives, a Center should negotiate a price giving untrammeled access to the use of the innovation. The course to be followed is to allow the least-cost supplier to do the research and into the cost-estimate will be built the speed, effectiveness and precision of the supplier. These principles should apply whether the materials or methods have already been created or whether they are to be developed at the request of a Center.

When the biotechnology requirement is perceived by an IARC or by developing countries, but not previously by any existing biotechnology laboratory, a Center should formulate a specification defining the requirements. It must be aware of the capabilities of public and private sector laboratories throughout the world. It will specify the needs, on a confidential basis, to a few potential suppliers and choose among the quotations received on the basis of price (if any), likely speed and efficiency. On this basis the intellectual property, the know-how, and agreement will be appropriate for earnings from any use of the findings in developed countries.

Such work may often be a joint venture with a private sector corporation or perhaps a contract made by an IARC, as customer, with a corporation, as contractor. When this is the case, two rules should apply:

- The property or materials resulting should not be protected in developing countries.
- The property may be protected in developed countries, when sharing the costs of patenting should be agreed between a Center and the contractor/collaborator. A Center and the contractor should agree on the nature of the license changes to be levied from exploiters (e.g., upfront payments, percentage of the sales price of each unit sold, rate of decline of the license fee with volume, etc.). Special arrangements will be necessary where the contractor becomes the exclusive exploiter in developed countries.

Again, I must emphasize that if a Center is to act in this entrepreneurial manner it must have well established biotechnological competence in-house.

## PRIVATE SECTOR

Much of the above implies the greater involvement of a Center with the private sector of agricultural research. This seems quite inevitable as agricultural biology becomes more sophisticated, complex, and expensive, so that most investments will be made by those who expect a fair return on their investment. But such investors may also need facilities which the IARCs can uniquely provide—indeed they may be the only source, for example, of a wide range of germplasm and of a great diversity of testing sites and environments. So the links between a Center and the private sector may be symbiotic and a Center should not feel threatened.

But IARCs must understand the ways in which plant material is afforded protection in developed countries, so I conclude by setting out the forms of protection available.

Many developed countries have legislation conferring the right to collect royalties from the users of varieties by the owners. This is *variety protection* (plant breeders' rights). Such rights will be available to the owners of varieties that are entered on the

official (country-specific) list of certain crop species. To be entered on the list, varieties must be distinct, uniform, and stable. Protection is for a limited period, often 20 years. The claim of plant breeders' rights is confined to commercial use (multiplication and sales of seed or vegetative propagules). There is no limitation on the use of protected varieties for the purpose of creating other varieties (research exemption) or for on-farm seed production (farmers' exemption). Member countries that have ratified the UPOV[a] convention mutually acknowledge the claim of plant breeders' rights issued in one of the member countries (UPOV states). By providing commercial incentives, plant breeders' rights have stimulated private sector investments in plant breeding and have extended the range of varietal choice available to farmers.

*Patent rights* were established in developed countries much earlier than plant breeders' rights with the aim of encouraging invention by offering protection covering the use of new processes or new products. Until recently, patent rights have applied mainly to industrial products or processes, including the use of microorganisms in fermentation processes. Patent protection is also confined to a limited period of time (in most countries much shorter than 20 years), but its applications are much more restricted and without the exemptions defined for the claim of plant breeders' rights. Neither the processes nor the products of conventional plant breeding, in general, would fit into the patenting process. With the progress made in molecular genetics, genes and gene products can now be defined with accuracy, equivalent to the products of synthetic chemistry—a classical area for the application of patent rights. The presence of patented genes in transgenic plants can be proven beyond doubt if earmarked by only a few base pairs. So, there is a collision of interests between the different levels of protection that can be achieved if a patented gene is incorporated into a variety that warrants no more protection than that of plant breeders rights. Would the research exemption normally applied to the use of a variety be nullified by patent protection?

Developing countries do not yet have variety protection so this is not an obstacle to the use of biotechnology. In addition, few owners of intellectual property would find it worthwhile to attempt to patent it in most developing countries. The income would be too small and the complexity of litigation against infringers of their patents would be prohibitive. Consequently, limitations to the use of biotechnology by the IARCs appear slight.

[a] UPOV is the "Union Protection Obtention Vegetale," which is the agency created to administer the international convention on plant breeders' rights.

**RESUMEN**

En este artículo se examina la organización científica en lo que respecta a la biotecnología derivada de las recientes experiencias del autor como presidente de la Revisión Externa de los Programas del Instituto Internacional de Investigación de Arroz (IRRI) en 1987, y del Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT) en 1988. No cabe duda de que cuando el trabajo primordial de un centro internacional de investigación agrícola es el mejoramiento de germoplasma (cuya disciplina fundamental es la genética), la biotecnología tiene la posibilidad de aumentar su eficiencia. Se presenta una definición detallada de la biotecnología relacionada con la manipulación de protoplastos, el cultivo de anteras, las sondas, la ingeniería genética y los comensales que han sido sometidos a la misma. La función esencial de los centros internacionales será la conversión de los hallazgos y materiales que produzca la biotecnología en formas que puedan usar los países en desarrollo. En cuanto a los derechos de patente, los centros deben estar conscientes de las maneras en que el material vegetal es protegido en los países desarrollados, y que se describen en este trabajo. Al parecer son pocas las limitaciones al uso de la biotecnología impuestas como resultado de las violaciones de patentes por parte de los centros internacionales.

# Rockefeller Foundation's international network on rice biotechnology[a]

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The goals of the Rockefeller Foundation's International Network on Rice Biotechnology are: 1) to ensure that new techniques for crop genetic improvement, based on advances in molecular and cellular biology, are developed for rice; 2) to facilitate the transfer of these biotechnologies to rice breeding programs in the developing world; and 3) to help build the scientific research capacity necessary for continued development and application of new rice genetic improvement technologies in selected developing countries. This paper discusses why rice was the crop focused upon for this network, provides details on the program's activities, and highlights recent accomplishments and future plans.

## FOCUS ON RICE

In selecting a focus for a network that would apply molecular biology to plant breeding and direct the benefits to the disadvantaged in developing countries, Rockefeller Foundation (RF) officers decided to single out a specific crop and to develop a fully integrated program ranging from fundamental research to the application of new techniques in breeding.

Table 1 shows the criteria used to determine support for a particular crop and most pointed toward rice. At present, rice is by far the most important crop in the developing world in terms of production and consumption. Projections indicate that, in the 1990s

[a] Adapted from paper presented at the USAID-sponsored conference on "Strengthening Collaboration in Biotechnology: International Agricultural Research and the Private Sector," April 17-21, 1988, Washington, D.C.

and beyond, the growth in demand for rice will surpass its supply in many developing countries. Rice breeding programs are now quite advanced at international centers such as the International Rice Research Institute (IRRI) and in several developing countries.

Rice also turned out to be a highly neglected crop with regard to biotechnology research. When RF conducted a survey in 1983, biotechnology-orientated research programs in the West were limited to a few modest research efforts on rice tissue culture. Even in Japan there was surprisingly little research on the molecular genetics of rice. Because of the dearth of biotechnology research efforts in rice, a considerable investment of time, effort, and resources would be necessary to realize significant results in the field. Because rice is less important than wheat, maize and many other crops in the developed world, it seemed unlikely that either public agencies or private firms in developed countries would invest heavily in rice biotechnology.

The inclination to support rice was fueled further by a very successful intercenter meeting on biotechnology, organized by IRRI, and attended by many scientists conducting biotechnological research on other crops.

The RF's Trustees approved the network in December 1984 fully realizing that a 10- to 15-year commitment of $3-5 million/year most likely would be required. Research at several laboratories began in 1985; other laboratories have been added as the network continues to evolve and expand. More than 50 projects are now supported with collaborative research conducted by scientists from throughout the world. Further expansion of the network in developing countries is anticipated. IRRI plays a key role in the identification of research priorities, the preparation and evaluation of germplasm, the provision of genetic stocks, cultivars and wild species, and by carrying out some of the research projects themselves in collaboration with other institutions.

A deliberate effort was made to balance priorities among traits for rice that would be applicable in poor production environments and traits that would contribute most to increasing production.

**Table 1. Criteria used to determine crop focus of RF biotechnology network.**

| Crop | Importance of crop in LDCs | Experience of IARC and LDC breeding programs | Lack of biotechnology research effort | Probability of near-term success |
|---|---|---|---|---|
| Rice | **** | **** | **** | ** |
| Cassava | ** | ** | **** | *** |
| Wheat | *** | **** | ** | ** |
| Millet | ** | ** | **** | * |
| Maize | *** | *** | * | ** |
| Sorghum | ** | *** | *** | * |
| Sweet potato | ** | * | *** | *** |
| Potato | * | *** | * | **** |

**** relatively high.
* relatively low.

It is not within the scope of this paper to describe precisely how the priorities were determined; this has been described in detail by Herdt and Riley (1987). However, the guiding principle was that traits easy to introduce using 'conventional' approaches probably should not have high priority for biotechnology because conventional methods are easier and cheaper. Conversely, traits difficult to manipulate were considered better candidates for biotechnology treatment, including genetic engineering.

Analysis results indicate that there is a reasonable chance that biotechnology can provide some solutions to widespread problems that highly limit yield in many of the agro-ecologies. Sixty-eight problems were identified and analyzed. Highest priorities among the insect pests include: yellow stem borer, gall midge, brown planthopper, leaf folder, storage pests, and striped stem borer; among the diseases: tungro virus, ragged stunt virus, blast, sheath blight, and bacterial blight; among physical environmental conditions: tolerances to submergence, stagnant flooding, upland drought, lowland drought at anthesis, and cold temperature at the seedling stage. Opportunities for raising potential productivity include greater lodging resistance, cytoplasmic male sterility, greater seedling vigor, and apomixis. These results are being used as a general guide for allocating RF resources aimed at identifying genes for rice improvement.

## WIDE HYBRIDIZATION

Wide hybridization is considered to be an important component of this program and the RF is supporting a significant expansion of this work at IRRI. Of the 22 *Oryza* species, only two are cultivated—*O. sativa* and *O. glaberrima*. The genus includes both diploid and tetraploid species and six different genomes (A-F).

The objectives of the wide hybridization program are to: 1) transfer useful traits from wild relatives to cultivated rice, 2) generate new variability for use in rice improvement, 3) transfer wide hybridization technology to the national rice improvement programs, and 4) lay the foundations for use of new molecular techniques at IRRI.

The wild *Oryza* species possess many agronomically useful traits, such as resistance to pests and diseases and tolerance to problem soils (Table 2). The ease with which crosses can be made between cultivated rice and the wild species depends on the species used. Crosses involving species with the AA genome, such as *O. nivara* or *O. rufipogon*, generally have high seed sets and well formed seeds. Crosses involving species with distinct genomes generally have low seed sets and poor seed development. Embryo rescue is routinely used in such crosses to ensure hybrid survival. Hybrid sterility is a problem in most intergenomic crosses, but can be reduced by the production of amphiploids. The trait is then transferred into the cultivated genotype by backcrossing and selection procedures.

Successful trait transfers, to date, include resistance to brown planthopper and whitebacked planthopper from *O. officinalis* (CC) and bacterial blight from *O. longistaminata* (AA). Hybrids have been made with the wild species *O. latifolia* (CCDD), *O. nivara* (AA), and *O. rufipogon* (AA); backcrossing is underway to hopefully increase the yield potential of cultivated rice. *O. perennis* and *O. rufipogon* are being used as potential new sources of cytoplasmic male sterility. *O. minuta* is being used as a source of resistance to brown planthopper, whitebacked planthopper, blast, and bacterial blight.

Strong barriers operate in some of the more distant crosses that prevent hybrid production and the transfer of desirable traits, such as salt tolerance from the related genus *Porteresia coarctata*. In attempts to overcome this type of incompatibility, IRRI and Dr. E.C. Cocking at the University of Nottingham are using *in vitro* pollination techniques and somatic hybridization, respectively.

Postdoctoral scientists from developing countries are now assisting IRRI in the wide hybridization research with the aim of transferring the technology to their home institutions.

## DEVELOPING A KNOWLEDGE BASE AND RICE BIOTECHNOLOGY TOOLS

Emphasis was initially placed on rice research aimed at developing the various molecular and cellular biology techniques, protocols, and materials that are the tools of biotechnology research. RF encouraged university-based scientists in the developed world, who were at the forefront of research on plant molecular and cellular biology of other crops, to make a significant commitment to research on rice. The first group of laboratories joined the network in 1985.

### Genetic maps and markers

One of the near-term uses of molecular biology in plant breeding will be the development of genetic maps and markers based on cloned DNA sequences. The identification of a molecular marker linked to a trait of agronomic importance will enable breeders to monitor desired introgression, and will be of particular value when

**Table 2. Agronomically important characteristics identified among wild *Oryza* spp.**

| Species | 2n | Genome | Characteristics |
|---|---|---|---|
| *O. nivara* | 24 | AA | Grassy stunt virus resistance |
| *O. rufipogon* | 24 | AA | Source of cytoplasmic male sterility, tolerance to stagnant flooding |
| *O. glaberrima* | 24 | AA | GLH resistance, early vegetative vigor |
| *O. barthii* | 24 | AA | Bacterial blight resistance |
| *O. longistaminata* | 24 | AA | Floral characteristics for out-crossing |
| *O. punctata* | 24,48 | BB,BBCC | BPH, WBPH, GLH resistance |
| *O. officinalis* | 24 | CC | BPH, WBPH, GLH resistance |
| *O. eichingeri* | 24 | CC | BPH, WBPH, GLH resistance |
| *O. minuta* | 48 | BBCC | BPH, WBPH, GLH, blast and bacterial blight resistance |
| *O. australiensis* | 24 | EE | BPH resistance, drought resistance |
| *O. brachyantha* | 24 | FF | Rice whorl maggot and rice stem borer resistance |
| *O. ridleyi* | 48 | | Rice whorl maggot resistance |

BPH = brown planthopper, WBPH = whitebacked planthopper, GLH = green leafhopper.

the linked agronomic trait is not expressed or is difficult to detect or when the pyramiding of genes is desired. RF-supported work in this area include the projects discussed below.

At Cornell University, Steven Tanksley and colleagues have recently developed a genetic map of rice chromosomes based on restriction fragment length polymorphism (RFLP) markers (McCouch *et al.* 1988). This RFLP map consists of 123 loci and was created using DNA clones of the Philippine variety IR36. Based on segregation analysis and use of primary trisomics provided by Gurdev Khush at IRRI, all 123 markers have been assigned to linkage groups and positioned on specific chromosomes. All 12 rice chromosomes are now marked by more than one RFLP marker.

We also hope to link the RFLP markers to genes for important quantitative traits, such as drought tolerance. Eventually, the RFLP map may allow important rice genes to be located and cloned, essential prerequisites for transferring them between genotypes by genetic engineering.

Gary Kochert at the University of Georgia is determining the types of repeated sequence DNA in rice and will add these sequences to the RFLP map.

The detection of introgression of alien chromatin in wide hybridization-derived progeny is difficult primarily because rice chromosomes are so small they do not show reliably characteristic banding patterns after Giemsa staining. Four research groups have joined the network to develop species-specific probes. Using *in situ* hybridization techniques, these probes can be used to study the extent and location of introgressed alien chromatin.

Two groups of French scientists, led by Gerard Second of the ORSTOM Institute in Montpellier and Michel Delseny at the University of Perpignan, are developing genome-specific probes within the *Oryza* genus.

Similarly, Lynne McIntyre, at the University of Missouri, and Rudi Appels, at CSIRO in Australia, are developing probes based on the ribosomal DNA spacer sequences. Appels and McIntyre have shown that, in *Triticum*, the DNA sequences in the spacer regions are highly variable and often this variation is species-specific. Preliminary evidence suggests that this is also true in *Oryza*.

Perry Gustafson and colleagues at Missouri are developing and using species-specific DNA probes to visualize alien introgressions under both light and scanning electron microscopes. The electron microscope studies may allow the detection and location of regions of alien chromatin which are too small to be detected under the light microscope.

**Protoplast techniques**

The genetic manipulation of individual cells is an important aspect of plant biotechnology, but it is of limited value unless whole plants can be regenerated from the cells and protoplasts. RF is funding the activities of five groups that are working on the development of rice tissue culture and protoplast techniques. The goal is to obtain efficient regeneration of whole plants from protoplasts so that the protoplasts can be used as a vehicle for various genetic manipulations.

In 1986 Dr. Cocking's group at the University of Nottingham was successful in regenerating plants from protoplasts of the japonica rice variety 'Taipei 309'. The protocol has been extended to several other japonica lines. In March 1987, 30 scientists

from most of the other laboratories in the RF Network attended a course at Nottingham that provided hands-on training in the regeneration protocol. Many laboratories have now reproduced and confirmed the Nottingham results.

Recently, Tom Hodge's group at Purdue University has obtained plant regeneration from protoplasts of indica lines IR54 and IR52 and from U.S. variety 'Calrose 76', which has both indica and japonica lines in its background. The plants still need to mature to test fertility, but they appear to be growing normally.

Hirofumi Uchimiya at the University of Tsukuba is using protoplast culture and regeneration as a vehicle for genetic transformation. He has shown transient expression of foreign genes in callus derived from transformed rice protoplasts (Uchimiya *et al.* 1986). Although early regenerants did not show expression of the foreign DNA, he has recently informed us that he has obtained transgenic rice plants.

**Genetic transformation**

The genetic transformation of rice through the introduction of alien or modified genes is one of the principal technological goals of the network. In addition to the protoplast work described above, RF is providing support for research on a variety of other techniques mentioned below.

Marc Van Montagu's group at the University of Ghent and Rob Schilperoort and colleagues at the University of Leiden are studying the suitability of *Agrobacterium* as a vector. Results, such as the induction of the "virulence" functions of the Ti-plasmid by rice cells and the stimulation of root proliferation at a wound site infected with *Agrobacterium rhizogenes*, suggest that *Agrobacterium* may serve as a vector for gene transfer to rice.

At Stanford University, Virginia Walbot's laboratory is perfecting an electroporation-mediated direct gene transfer system for rice. She is also introducing DNA and obtaining foreign gene expression in rice mitochondria by the same method.

At Cornell University, Ray Wu's laboratory is experimenting with protoplast uptake of DNA and the use of a particle gun to deliver DNA to intact cells. Like Dr. Uchimiya, Dr. Wu recently obtained transgenic rice after the regeneration of transformed protoplasts.

Recent projects added to the network include studies on the use of microinjection as a tool for transforming protoplasts, embryoids, and inflorescence. William Lucas (University of California, Davis), Horst Lörz (Max-Plank-Institute), and Robert Schilperoort (University of Lieden) are conducting these studies. Lucas is also exploring the possibilities of using liposomes as a transformation tool.

Although genetic transformation of rice has been demonstrated in at least two laboratories, further refinement of the protoplast technique and development of other techniques will be required before rice genetic engineering becomes a routine process.

**Cloning and characterizing rice genes**

The application of genetic engineering to genetic improvement in rice will involve the introduction of novel genes and/or the modification of existing rice genes and genetic systems. RF is funding a number of projects aimed at obtaining greater fundamental knowledge of rice gene regulation and expression. These projects are listed in Table 3, two of which are discussed in more detail below.

In the work on genes for carotenoid biosynthesis, Alice Cheung's group at Yale University is looking to improve the nutritional value of rice by making the grain a new source of pro-vitamin A compounds, such as Beta-carotene. The genes for carotenoid synthesis are present in rice, as in all photosynthetic plants, but they are expressed in photosynthetic tissue and not in the endosperm. Through genetic engineering, it may be possible to modify the regulation of these genes so that carotenoid biosynthesis will occur in rice endosperm, as it does in yellow maize endosperm.

In the waxy gene research, Susan Wessler (University of Georgia) is studying possible indigenous and introduced transposable elements in rice. The identification or introduction of a transposable element would provide a powerful tool for tagging and isolating important rice genes.

**Diagnostic tools and the study of host-pathogen interaction**

Bacterial blight and blast are two important diseases of rice throughout the world. Resistance is often short-lived due to changes in the pathogens. RF is supporting collaborative research with IRRI conducted by Jan Leach at Kansas State University

**Table 3. RF-funded projects on cloning and characterizing rice genes.**

| | |
|---|---|
| ABA-induced genes,<br>Rice phytochrome genes,<br>Photochlorophyll reductase genes | Rockefeller University,<br>Nam-Hai Chua |
| Plasmamembrane ATPase genes,<br>Protein kinase genes | Salk Institute,<br>Chris Lamb |
| Genes for carotenoid biosynthesis | Yale University,<br>Alice Cheung |
| Glutelin genes,<br>Prolamine genes,<br>ADP glucose pyrophosphorylase genes | Washington State University,<br>Tom Okita |
| Prolamine genes,<br>Glutelin genes | Kyoto Prefectural University,<br>K. Tanaka |
| Waxy genes | University of Georgia,<br>Susan Wessler |
| Nitrate reductase genes | Washington State University,<br>Gynheung An |
| Cold shock genes | Standford University,<br>Virginia Walbot |
| Actin genes,<br>Alpha-amylase genes | Cornell University,<br>Ray Wu |

and Sally Leong at the University of Wisconsin. They are studying the pathogens that cause bacterial blight and blast, respectively. Near-term goals are to: 1) study the genetics of these pathogens and their ability to overcome resistance, 2) develop race-specific probes for rapid race diagnosis, and 3) better understand the molecular mechanisms controlling pathogenesis. The long-term goal is to use the knowledge generated to produce more durable resistance in rice.

### Novel genes for rice improvement

Genetic engineering should make it possible to incorporate essentially any gene from any source into rice. Table 4 lists relevant RF-suggested research projects involving the search for and incorporation of such novel genes.

Powell *et al.* (1986) recently demonstrated that resistance or tolerance to viral infection can be conferred by the integration of the virus coat protein gene into the plant genome. Research has been initiated to characterize the molecular structure of the rice tungro viruses and rice ragged stunt virus with the aim of creating genes for resistance to these viral infections.

**Table 4. RF-funded projects on novel genes for rice improvement.**

| | |
|---|---|
| Viral genes for resistance to rice tungro virus | John Innes Institute, Roger Hull |
| | Washington University, Roger Beachy |
| | IRRI, H. Hibino |
| *B.t.* toxin genes for resistance to yellow stemborer and other insects | IRRI and collaborators |
| Maize gene for endosperm carotenoid biosynthesis | Iowa State University, Don Robertson |
| Wheat genes for inhibitors of rice weevil amylase | Kansas State University, Gerald Reeck |
| Apomixis in wild *Oryza* spp. | Univ. of Calif., Davis, Neil Rutger |
| *Pennesitum* genes for apomixis | Univ. of Georgia, Wayne Hanna |

Barton *et al.* (1987) have demonstrated that transgenic plants expressing toxin genes from *Bacillus thuringiensis* are resistant to insect feeding. The Entomology Department at IRRI is now screening *B. thuringiensis* strains for toxicity to the yellow stem borer and other insect pests of rice. Once toxic strains are identified, IRRI entomologists will collaborate with Marc Van Montagu and colleagues at the University of Ghent to isolate the toxin genes for transfer to rice.

## BUILDING CAPACITY IN DEVELOPING COUNTRIES

Initially, emphasis was placed on the development of the knowledge base and technologies required for the genetic engineering of rice. Now, RF intends to use a greater portion of available funds to facilitate transfer and use of new rice improvement technologies in the developing world and to help build the scientific capacity of national research programs in selected developing countries.

Since 1985, RF has provided support to rice breeding programs at the following institutes to conduct research on the use and improvement of currently applicable biotechnologies such as anther culture:

- China National Rice Research Institute, Hangzhou
- Korean Rural Development Administration, Research Bureau
- Shanghai Academy of Agricultural Sciences
- International Rice Research Institute, Philippines
- International Center for Tropical Agriculture (CIAT), Columbia

In 1987 RF began discussions on significantly increasing support for rice biotechnology research in China and India that would concentrate on knowledge-generation and technology development, as well as applications in rice breeding. RF believes that Chinese and Indian scientists will be making important contributions to the network and anticipates that their national rice biotechnology programs will, as they gain experience, assume more and more responsibility for future development and refinement.

Progress is proceeding most rapidly in China where, to date, 23 research proposals at 15 institutions have been approved for funding. Participating institutions are eligible to nominate their best young scientists for RF predoctoral, postdoctoral, and career development fellowships.

In India, officials of the Department of Biotechnology in the Ministry of Science and Technology and of the Indian Council of Agricultural Research have expressed enthusiasm for RF assistance in establishing a similar coordinated national program on rice biotechnology. Discussions are currently underway concerning the details of the Indian program.

## CONCLUSIONS

Since the initiation of this network, considerable advances have been made in many research areas, such as the completion of the first molecular map of rice, regeneration from rice protoplasts, and transformation of rice. During the next 5 years, there will be significant accomplishments in the transformation of rice for traits such as virus resistance (conferred by the insertion of viral coat protein genes) and of insect resistance (by the insertion of the *B. thuringiensis* toxin gene).

Transformation protocols are being developed very quickly. At present, there are few cloned genes available for transformation. The application of these techniques will soon be limited by the absence of cloned genes of agronomic importance. At this stage, priority is now being given to the identification and isolation of genes of agronomic importance, so that we can make full use of the available techniques.

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## RESUMEN

Los objetivos de la Red Internacional de la Fundación Rockefeller sobre la Biotecnología en el Arroz son: 1) garantizar la creación de nuevas técnicas para el mejoramiento genético del arroz, basadas en los avances realizados en la biología molecular y celular; 2) propiciar la transferencia de estas biotecnologías a los programas de mejoramiento de arroz de los países en desarrollo, y 3) colaborar en la creación de la capacidad de investigación científica necesaria para el constante desarrollo y aplicación de las nuevas tecnologías de mejoramiento genético del arroz en algunos países en desarrollo. En este artículo se analizan las razones por las cuales se eligió el arroz como objetivo de las actividades de esta red, se dan detalles sobre las actividades del programa y se reseñan los avances más recientes y los planes para el futuro.

# Collaborative research at the International Potato Center on genetic manipulation for potato improvement

J.H. Dodds
*International Potato Center (CIP), Lima, Peru*

Over the last few years, the International Potato Center (CIP) has set up a network of collaborators for studying genetic engineering of potatoes. The projects, some from special projects and some from core budget, have concentrated on the incorporation and expression of genes identified to be important to national programs. Initial studies concentrated on the use of synthetic genes were aimed at further improving the nutritional value of potato. The synthetic genes code for synthetic proteins containing a high content of essential amino acids (particularly lysine and methionine) has been identified. We have been successful in inserting the genes and at obtaining organ-specific (tuber) expression. This work involved the cooperation of a number of laboratories. On the basis of these initial studies, we are now using synthetic and natural genes to attempt to confer resistance to pests and diseases in potato. To date, a number of genes and a range of promoters are available. These studies may offer a specific way to confer novel resistances to potato.

From its founding, the International Potato Center (CIP) has had a policy of collaborative research with other institutions to use the comparative advantage of each institution to pursue research on potato improvement. These types of collaborative agreements have allowed CIP to reach research objectives more rapidly and at a lower cost. Over the past few years, CIP has entered into several such collaborative arrangements to develop new areas of research in plant tissue culture and genetic engineering. Plant biotechnology is an area where CIP has been active for many years, for example, germplasm conservation and *in vitro* distribution of pathogen-tested materials (Tovar *et al.* 1985).

In recent years, CIP has developed a number of new and exciting areas of research in tissue culture and genetic engineering in collaboration with research institutions in

different parts of the world. Some of these research activities are still in the initial phase and will need several more years of development until the resulting technologies can be transferred to practical use in farmers' fields. It is important, however, to explain how this type of research is progressing, what CIP's objectives are, and how this type of work will help CIP's current research programs and research within national potato programs. In some cases, even though the technologies themselves may be inappropriate for direct transfer to national programs, the improved germplasm resulting from these technologies may be of fundamental importance in breeding, leading to the release of new improved materials for national programs.

## SYNTHETIC PROTEIN GENES

In collaboration with Dr. Jesse Jaynes, Biochemistry Department, Louisiana State University, CIP has developed a project to insert synthetic genes into potato plants. In this project, we use *Agrobacterium* plasmid vectors (Espinoza *et al.* 1986), a system of gene transfer now used by several laboratories worldwide. The primary objective of the project is to enhance the nutritional value of the potato by obtaining the supplementary production of a synthetic protein rich in essential amino acids. The synthetic protein is produced from a synthetic gene that can be synthesized in the laboratory of Dr. Jaynes.

Through this research collaboration, CIP has been successful in inserting the synthetic gene into potato plants. We have also proved that the gene is transcribed and produces a corresponding messenger RNA (mRNA) molecule, which is then translated in the plant to produce the synthetic protein (Fig. 1). This synthetic gene project has shown the technology now exists for the genetic engineering of potato plants (Fig. 2). These genes can be expressed and can be shown to contain significant amounts of essential amino acids (Fig. 3). The expression of a relatively low percentage of these proteins can significantly affect the protein nutritional value of potatoes. Much more research, as well as the appropriate legislative measures, will be needed before these genetically engineered plants can be released to national potato programs.

## ENGINEERED PEST AND DISEASE RESISTANCE

### Bacterial disease of potato

Although there are a number of bacterial diseases of potato, the majority of the economic losses can be attributed to just two of them. A short description of the etiology of these diseases follows.

*Bacterial soft rot (blackleg).* The causal agent of this disease is *Erwinia carotovora*. Symptoms occur at any stage of plant development and the stems of infected plants typically exhibit an inky black decay that usually begins at the decaying seed piece (portion of the seed tuber) and may extend up the stem. Stem pith (inner part of the stem) is often decayed above the black discoloration, and vascular tissues in the stem are generally discolored. Infected plants are commonly stunted and have a stiff, erect growth habit, particularly early in the season. Foliage becomes chlorotic, and leaflets tend to roll upwards at the margins. Leaflets and, later, entire plants may wilt and slowly die. Young shoots may be invaded and killed before emergence. Tubers can be affected with soft rot while in storage or in the soil before harvest, and seed tubers

decay after planting. Infection occurs through lenticels and wounds or through the stolon end of the tuber in the infected mother plant.

*Brown rot (bacterial wilt).* The causal agent is *Pseudomonas solanacearum*, which is infectious at any stage of the plant's growth. Wilting of leaves and collapse of stems may be severe in young, succulent plants of highly susceptible varieties. Initially only one branch in a hill may show wilting. If disease development is rapid, all leaves on the plants in a hill may wilt quickly without much change in color. Tubers are also affected by a general decay and soft rot.

Total losses from bacterial infections in developing countries can amount to hundreds of millions of US dollars annually and, in particularly bad years, it can approach several billion dollars worldwide. The extent of loss varies greatly from

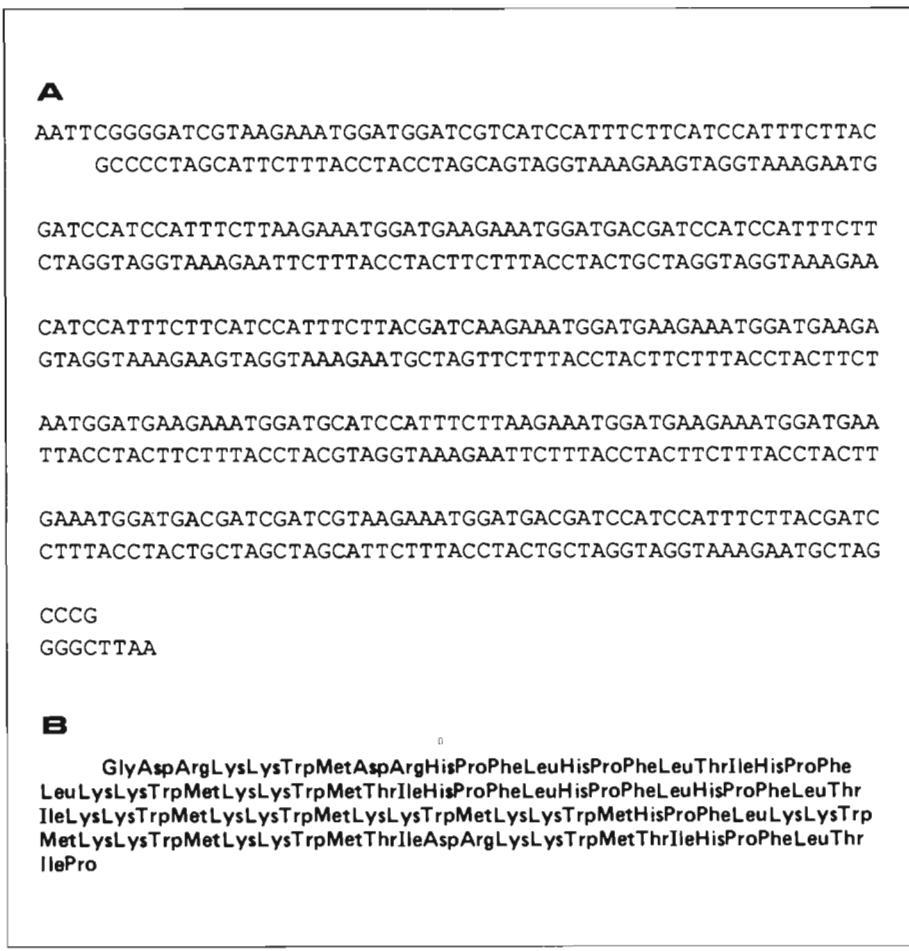


**Figure 1. A synthetic DNA sequence coding for a protein rich in essential amino acids (A) that can be translated to give the protein molecule shown in (B).**

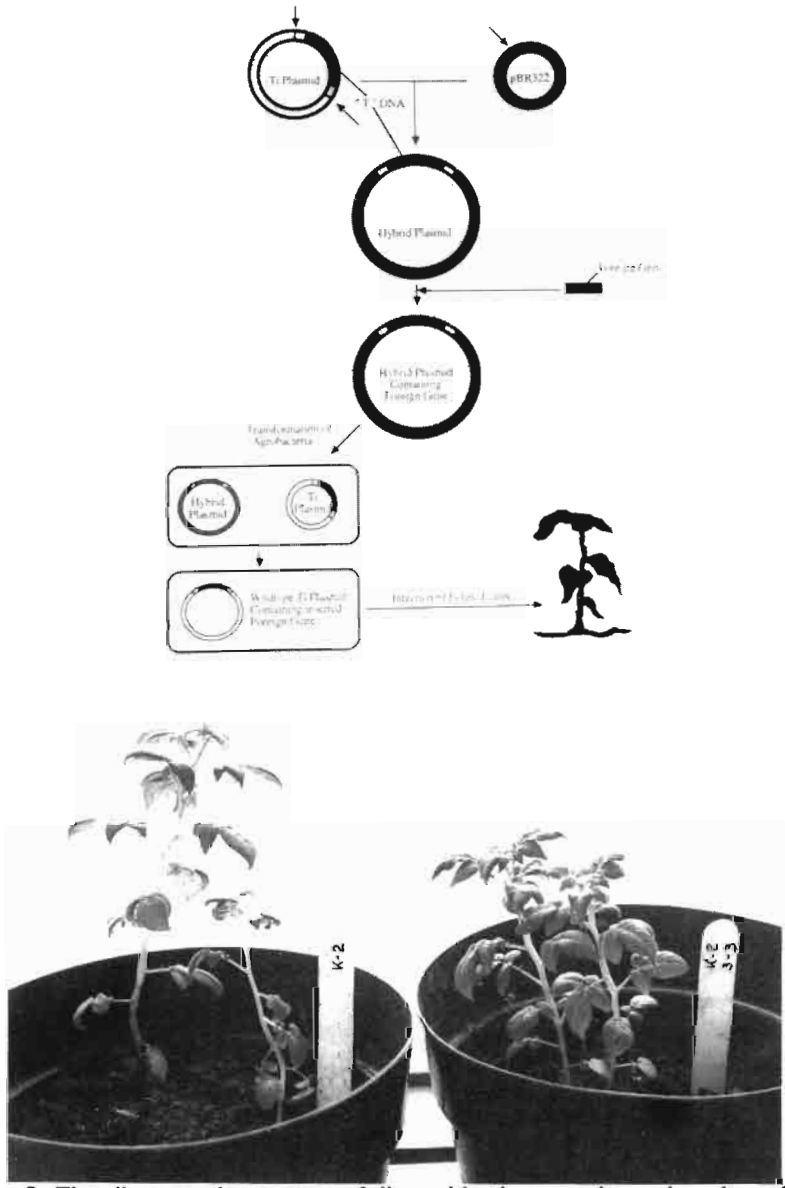


**Figure 2. The diagram shows steps followed in the genetic engineering of potato plants. A hybrid plasmid is constructed, the foreign gene is inserted, and the plasmid is introduced into *Agrobacterium*. The *Agrobacterium*, containing the foreign gene, will be used to inoculate the plant in which the synthetic protein is to be produced. The two pots containing potato clone K-7: genetically engineered plant containing the synthetic gene (right), control plant, nonengineered (left).**

country to country and is influenced by climate and conditions of growth and storage. Bacterial soft rot is a major factor limiting the storage of potato tubers under tropical conditions. Losses can be between 30 and 100% during the 2- to 6-month storage period, where temperatures can be between 27 and 32 °C.

As new heat-adapted clones are produced, allowing the potato to be grown in lowland tropical conditions, control of bacterial disease becomes a high priority. The cultivation of genetically resistant material has so far been the only effective method for the control of bacterial wilt. However, experience has shown that the available genetic resistance has a tendency to break down if the material is moved into climatic zones for which it is ill-adapted. Moreover, the genetic basis of traditional sources of resistance is limited to two Colombian clones of *Solanum phureja*. The reliance on this small gene pool has been a concern to breeders and plant pathologists for a very long time. Because of this we have given attention to the identification of innovative methods for bacterial disease resistance.

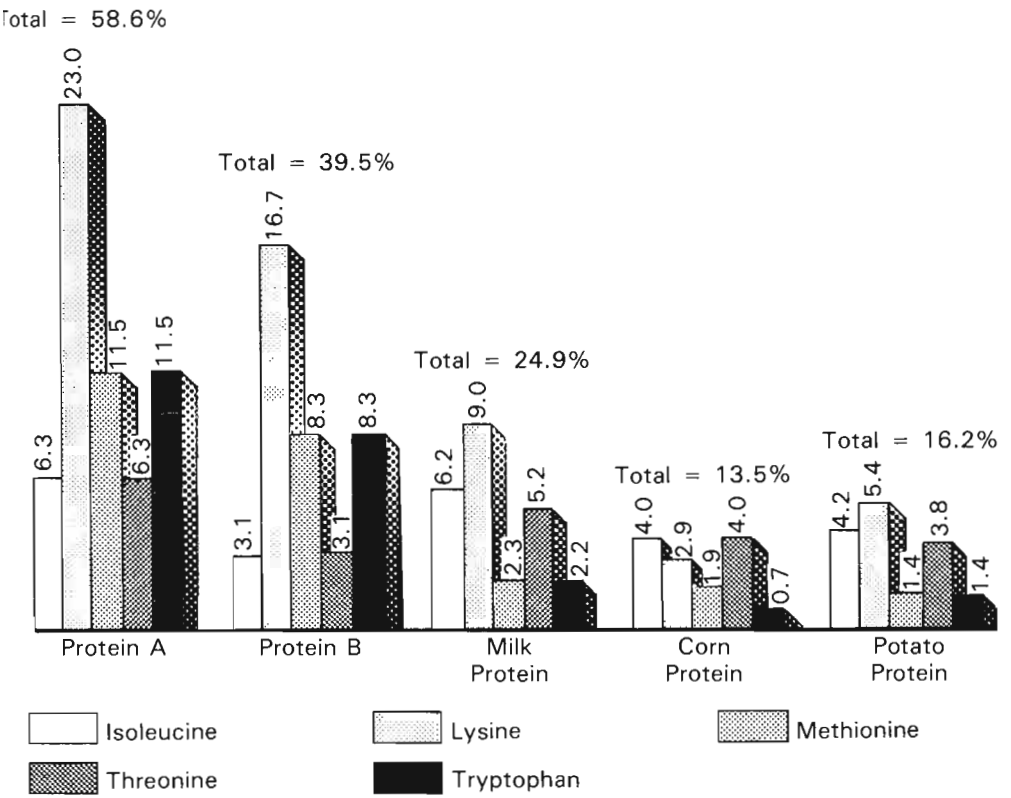


**Figure 3. The percent composition found in the synthetic proteins A and B, for the five most limiting essential amino acids of plant-derived proteins (isoleucine, lysine, methionine, threonine, and tryphophan), is compared with the content of proteins from some common foods. Synthetic protein A contains almost three times the amount of these essential amino acids than beef protein.**

## Use of genes encoding for material proteins to confer resistance to bacterial disease

The hemolymph of an immunized giant silk moth (*Hyalophora cecropia*) pupa contains at least three groups of antibacterial proteins: lysozyme, the antibacterial protein found in egg white and human tears; and two other classes of antibacterial peptides: cecropins and attacins. Thus, these insects have evolved a rather successful but apparently nonspecific means of fighting bacterial infections.

These antibacterial proteins have a rather broad spectrum activity, being effective in killing many different types of plant pathogenic bacteria (Table 1). With this array of antibacterial proteins, a genetically engineered plant would possess a rather potent arsenal composed of three different antibacterial proteins that appear to work in different ways to actively destroy the bacterial pathogen. This multi-level defense system would present a formidable challenge to the invading bacteria. The probability that a pathogen would become naturally resistant to all three toxins at once is rather remote (about 1 in 108). Also, it has been found that, when used together, there is a synergistic effect exerted which will make it even more difficult for the bacterial pathogen to compete. In essence, it was demonstrated that attacin enhanced the activity of cecropin and lysozyme when tested together on *Escherichia coli*. Primary results obtained in our laboratory seem to indicate that, at least for the plant pathogens listed in Table 1, there is a measurable synergism between cecropin and chicken egg-white lysozyme (unpublished results). While the exact modes of action of these antibacterial proteins have not been conclusively determined, it is clear that their actions are specific.

When *H. cecropia* pupae are immunized, they produce a set of proteins that are normally not present in the hemolymph of the insects, and are therefore an ideal biological system for the enrichment of the RNA and proteins that are synthesized from the inductive genes for immunity. Boman and his co-workers have taken advantage of this fact, both in the purification of 15 different immune proteins and in the isolation of immune RNA, later to be used for the preparation of a cDNA bank. After a short period of RNA synthesis, the insects respond to the exposure of live bacteria by the production of a potent antibacterial activity, which is due to the synthesis of at least three novel classes of bactericidal proteins: lysozyme, cecropins, and attacins.

**Table 1. Antibacterial activity on representative plant pathogenic bacteria.**

| | $LD_{50}$ (μM) | | | | | |
|---|---|---|---|---|---|---|
| Bacteria | SB-37 | SB-37+[a] | Synergy | Shiva-1 | Shiva-1+[a] | Synergy |
| *P. syringae* pv. *tabachi* | 5.2 | 0.19 | 27x | - | - | - |
| *P. solanacearum* | 64 | 16 | 4x | - | - | - |
| *E. carotovora* cr. | 1.5 | 0.44 | 3.3x | 0.5 | 0.26 | 1.9x |
| *X. campestris* cm. | 0.57 | 0.03 | 21x | 0.36 | 0.07 | 5.4x |
| *C. sepidonicum* mi. | 2.7 | - | - | 10 | 5.3 | 1.9x |

[a] + signifies the presence of lysozyme.

*Lysozyme. H. cecropia* lysozyme was purified from immune hemolymph and was identified as a chicken-type lysozyme. The primary structure of this enzyme was elucidated by determination through amino acid sequence analysis. A synthetic oligonucleotide probe was constructed, based on the protein sequence, and used to isolate cDNA clones. One clone, pCP701, with the largest insert size of about 420 base pairs (bp), was sequenced and found to include the full structural information for the mature enzyme (Fig. 4). The primary sequence of the mRNA that corresponds to clone pCP701 and the amino acid sequence obtained were in agreement except for one residue. More detailed studies, which included repeated amino acid sequence determinations from different lysozyme preparations, identified three variants of enzyme. It is surmised that these are allelic variants and that all could have originated from point mutations in their respective codons.

*Attacins.* Attacins are the largest antibacterial molecules found in the hemolymph of immunized *H. cecropia* pupae, with a molecular weight of about 20,000 daltons (20 kDA). They are comprised of six different forms, which can be fractionated according to their isoelectric point. The results from the amino acid sequence of the N-terminus of five of the attacins indicates the presence of three basic and two acidic forms that are slightly different from each other. It has been suggested that they are the products of two related genes. Two cDNA clones, pCP517 and pCP521, were isolated that correspond to these two genes. Comparison of the two clones has revealed 76% homology in the coding region (Fig. 5) and it has become evident that the genes originated from a common ancestral gene. The six attacin forms found in the hemolymph are probably products of secondary modification of the two precursor molecules.

*Cecropins.* The cecropins are the most potent group of antibacterial factors, with a broad spectrum of antibacterial activity against both Gram-negative and Gram-positive bacteria. They are small (around 4 kDA) and basic. They are comprised of three major forms (A, B, and D). Comparison of the amino acid sequences of the different forms has revealed a high degree of homology. They all have a basic N-terminal region and a hydrophobic stretch in the C-terminal part of the molecule. It seems that the cecropins are products of three related genes that, as in the case of the attacin genes, have originated by gene duplication. Recombinant cDNA clones corresponding to the cecropin-B form have been isolated which, when analyzed together, include information for the entire coding region of the cecropin-B molecule.

## INSECT RESISTANCE

Insect attack of food crops can lead to tremendous losses either by direct destruction of the plants or by causing insect damage that allows the infection and spread of other pathogens. Any possible means to increase a plant's tolerance to insect damage would thus be of significant value, especially to farmers in the developing world, who often lack the financial resources to protect their crops.

Several commercial laboratories are exploring the use of a natural insecticide to protect plants from insect damage. This natural insecticide, isolated from *Bacillus thuringiensis*, is actually a protein that forms a high molecular weight crystal. These

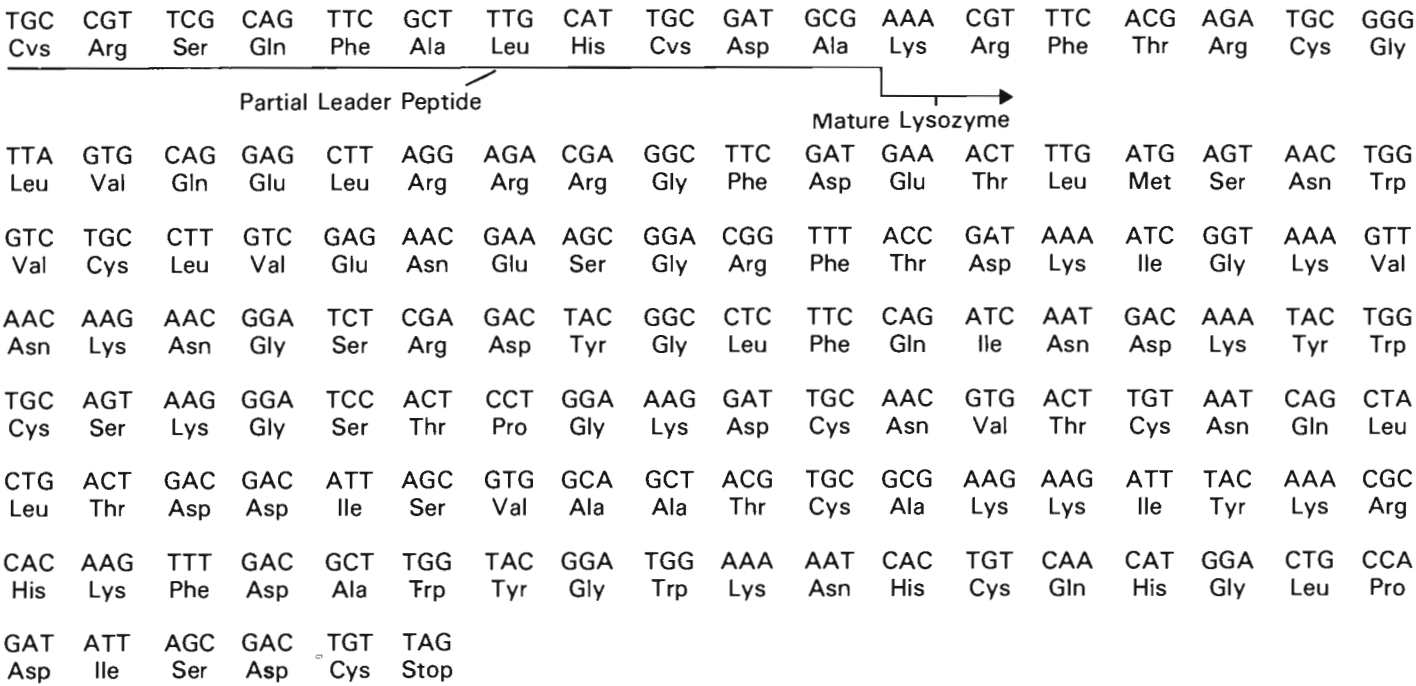


**Figure 4. Nucleotide and deduced amino acid sequences of the *H. cecropia* lysozyme. Part of the leader peptide is indicated by a line under the respective sequence.**

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GAC | GCG | CAC | GGA | GCC | CTT | ACG | CTC | AAC | TCC | GAT | GGT | ACC | TCT | GGT | GCT | GTG | GTT |
| Asp | Ala | His | Gly | Ala | Leu | Thr | Leu | Asn | Ser | Asp | Gly | Thr | Ser | Gly | Ala | Val | Val |
| AAA | GTA | CCC | TTT | GCT | GGT | AAC | GAC | AAG | AAT | ATA | GTA | AGC | GCT | ATC | GGT | TCC | GTA |
| Lys | Val | Pro | Phe | Ala | Gly | Asn | Asp | Lys | Asn | Ile | Val | Ser | Ala | Ile | Gly | Ser | Val |
| GAC | TTA | ACT | GAT | AGG | CAG | AAA | CTA | GGC | GCT | GCA | ACC | GCT | GGA | GTG | GCA | CTG | GAT |
| Asp | Leu | Thr | Asp | Arg | Gln | Lys | Leu | Gly | Ala | Ala | Thr | Ala | Gly | Val | Ala | Leu | Asp |
| AAT | ATA | AAC | GGT | CAC | GGA | CTA | AGT | CTC | ACG | GAT | ACA | CAC | ATC | CCC | GGG | TTC | GGA |
| Asn | Ile | Asn | Gly | His | Gly | Leu | Ser | Leu | Thr | Asp | Thr | His | Ile | Pro | Gly | Phe | Gly |
| GAC | AAG | ATG | ACA | GCA | GCC | GGC | AAA | GTG | AAT | GTC | TTC | CAC | AAT | GAT | AAC | CAC | GAC |
| Asp | Lys | Met | Thr | Ala | Ala | Gly | Lys | Val | Asn | Val | Phe | His | Asn | Asp | Asn | His | Asp |
| ATC | ACA | GCG | AAG | GCT | TTC | GCC | ACC | AGA | AAC | ATG | CCG | GAT | ATT | GCT | AAT | GTA | CCT |
| Ile | Thr | Ala | Lys | Ala | Phe | Ala | Thr | Arg | Asn | Met | Pro | Asp | Ile | Ala | Asn | Val | Pro |
| AAT | TTC | AAC | ACT | GTC | GGT | GGC | GGA | ATA | GAC | TAT | ATG | TTC | AAA | GAT | AAG | ATT | GGT |
| Asn | Phe | Asn | Thr | Val | Gly | Gly | Gly | Ile | Asp | Tyr | Met | Phe | Lys | Asp | Lys | Ile | Gly |
| GCA | TCT | GCG | AGC | GCC | GCT | CAC | ACG | GAC | TTT | ATC | AAT | CGC | AAC | GAC | TAC | TCT | CTT |
| Ala | Ser | Ala | Ser | Ala | Ala | His | Thr | Asp | Phe | Ile | Asn | Arg | Asn | Asp | Tyr | Ser | Leu |
| GAC | GGG | AAA | CTG | AAC | CTC | TTC | AAG | ACT | CCT | GAT | ACC | TCG | ATT | GAT | TTC | AAC | GCC |
| Asp | Gly | Lys | Leu | Asn | Leu | Phe | Lys | Thr | Pro | Asp | Thr | Ser | Ile | Asp | Phe | Asn | Ala |
| GGT | TTC | AAG | AAG | TTC | GAT | ACA | CCT | TTC | ATG | AAG | TCC | TCT | TGG | GAG | CCT | AAC | TTC |
| Gly | Phe | Lys | Lys | Phe | Asp | Thr | Pro | Phe | Met | Lys | Ser | Ser | Trp | Glu | Pro | Asn | Phe |
| GGA | TTC | TCA | CTT | TCT | AAA | TAT | TTC | TGA | TTA | | | | | | | | |
| Gly | Phe | Ser | Leu | Ser | Lys | Tyr | Phe | Stop | Stop | | | | | | | | |

**Figure 5. Nucleotide and deduced sequences of the clone encoding the mature form of the basic attacin.**

crystals are toxic to the larvae of a number of lepidopteran insects. Work is currently in progress to insert the gene for the *B. thuringiensis* toxin protein into suitable *Agrobacterium* vectors for the eventual introduction of this gene into potato plants.

In addition to this toxin, we are currently characterizing a gene which encodes an enzyme called chitinase, which we believe will prove to be a potent anti-insect compound. The chitinase enzyme will attach to the skeleton and gut of the insect allowing it to contract bacterial infections leading to its eventual death. This gene was isolated from a bacterium called *Vibrio parahemolyticus*. We have already purified the chitinase gene and are now inserting this into *Agrobacterium* plasmids ready to transfer the gene to potato plants. These transformed plants will then be screened for resistance to insect damage and to *Phytophthora*. Fungal cells also contain chitin and may be sensitive to lysis by the plant-produced chitinase enzyme.

## RESEARCH NETWORK

From the preceding sections, it can be seen that a wide range of options exist to attempt potato improvement by use of genetic engineering technology. The approach taken by CIP to develop this type of work has been based on our use of comparative advantages. CIP has a comparative advantage in germplasm availability and tissue culture technology, whereas, gene synthesis work is best done in institutions fully equipped for that type of work. In the case of genetic engineering, some laboratories specialize in gene synthesis, some in promoter isolation, some in transformation, etc. Thus over the year's CIP has built up a substantial network (Fig. 6). The use of these collaborative bridges should enable us to serve the needs of national programs in the most efficient and cost effective manner.

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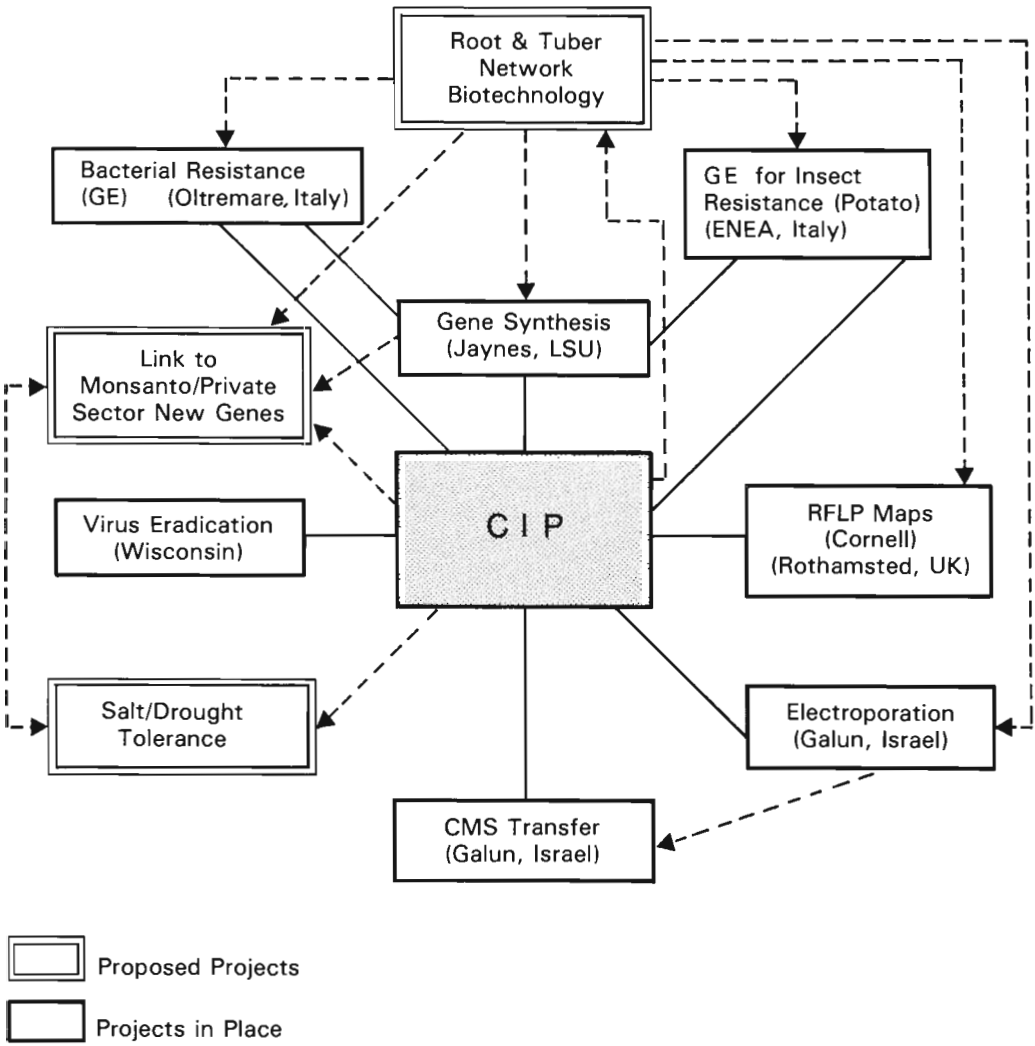


**Figure 6. Network of biotechnology research projects currently underway in CIP's tissue culture laboratory.**

**RESUMEN**

En los últimos años, el Centro Internacional de la Papa (CIP) ha creado un sistema de colaboradores para estudiar la ingeniería genética de la papa. Los proyectos, algunos de ellos financiados como proyectos especiales y otros con fondos del presupuesto básico, se han concentrado en la incorporación de una expresión de genes que se considera importante para los programas nacionales. Los primeros estudios, basados en el empleo de genes sintéticos, tenían por objeto mejorar lo más posible el valor nutritivo de la papa. Los genes sintéticos codifican proteínas sintéticas con un alto contenido de aminoácidos esenciales (sobre todo lisina y metionina). Se ha tenido éxito en cuanto a la inserción de los genes y a la obtención de una expresión específica del órgano (tubérculo). En esta tarea participaron diversos laboratorios. Tomando como base estos estudios iniciales, actualmente se emplean genes naturales y sintéticos para tratar de crear en la papa resistencia a las plagas y a las enfermedades. Hoy día se dispone de diversos genes y de una amplia gama de promotores. Estos estudios ofrecen un método específico para dar a la papa nuevos tipos de resistencia.

# Recommendations and plans for a third symposium

A planning meeting was held Tuesday evening, August 30, 1988, to discuss the follow-up of the Second Genetic Manipulation Symposium. The following recommendations were made and later approved by all the participants at the closing session on August 31.

## ARRANGEMENTS FOR THE THIRD INTERNATIONAL SYMPOSIUM ON GENETIC MANIPULATION IN CROPS

It is desirable to continue this series of symposia so that issues relating to the application of recent advances in molecular biology and genetic engineering to crop improvement can be addressed. It was agreed that the third symposium should be held at a suitable city in Africa sometime in 1991. The following locations are being considered:

- Dakar, Senegal
- Harare, Zimbabwe
- Ibadan, Nigeria
- Nairobi, Kenya

To ensure effective local participation, the following organizations may be involved in the planning of the third symposium from the beginning, including the choice of the location and dates:

- African Academy of Science (President: Prof. T. Odahiambo)
- African Genetics Society (President: Prof. O. Aromose)
- Overseas Agency for Scientific and Technological Research (ORSTOM), France

### Co-sponsors of the Third Symposium

Academia Sinica and CIMMYT have agreed to serve as co-sponsors. A request will be made to the Director General of IRRI for IRRI to also continue as a co-sponsor of this symposium series. In addition to these three, it was suggested that the following organizations might want to consider co-sponsoring the third symposium:

- International Agricultural Research Centers working in Africa (IITA, ILARD, ILCA, ICRISAT, WARDA, ICARDA, ICIPE).

- International Centre for Genetic Engineering and Biotechnology (ICGEB).
- The Rockefeller Foundation Network on Rice Biotechnology.
- International Genetics Federation (IGF)
- United Nations University

**Wide participation**

For such events to be truly worthwhile, there should be greater participation of scientists and young scholars from the countries of the region in which the symposium is held. Important purposes of the meetings are education and the exchange of information where the latest findings and techniques are made available to the scholars of the region. Therefore, the third symposium in this series should be organized in such a manner that a wide participation becomes possible.

**Steering committee**

A steering committee was set up to plan and organize the third international symposium (at a suitable location in Africa sometime in 1991). The members include: M.S. Swaminathan, chairman; Q.Q. Shao, A. Mujeeb-Kazi, and L.A. Sitch, joint secretaries; R. Chavez, V.L. Chopra, E.C. Cocking, G.E. Hanning, H. Hu, Y.F. Li, H. Lörz, Sir Ralph Riley, M.D. Winslow, and A.H. Zakri

A local organizing committee will be set up when the location and dates have been settled.

## GENETIC MANIPULATION NEWSLETTER

Gratitude was expressed to Academia Sinica and to Professors H. Hu and Q.Q. Shao for producing very informative newsletters, in accordance with a request made at the First Symposium in China. These newsletters (four have been published so far) have been particularly helpful in spreading information on the recent work of Chinese geneticists. Prof. Shao announced that from January 1989 the Newsletter will be converted into an *International Journal of Plant Genetic Manipulation*. An International Editorial Advisory Committee has been established for this Journal.

## ACKNOWLEDGMENTS

Gratitude was expressed to the Director General and Staff of CIMMYT for the excellent arrangements made for the Second Symposium. Also, the financial assistance of UNDP, Rockefeller Foundation, USAID, and the Third World Academy of Sciences was gratefully acknowledged.

# Fertile revertants of male sterility in somaclones from somatic cell culture of rice

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Male sterile mutants were induced from somatic cell culture in indica rice and fertile revertants were obtained from *in vitro* culture of S-type and T-type male sterile maize. It might be possible that tissue culture is an effective method for inducing male sterility and causing its reversion.

Male sterile (ms) mutants were induced from somatic cell culture in indica rice (Ling *et al.* 1987) and fertile revertants were obtained from *in vitro* culture of S-type (Earle *et al.* 1987) and T-type male sterile maize (Umbeck and Gengenbach 1983). This experiment attempts to answer the question: in rice, can a reverse mutation of male sterility be obtained by somatic cell culture?

## MATERIALS AND METHODS

### Male sterile plants

Two kinds of ms materials were used. One was the indica male sterile line, 194A, developed by transferring the BT male sterile cytoplasm from japonica rice (Shinijyo 1969). The other represents ms mutant plants obtained as somaclonal variants from callus cultures of IR8, IR24, and IR54 (Table 1).

### Tissue culture

The young panicles of the ms plants, 0.5-1.0 cm in length, were cultured in MS (Murashige and Skoog 1962) medium with 2 mg/L of naphthalene acetic acid (NAA) and 2 mg/L of Kinetin (KT) and LS (Linsmaier and Skoog 1965) medium with 1 mg/L of 2,4-Dichlorophenoxy acetic acid (2,4-D) and 1 mg/L of KT. In the first medium, the spikelets of the young panicles regenerated directly without a callus stage to produce plants (Ling *et al.* 1983). In the second medium, calluses were induced and plant regeneration was stimulated when the calluses were transferred to a regeneration medium.

## RESULTS AND DISCUSSION

### Fertile reverse mutation

A total of 126 regenerants (R1 generation) was obtained and reached maturity. Among them, 94 regenerated directly from the spikelets from five ms somaclones of IR8 (91, 203, and 54072), IR24 (826), and IR54 (457), without a callus stage (Table 1); 32 were regenerated plants from the ms-line 194A, following callus induction. Of the 126 regenerants, two plants from the 91-20 ms-plant and one from 194A were found to be fertile and given the codes '91-20RVT' and '194RVT', respectively. The fertilities of 91-20RVT and 194RVT were 87.5 and 78.1%, respectively; those of the original ms-plants, 91-20 and 194A, were 0 and 1.5%, respectively.

In addition, one of the plants regenerated directly from the spikelets of ms-line 91-20 and was found to have a modified form of male sterility. The original ms-plant (91-20) had no pollen in its anthers (Ling *et. al.* 1987), whereas the anthers of ms-plant 91-20P possessed pollen, but it was not viable, and hence was not stained by I-KI solution.

Figure 1 illustrates the process of the induction of male sterility in IR54 and reversion to fertility, thus producing 91-20RVT.

### Expression of the offspring in revertants

The progeny of the revertant 194RVT (the R2 generation) was found to segregate for fertility. Of 22 progeny of 194RVT, 15 were fertile and 7 were sterile. The hybrids produced from the test cross 194A/194RVT also segregated for fertility. These results showed that the reverse mutation conferring fertility was dominant and that the revertant (194RVT) was heterozygous for this mutation. The genetic control of fertility in the revertant (91-20RVT) has not yet been determined because, as of this writing, the R2 generation derived from 91-20RVT has not flowered.

**Table 1. Number of fertile and male sterile (ms) plants regenerated directly from cultured spikelets.**

| Source cultivar | IR54 | | | | | | | | IR8 | IR24 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ms mutant code no. (generation) | 91-12 ($R_2$) | 91-20 ($R_2$) | 91-1 ($R_3$) | 91-5 ($R_3$) | 91-8 ($R_3$) | 91-13 ($R_3$) | 203-1 ($R_1$) | 54072 ($R_1$) | 826 | 457 | |
| No. of plants regenerated directly from spikelets | 5 | 15 | 8 | 10 | 3 | 5 | 22 | 3 | 3 | 20 | 94 |
| No. of ms plants | 5 | 13[a] | 8 | 10 | 3 | 5 | 22 | 3 | 3 | 20 | 92 |
| No. of fertile plants | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Including a plant showing alteration in nature of ms (with abortive pollen, 91-20P).

In our laboratory, 12 ms mutants were obtained from somatic cell culture of nine rice cultivars between 1984-1987. This shows that tissue culture may be an effective method for inducing male sterility and causing its reversion.

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## RESUMEN

Se indujeron mutantes masculinos estériles a partir del cultivo de células somáticas del arroz indica y se obtuvieron invertidores fértiles del cultivo *in vitro* del tipo S y del maíz masculino estéril tipo T. Este experimento trata de resolver la siguiente pregunta: ¿Puede obtenerse una mutación inversa de la esterilidad masculina en el arroz mediante el cultivo de células somáticas?

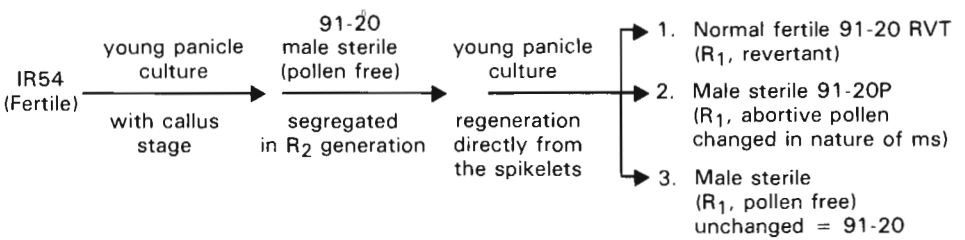


**Figure 1. The process of induction of male sterility in IR54 and reversion of fertility through *in vitro* culture.**

# Diagnostic markers in wheat wide crosses

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It is important to identify alien chromatin accurately and rapidly in a wide hybridization program. Markers are heritable characteristics associated with and useful for the identification of specific genotypes. Markers of significance usually show high polymorphism, few epistatic or pleiotropic effects, exhibit co-dominant inheritance, and are free from environmental and developmental influence.

Markers are heritable characteristics associated with specific genotypes and hence often used for genotypic characterization. Among other things, markers are used to describe parental lines and populations, assess genetic variability and phylogenetic relationships, identify gene blocks contributing to important agronomic characters, tag genes of agronomic traits, test for normality of gene flow in interspecific or intergeneric crosses, confirm hybrids, identify aneuploids, establish wheat-alien chromosome homoeologies, and track alien chromosome segments in wheat backgrounds. Important features of a useful marker include a high level of polymorphism, speed of assay, few epistatic or pleiotropic effects and a co-dominant mode of inheritance. Markers may be categorized as morphological, cytological, or biochemical.

## MORPHOLOGICAL MARKERS

Morphological markers are often easily identified but are limited by environmental influences, exhibit dominant/recessive inheritance and relatively low polymorphism. Characters such as pubescence, presence of anthocyanin pigmentation (Fig. 1a), grain color (Fig. 1b), stem solidity (Fig. 1c), and spike shape (Fig. 1d) have been often used in the Triticeae to indicate the presence of their respective genes. Wheat plants are often modified in terms of such characters upon introgression of alien chromatin, thus assisting characterization of the introduced segments since genetic control for most of these characters has already been attributed to specific homoeologous groups.

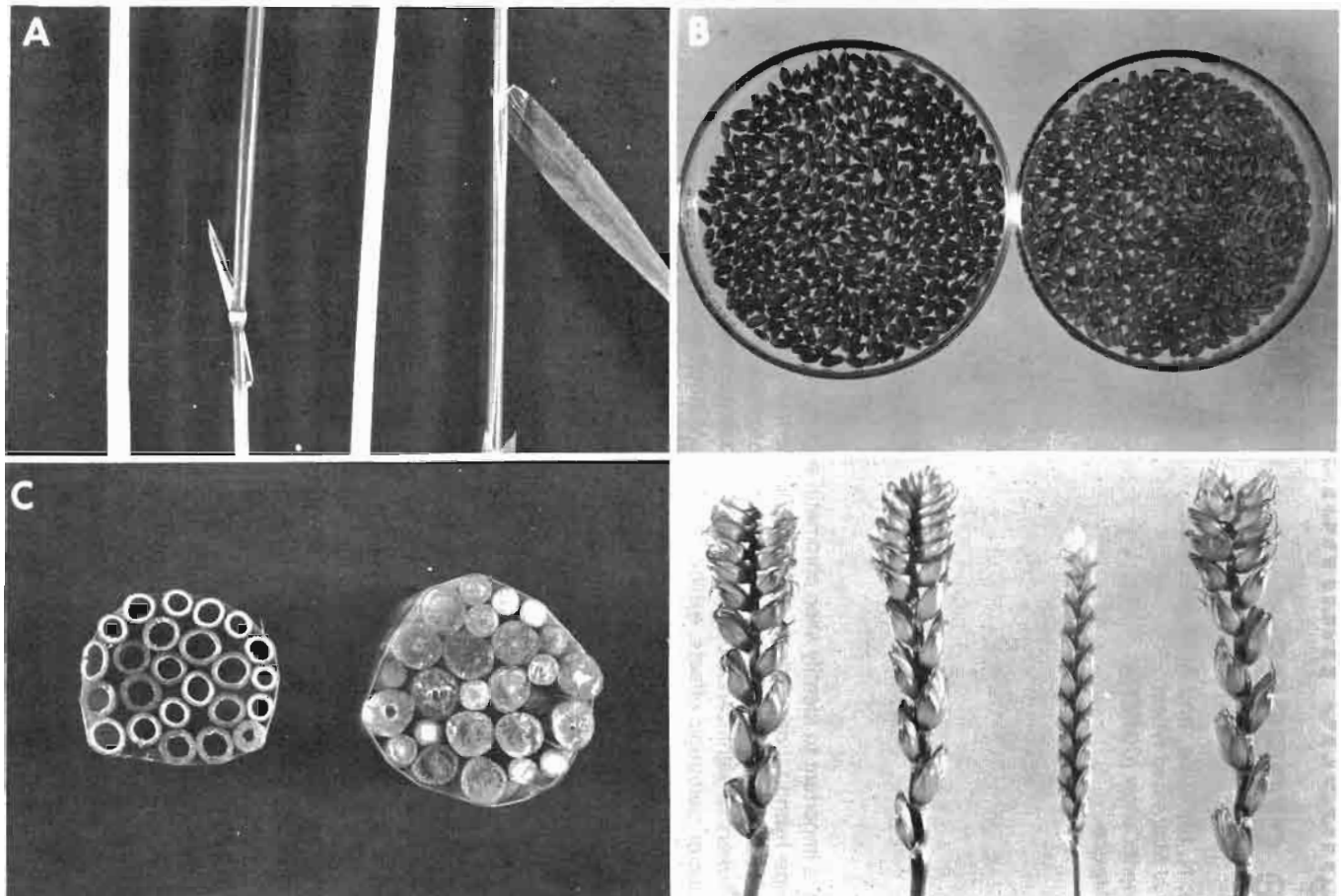



**Figure 1. Morphological markers showing in: A) presence of anthocyanin (segregation for color); B) seed color (blue aleurone versus normal color); C) stem solidity (normal versus solid stem); and D) spike morphology, left to right, 'Chinese Spring' (CS) + 5R (from *Secale cereale)*, CS + 5J *(Thinopyrum bessarabicum, Agropyron junceum)*, CS, CS + 5 UM *(Aegilops umbellulata).***

## CYTOLOGICAL MARKERS

Cytological markers include features such as chromosomal size/arm ratio (Fig 2a; Mujeeb-Kazi and Miranda 1985), satellites/secondary constrictions (Fig. 2b; Mujeeb-Kazi and Miranda 1985), and differential C- and N-banding staining patterns (Figs. 2c and d; Mujeeb-Kazi 1982; Mujeeb-Kazi *et al.* 1989) that are useful in identification of wheat and alien chromosomes. Many different stains have been used to facilitate such identification, the more common being feulgen, acetocarmine, aceto-orcein, giemsa, and Leishman. Recently radioactive- and biotin-labelled DNA sequences have been introduced as markers through their *in situ* hybridization to complementary DNA sequences in cytological preparations and the detection of the resulting duplexes by autoradiography, fluorescence, or through enzymatic procedures. The application of biotin labelled probes is projected to detect D genome/alien genome chromosome translocations; stocks that are being developed at CIMMYT (Mujeeb-Kazi 1984).

## BIOCHEMICAL MARKERS

Biochemical markers may be subdivided on the basis of whether they are the gene products (proteins) or DNA sequences. Differential characteristics of proteins and DNA have been used to distinguish among members of the Triticeae and their progenies. Protein markers have been used more extensively than DNA markers and involve analyses of isozymes and/or storage proteins. There are currently over 100 structural gene loci in wheat identifiable by protein markers. Homoeoallelic loci have been identified in related species. Only the short arms of homoeologous group 2 chromosomes have not been reliably marked so far in this way. The protein separation techniques used in the wheat wide crosses program at CIMMYT are: cellulose acetate electrophoresis, polyacrylamide gel electrophoresis (PAGE) under native conditions, sodium dodecyl sulfate (PAGE), and polyacrylamide gel isoelectricfocussing (PAG.IEF).

Characteristic separation patterns have been established of many isoenzymes and storage proteins for many spring wheats and alien species. Consistent differences between the patterns of the alien species as compared to those of wheat, especially as confirmed in the wheat-alien species hybrid, serve as useful markers. The enzymes analyzed most often in our program are acid phosphatase, aconitate hydratase, aliphatic alcohol dehydrogenase, esterases (Figs. 3a and b), glucose phosphate isomerase, glutamate oxaloacetate transaminase, isocitrate dehydrogenase, malate dehydrogenase, phosphoglucomutase (Fig. 3c), aspartate aminotransferase (Fig. 4a), 6-phosphogluconate dehydrogenase (Fig. 4b), shikimate dehydrogenase and superoxide dismutase. DNA markers termed restriction fragment length polymorphisms (RFLPs) have the potential to saturate the genetic maps of most crop plants as they have the capacity to detect differences in the DNA sequence which may not result in detectable gene products or changes in gross morphology. RFLP technology is yet to be extensively employed in wheat wide hybridization owing to the lack of suitable probes in the public sector.

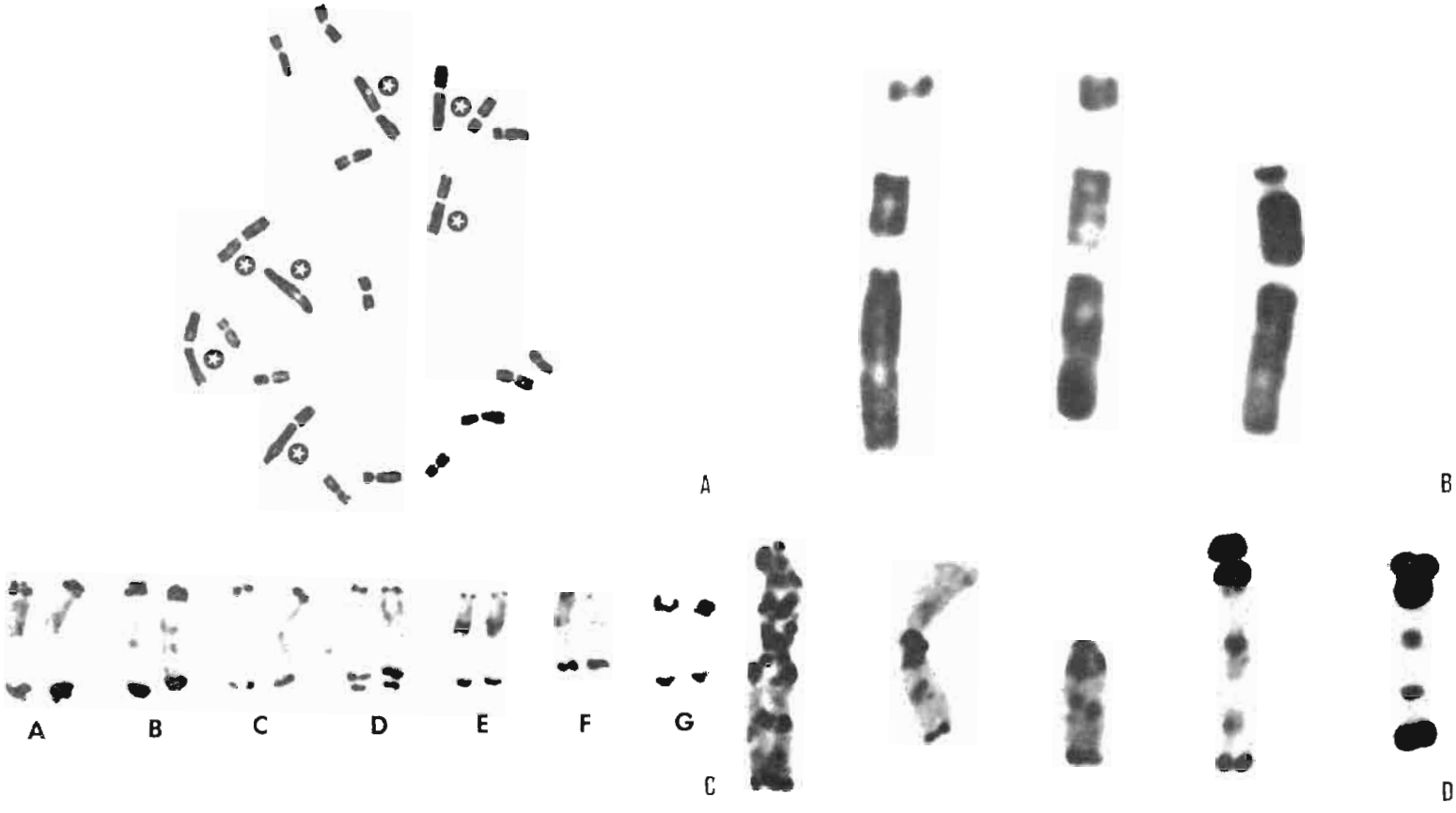
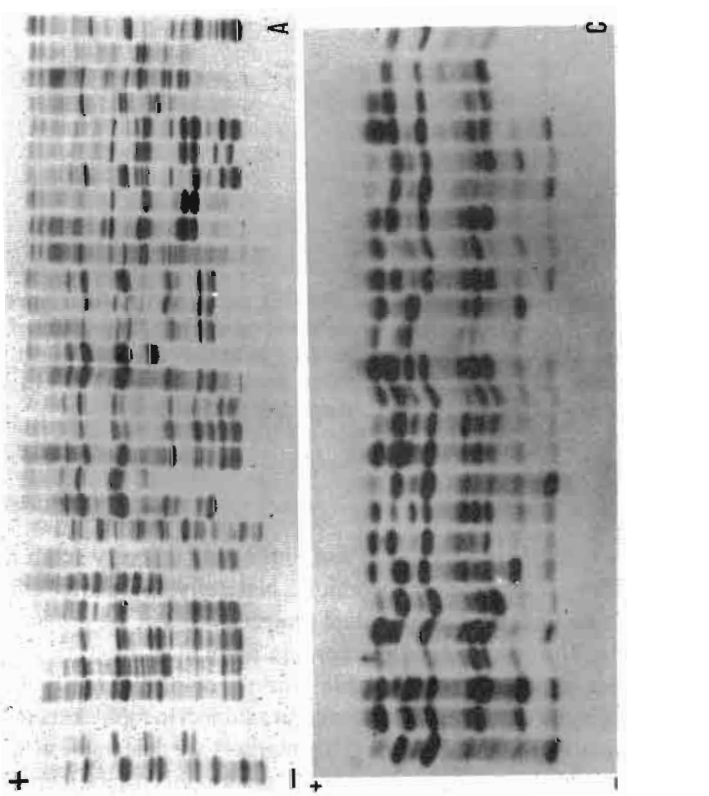



Figure 2. Chromosome structure showing size/arm ratio, satellites/secondary constrictions, differential staining (C and N banding) as follows: A) chromosomes of *Agropyron elongatum/Secale cereale* $F_1$ hybrid (* = *S. cereale*) (Mujeeb-Kazi and Miranda 1985); B) left to right: satellited chromosomes 1B, 6B, and 5D of *Triticum aestivum* (Mujeeb-Kazi and Miranda 1985); C) C-banded karyotype of *Haynaldia villosa* (Mujeeb-Kazi 1982); and D) left to right: N-banded chromosomes of 1B, 1BL/1RS, 1RL, and C-banded 1BL/1RS, 1R. (Mujeeb-Kazi 1982, Mujeeb-Kazi *et al.* 1989).



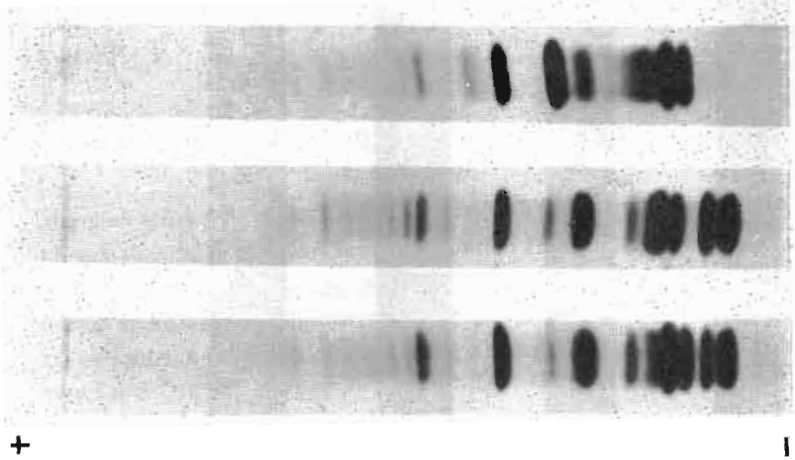




Figure 3. Polyacrylamide gel isoelectric focussing (PAG.IEF) patterns of the following: A) Esterase (E.C.3.1.1.2) for three accessions of *Thinopyrum (Agropyron) intermedium* (10 tracks/accession); B) Esterase showing polymorphism for different CIMMYT spring wheats *(Triticum aestivum)*; C) Phosphoglucomutase (E.C.2.7.5.1) for 25 accessions of *Th. (A.) intermedium*.

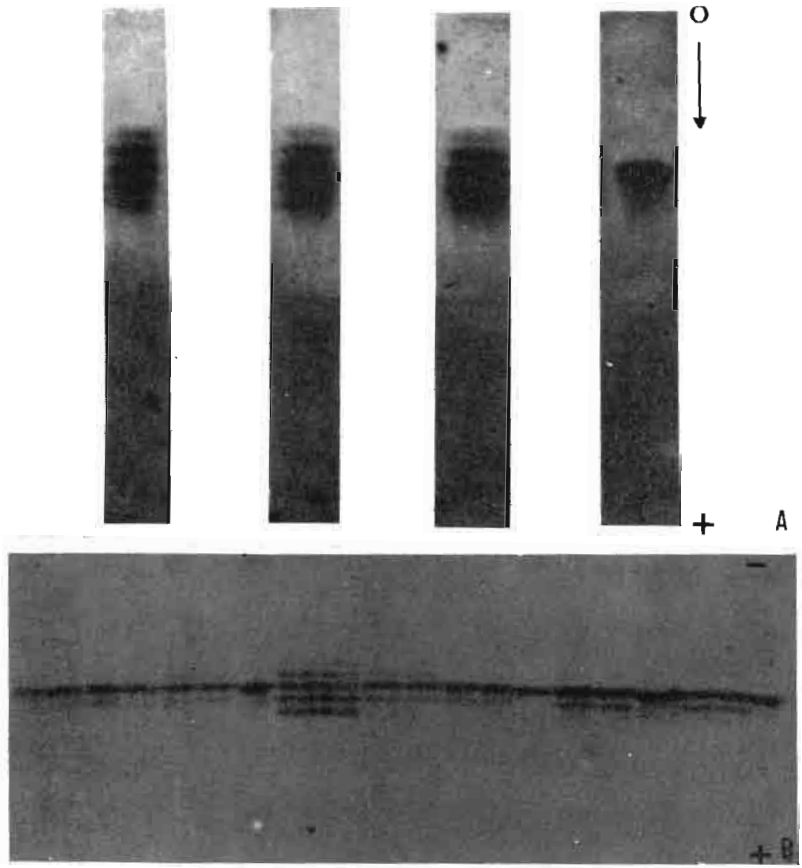



**Figure 4. Polyacrylamide gel electrophoresis (PAGE) patterns of: A) aspartate amino transferase (E.C.2.6.1.1) for, left to right, *Thinopyrum (Agropyron) acutum, Triticum turgidum* L. cv. Yavaros/*Th. (A.) acutum* (2 tracks) and *T. turgidum* L. cv. Yavaros; B) 6-phosphogluconate dehydrogenase (E.C.I.I.I.44) for backcross derivatives of *T. aestivum* L./*Aegilops variabilis*.**

## CONCLUSION

All classes of markers have their advantages and limitations but very often marker combinations from different categories are beneficial. Naturally, the relative emphasis on the different classes depends on the individual project, stages of the project and/or the resources available. The biochemical markers, in particular, are very versatile. They have an abundance of polymorphism, exhibit little to no pleiotropic or epistatic effects, are less affected by environmental influence, are subject to rapid detection, and are inherited co-dominantly. Furthermore the DNA markers are detectable at all plant developmental stages and all the polymorphisms at all marker loci can be identified by a single technique.

## ACKNOWLEDGMENT

The authors are grateful to Drs. E.R. Sears (University of Missouri, USA), B. Forster, and C. Law (PBI now AFRC-IPSR, England) for the generous supply of Imperial rye (*Secale cereale*) addition lines, 5J disomic addition of *Thinopyrum bessarabicum*, and 5UM disomic addition of *Aegilops umbellulata* in 'Chinese Spring' background, respectively.

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## RESUMEN

En un programa de hibridización amplia, es de vital importancia identificar con precisión y rapidez la cromatina extraña. Los marcadores son características hereditarias que son de gran utilidad en la identificación de genotipos específicos. Los marcadores de mayor importancia suelen presentar un alto nivel de polimorfismo, escasos efectos epistáticos o pleiotrópicos, muestran herencia codominante y no se ven afectados por las influencias ambientales y del desarrollo.

# *In vitro* culture for the conservation of wild *Manihot* species

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The wild relatives of cassava, species from the genus *Manihot*, are receiving increasing attention due to their source of genes for resistance to several pests and diseases, and for tolerance to common abiotic stresses. During the last decade, a considerable amount of work has been done on taxonomy and ecology of wild *Manihot* species. Nearly 100 species related to cassava have been described. The urgent need to build up a collection and preserve wild germplasm is supported by the fact that considerable genetic erosion currently takes places in nature among wild species of *Manihot*. In 1987, a research project was started at the International Center for Tropical Agriculture (CIAT) to develop techniques for the conservation and utilization of wild *Manihot* species. A standardized system of abbreviation for 99 wild species and 18 sections has been developed and implemented at CIAT.

The wild relatives of cassava, *Manihot* spp., are receiving increasing attention due to their source of genes for resistance to several pests and diseases, and for tolerance to common abiotic stresses. It has also been hypothesized that the wild *Manihot* gene pool may have a high genetic variability for important biochemical traits, such as low cyanide content and high protein content and for improved C4 photosynthetic mechanisms. However, very little is known about the intra- and inter-specific variation, reproductive biology, genome constitution, and phylogenic and crossability relationships with cultigens of *M. esculenta*. This information could be very useful for the successful gene transfer for crop improvement.

During the last decade, a considerable amount of work has been done on taxonomy and ecology of wild *Manihot* species.

Rogers and Appan (1973) described 98 species related to cassava; recently another new species, *M. neusana*, has been described (Nassar 1985) and at present there are at

least five new species of *Manihot* being described (Allen, per. comm.). Nearly every species possesses traits of potential interest to plant breeders as well as a high range of interspecific variation in morphological and physiological traits (Fig. 1).

The wild *Manihot* species offer sources of useful genes, for example, low cyanide content from *M. pringlei* and resistance to African cassava mosaic virus from *M. glaziovii*, resistance to cassava bacterial blight from *M. pseudoglaziovii* and *M. reptans*, high starch content from *M. tristis* and *M. angustiloba*. Genetic resistance to the most common cassava pests has been found among genotypes of *M. glaziovii* and *M. dichotoma* including high levels of resistance to mealybug, a very dangerous pest in West Africa, and resistance to stem borer in *M. neusana*, *M. pohlii* and *M. grahami* (Allen 1984). New genes for disease resistance to African mosaic virus have been incorporated from *M. glaziovii* to *M. esculenta*.

Many species show adaptation and tolerance to different abiotic stresses. For example, *M. chlorosticta* grows mainly in saline soils, and *M. pseudoglaziovii*, *M. carthaginensis*, and *M. dichotoma* are well adapted to drought conditions. Another striking example of adaptation is displayed by *M. attenuata* and *M. rubricaulis* which may provide useful genes for tolerance to cool temperatures because they grow at elevations of about 1600-1700 masl. Outstanding adaptation to acid soils with aluminum toxicity and low phosphorus content, a desirable trait in cassava, has been found among wild populations of *M. irwinii*, *M. tripartita*, and *M. orbicularis* from Brazil. The availability of a wide spectrum of wild *Manihot* germplasm is needed to broaden the genetic base of cultivated cassava.

The urgent need to build up a collection and preserve wild germplasm is also supported by the fact that considerable genetic erosion currently takes places in nature among wild species of *Manihot*. For example *M. walkerae*, *M. guaranitica*, *M. subspicate*, *M. angustiloba*, *M. longipetiolata* and *M. pringlei* have been reported to be extremely endangered species (Nassar and Cardenas 1985, Allen 1984).

A research project was started at CIAT in 1987 to develop techniques for the conservation and utilization of wild *Manihot* species. Since germination trials showed very low seed viability, it was necessary to carry out *in vitro* embryo culture as a means to recover plants from seeds unable to germinate under standard conditions (Biggs *et al.* 1986). Shoot tips from seedlings have also been used for *in vitro* maintenance of wild species. Twenty-one species of *Manihot* have been cultured *in vitro*; in addition, 26 genotypes of the putative wild cassava *M. esculenta* ssp. *flabellifolia* (Allen 1984), collected in Brazil, have also been cultured. Eleven species are being tested for adaptation to field conditions.

Using the drought-tolerant species *M. carthaginensis*, the effect of culture medium composition on embryo growth has been investigated (Table 1). Explant source and medium composition were shown to influence culture success. A standardized system of abbreviation for 99 wild species and 18 sections has been developed and implemented at CIAT (Chavez *et al.* 1987).

## SAMPLING OF MATERIAL

During the last 10 years, there have been several expeditions supported by the International Board for Plant Genetic Resources (IBPGR) and CIAT to collect wild *Manihot* species throughout the main centers of diversity. As a result, some living collections were raised and are currently maintained at CIAT, and in Brazil, Mexico, and Paraguay. Recently, with CIAT support, more than 3000 seeds of *M. carthaginensis* were sampled from more than 100 parental female genotypes in the semiarid


**Figure 1. Genetic diversity among wild species of *Manihot* from Brazil: a) natural habitat (savanna) of *M. orbicularis* and about 13 species; b) *M. irwinii;* c) *M. pentaphylla*; d) *M. anomala*; e) *M. peltata*; and f) *M. tristis*, closely related to the cultivated species.**

ecosystem of the Atlantic Coast of Colombia. Collection expeditions were made in Brazil with IBPGR, CIAT, and Brazilian support. Seed sampling was combined with *in vitro* shoot-tip sampling in the field (Withers and Williams 1985).

## GERMINATION

Seeds of wild *Manihot* species normally have severe dormancy or are in most cases nonviable. For this reason, difficulties have been encountered in raising plants from seeds. Attempts to germinate 21 wild *Manihot* spp. seeds resulted in 0-15% germination. In one experiment, seeds were subjected to temperatures of 35 °C for 8 hours and 25 °C for 16 hours with light provided during the 16-hour phase (Ellis and Roberts 1979). Although a few seeds germinated within 7 days, most did not germinate even after 21 days of treatment.

In another experiment, stored seeds from the cool room were divided into two groups based on whether they floated or sank in the water. The two seed groups were placed on paper towels (25 seeds on each), wetted in an aqueous solution of 0.2% phosphorus-rich fertilizer and 0.1% fungicide, and put in plastic bags with holes. The bags were placed in an incubator (25 °C/16 hours and 35 °C/8 hours) for a period of 21 days. More than 90% of the seeds germinated between 5 and 7 days later. Nongermination and low-viability were correlated with longer storage periods and with seeds that floated. Seed escarification did not enhance germination of wild *Manihot* seeds.

**Table 1. *In vitro* embryo culture of M. *carthaginensis* in different media.**

| Characteristic | No. of cultures in initial medium[a] | | | | | | No. of cultures in secondary medium | | Total No. |
|---|---|---|---|---|---|---|---|---|---|
| | CEC | 4E | M1 | R1 | W1 | W2 | 4E | 17N | |
| Normal seedling | 22 | 26 | 21 | 2 | 11 | 1 | 21 | 59 | 80 |
| Normal plant + callus | 6 | 4 | 6 | 1 | 2 | - | 13 | 4 | 17 |
| stem + callus, no root | 14 | 15 | 11 | 1 | 6 | 8 | 43 | 17 | 60 |
| Callus + root, no stem | 3 | 3 | 2 | - | - | - | - | - | 9 |
| Callus only | 11 | 12 | 2 | 5 | - | 1 | - | - | 31 |
| Stem only | 25 | 22 | 12 | 1 | 10 | 3 | 43 | 30 | 73 |
| Total nongerminated embryos | 19 | 28 | 46 | 90 | 71 | 87 | | | |

[a] 100 embryos were used in each culture medium.
CEC = 1/2 MS + sucrose (4%) + Inositol (100 mg/L) + thiamine (1 mg/L) + 1.23 mm IBA.
4E = MS + sucrose (2%) + Inositol + 0.04 BAP + 0.05 GA + 0.02 NAA.
M1 = MS + sucrose (3%) + Inositol (100 mg/L) + thiamine (1 mg/L) + Nicotimic acid (5 mg/L) + pyridoxine (0.5 mg/L) + Glutamine (50 mg/L) + GA (0.035).
R1 = 1/2 MS + sucrose (3%) + Inositol (100 ml/L) + thiamine (1 ml/L) + Thiourea (0.15%) + Glutamina (20 mg/L) + hydrolized caseina (100 mg/L).
W1 = 1/4 MS + sucrose (4%) + coconut milk (10%) + Glutamine (200 ml/L).
W2 = 1/2 MS + sucrose (6%) + coconut milk (20%) + Glutamine (100 ml/L).
17N= 1/3 MS + sucrose (2%) + ANA (0.01 mg/L) + GA (0.01 mg/L) + plant prod. (25.0 mg/L).

## VIABILITY TEST

Seeds of *M. carthaginensis*, *M. esculenta*, and *M. esculenta* ssp. *flabellifolia* in the above experiment that did not germinate after 21 days were selected and halved for viability tests. Embryos, cotyledons, and endosperm were soaked in an aqueous solution of 1% tetrazolim chloride for 24 hours at 30 °C. About 10-30% of the embryos were found viable. However, endosperm tissues in about 95% of the cases did not pick up the dye, indicating endosperm inviability and that embryo culture was needed to increase the rate of germination.

## EMBRYO CULTURE

Mature sexual seeds of 23 wild *Manihot* species were used as sources of embryos for *in vitro* culture. For embryo germination and plant development, MS (Murashige and Skoog 1962) medium was used supplemented with gibberellic acid (GA3), thiamine, sucrose, Indole-3-butyric acid (IBA) and napthalene acetic acid (NAA). Results suggest that embryos should be germinated at alternating temperatures (25 °C/8 hours and 35 °C/16 hours for 7 days prior to incubation at 32 °C during the day and 27 °C at night with a photoperiod of 12 hours and an illumination of 5000 lux). Compared with conventional seed germination techniques, a marked improvement in recovery was obtained with embryo culture for every accession and species tested.

## MICROPROPAGATION

Seedlings derived from embryo culture were micropropagated and stored *in vitro* in slow growth medium and subsequently grown in the greenhouse for morphological and biochemical characterization using IBPGR descriptors for cassava.

## *IN VITRO* COLLECTION AND EXCHANGE

Because of quarantine restrictions, plants can not be moved easily from country to country. If sexual seeds of wild *Manihot* species are not available, *in vitro* shoot-tip culture techniques can be applied to field collections. This technique has been developed at CIAT and used to collect 15 wild species from Brazil. Through collaborative efforts of CIAT and IBPGR over the last 8 years, a similar technique has been used to transfer over 2000 *M. esculenta* cultivars to CIAT.

## CURRENT STATUS OF WILD GERMPLASM

A total of 42 wild species of *Manihot* are maintained using *in vitro*, field, glasshouse, and seed techniques. Accessions stored under *in vitro* culture are available and can be sent upon request. A gradual conversion to *in vitro* culture for maintenance of all the wild germplasm is underway.

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## RESUMEN

Los parientes silvestres de la yuca (especies del género *Manihot*) reciben cada vez más atención debido a que son una fuente de los genes de resistencia a varios insectos y enfermedades y de tolerancia a factores abióticos adversos comunes. En los últimos 10 años se ha trabajado intensamente en la taxonomía y ecología de las especies silvestres de *Manihot*. Se han descrito cerca de 100 especies emparentadas con la yuca. La urgencia de establecer una colección y conservar el germoplasma silvestre es respaldada por el hecho de que en la actualidad se está dando en la naturaleza una gran erosión genética en las especies silvestres de *Manihot*. Se inició un proyecto de investigación en el Centro Internacional de Agricultura Tropical (CIAT) en 1987 con el fin de crear técnicas para la conservación y uso de esas especies. Se ha creado e implantado en CIAT un sistema estandarizado de abreviatura para 99 especies silvestres y 18 secciones.

# *In vitro* selection for cold and salt tolerance in rice

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The cellular nature of tolerance to cold and salt was demonstrated by the different reactions of cell lines derived from rice cultivars with different tolerance levels to these stresses. Calluses were induced from mature seeds of several genotypes; their growth and regeneration efficiency through somatic embryogenesis were quite variable. Somaclonal variation was used for the selection of tolerant cell lines. Genotypic differences in the temperatures necessary to completely prevent callus growth were noted. After 3 to 6 months at the sublethal stress temperature range (13-15 °C), some cell lines resumed their growth and gave rise to healthy calluses and plantlets. Similarly, calluses were maintained for 3 to 6 months on culture media containing sublethal stress concentrations (1.65-1.75%, depending on genotype) of sodium chloride (NaCl). After 6 months, some cell lines survived and regenerated plantlets after transfer to a medium without NaCl.

Rice is cultivated in very diverse ecological conditions, where the crop encounters various stresses that reduce yield. This is particularly true in Africa where rice expansion is relatively recent in many regions and the crop management is often rudimentary. The presence of sodium chloride (NaCl), and less frequently other salts, has reduced successful rice expansion in the large coastal areas and dry plains in West Africa. There is also a need for rice cultivars adapted to cold climates of the mountains in such countries as Madagascar, Rwanda, and Burundi. Genotypes introduced from high altitude areas of Asia were generally not very successful in these locations.

According to Swaminathan (1982), induction and selection of mutants at the cellular level are probably the most useful applications of the tissue and cell culture techniques. Tolerance to salt and aluminum toxicities is being studied at the International Rice Research Institute (IRRI); the salt problem is also being considered at

several other institutes. Cell selection for cold tolerance has not yet been attempted in rice breeding programs, however several successful experiments were reported for ornamental species (Preil *et al.* 1983).

The research on cell selection for cold and salt tolerance reported here was supported by a European Economic Commission (EEC) grant and directed toward breeding cultivars adapted to African conditions.

## MATERIAL AND METHODS

After preliminary trials with various genotypes, later experiments were conducted with cultivars from IRRI, Senegal, and Burundi. Calluses were primarily derived from mature embryos (Fig. 1), but immature embryos and fragments of plantlets were also used.

Several classical culture media were compared and the LS basic medium (Linsmaier and Skoog 1965) was selected. Different combinations of growth substances were tested for callus induction and plantlet regeneration. Callogenesis was induced in the dark. The addition of 3% sucrose, 0.5 mg/L of 2,4-Dichlorophenoxy acetic acid (2,4-D), 1 mg/L of Naphthalene acetic acid (NAA), 0.5 or 1 mg/L of 6-Benzlaminopurine (BAP), and 5% coco milk promoted callus induction. For regeneration, the same mineral solution was used after dilution (1/2), and was supplemented by 2% sucrose, 2,4-D (0.01 mg/L), NAA (1 mg/L) and BAP (1 mg/L). The cultures were left in the light. Rooting of the plantlets was more successful when they were transferred to the LS medium for 10 days with 1 mg/L of Indole-3-acetic acid (IAA) before planting in soil.

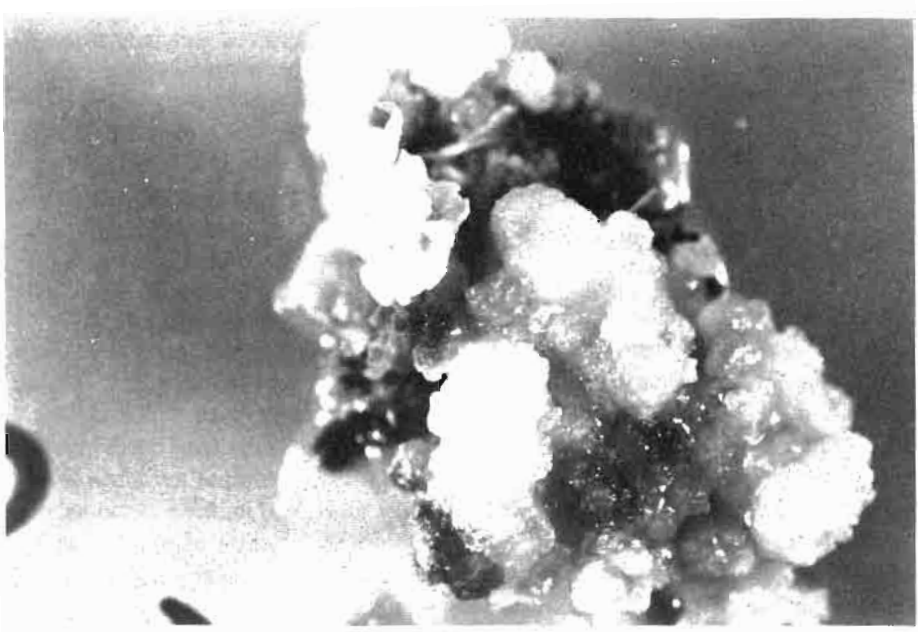

**Figure 1. Callus derived from mature embryo.**

Some experiments involving cell suspensions were also attempted. After plating on a solid medium, such cells proliferated to form microcalluses, but larger calluses and plantlets were not obtained.

## CELL CULTURE AND PLANT REGENERATION

Calluses were induced in the scutellum parenchyma and in the procambial tissues of the mesocotyl. In some genotypes, all the embryos gave rise to calluses and cell proliferation was very efficient. Callogenesis was less frequent and the callus growth was much slower in other cultivars. After transfer on the regeneration medium, genotypic differences were apparent in the proportion of embryogenic calluses as shown below.

- Srimalaysia 2: 20-50%
- I Kong Pao: 80-84%
- Nona Bokra: 45-60%
- Yunnan III: few plants
- Tatsumi Mochi: 36-50%
- KH 998: few plants

The highest proportion of embryogenic calluses correlated with a larger number of regenerated plantlets.

Differentiation started on the diluted medium, and sometimes earlier, on the callus-inducing medium. Somatic embryos appearing superficially on the callus tended to be abnormal. Their size was variable and their germination asynchronous. For some genotypes, 50 to 100 plantlets could be obtained from a single callus after 2 months (Fig. 2).

Samples of regenerated plants were cultivated in the greenhouse; they showed a broad variation for several agronomic characters, such as the size of the shoots and panicle, date of flowering, exsertion and form of the panicle or seed fertility. A high level of somaclonal variation was also noted among some progenies cultivated in the field in Africa.

## CELL SELECTION

Oono (1982) reported the occurrence of a large number of mutations induced in rice by culture of haploid and diploid tissues. That was confirmed later by several other authors. Such a broad somaclonal variation justified the application of selection pressures on tissue culture without mutagenic treatments, in order to isolate tolerant cell lines.

*In vitro* selection implies the determination of sublethal levels for the stresses and the application of the selection pressures during a sufficiently long period to eliminate, as much as possible, the majority of normal cells, and to keep tolerant ones alive. When the pressure is moderate, the physiological adaptation of the calluses could result in a general recovery of their growth. More drastic treatments involve the necrosis of most cultures, while rare cell lines start to proliferate after several months. Important cell cultures are, therefore, those that are able to regenerate plantlets after several months of *in vitro* culture under stress.

### Salt tolerance

'Nona Bokra' is well known as a salt-tolerant cultivar; it was compared with 'I Kong Pao'. Callus growth of both genotypes was progressively reduced on culture media containing 1.5 to 1.75 % NaCl, and, after 1 month, 'I Kong Pao' was more affected than 'Nona Bokra'. After 6 months in the presence of 1.65 or 1.75% NaCl, calluses of 'Nona Bokra' were still growing. On the other hand, only one cell line of 'I Kong Pao' continued to grow and subsequently generated plantlets after transfer to a normal medium. Three cell lines were selected from 'Nona Bokra' calluses cultured for 6 months in presence of a concentration of 1.75% NaCl (Fig. 3); plants were recovered.

Seeds were harvested on the plants derived from selected calluses of both genotypes. The number of plants recovered after selection over 6 months was not sufficient to test their tolerance. The characteristics of these lines will be studied during the next generations. Larger samples were obtained after 3 months of culture on a sublethal stress medium. Some of these plantlets were cultivated on a medium containing 1.5% NaCl and their survival was compared with that of plants derived from unselected calluses. The initial growth of the unselected population was affected more by salt than that of the selected population. All 150 plantlets derived from unselected calluses died after 3 weeks while, out of 180 plants derived from selected calluses, 20 survived.

Figure 2. Regeneration of plantlets on a callus.

### Cold tolerance

Two cold-tolerant cultivars ('Tatsumi Mochi' and 'Yunnan III') and two others ('Srimalaysia' and 'KH 998') were used in these experiments. Callus growth was almost completely stopped at 13 °C for the first two genotypes; the other two were more sensitive and their growth stopped at 15 °C. When calluses were maintained for a long time at these temperatures, most of them died, but some small cell clumps appeared among the necrotic tissues and proliferated normally (Fig. 4). In an experiment involving 121 calluses of 'Yunnan III', four survived after 6 months at 13 °C and generated plantlets. Five of 70 calluses of 'Tatsumi Mochi' also regenerated, while only one could be recovered after a treatment of 6 months at 11 °C. Regenerated plants were cultivated in the greenhouse and their progenies will be tested for cold tolerance.

## DISCUSSION AND CONCLUSIONS

Plants derived from rice calluses at sublethal stress temperatures selected from cell lines appeared to grow normally. The progeny of the plants derived from these cells will be grown at low temperature and their growth and fertility will be compared with that of the original cultivars. In some other species, cell selections for cold tolerance gave only a very low percentage of modified plants or were unsuccessful. The frequent ineffectiveness of the method has been explained by the chimeric structure of the plants and by the elimination of tolerant tissues in the absence of a selection pressure, or by a physiological and transitory adaptation of the cells. These problems could be avoided by a long-term selection of the calluses at the lowest tolerated temperature.

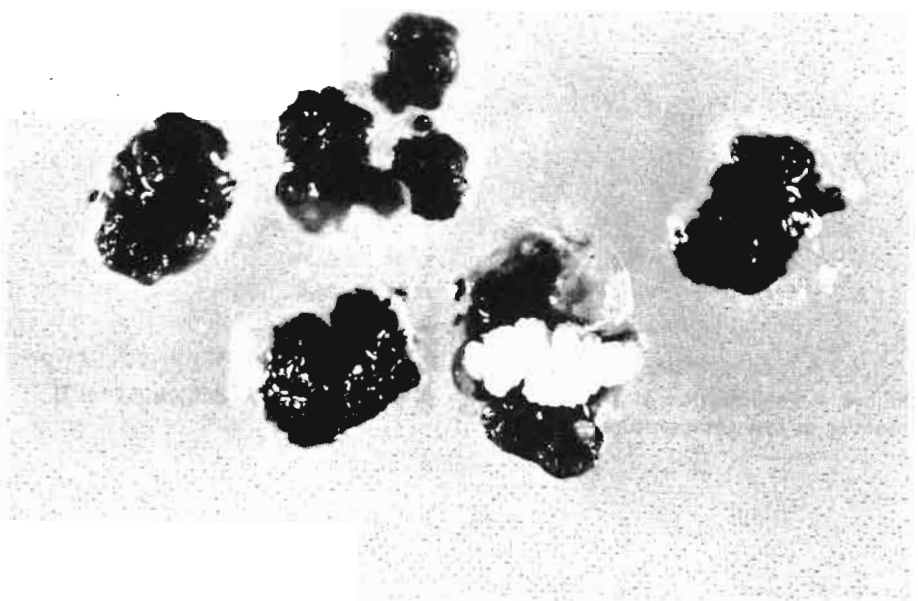

**Figure 3. Proliferation of a tolerant cell line on calluses cultivated for 6 months on a sublethal concentration of NaCl.**

Cell selection for salt tolerance and identification of mutated plants in the progenies are easier, and a rather high number of experiments has been reported on several crops, including rice. Salt-tolerant cell lines of rice showed better growth after plating on a medium containing 1.5% NaCl than on a normal medium (Croughan *et al.* 1981). In some experiments, mutagenic treatments were applied on the seeds before callus induction. In such a case, Woo *et al.* (1985) reported the presence of a number of highly tolerant plantlets in the progeny of plants regenerated from calluses selected by NaCl concentrations of between 0.6 and 2.2%.

Up to now, the results of cell selection for cold and salt tolerance are still fragmentary, at least for rice and other crops. Nevertheless, it seems that the method will lead to applications if applied on a large scale, after a thorough determination of the sublethal level of the stresses. Mutagenic treatments could raise the number of modified genes and a long-term culture in selective conditions will increase the proportion of tolerant cells. Finally, the observation of the seed progeny is necessary to confirm the stability and to elucidate the genetic basis of the selected characters.

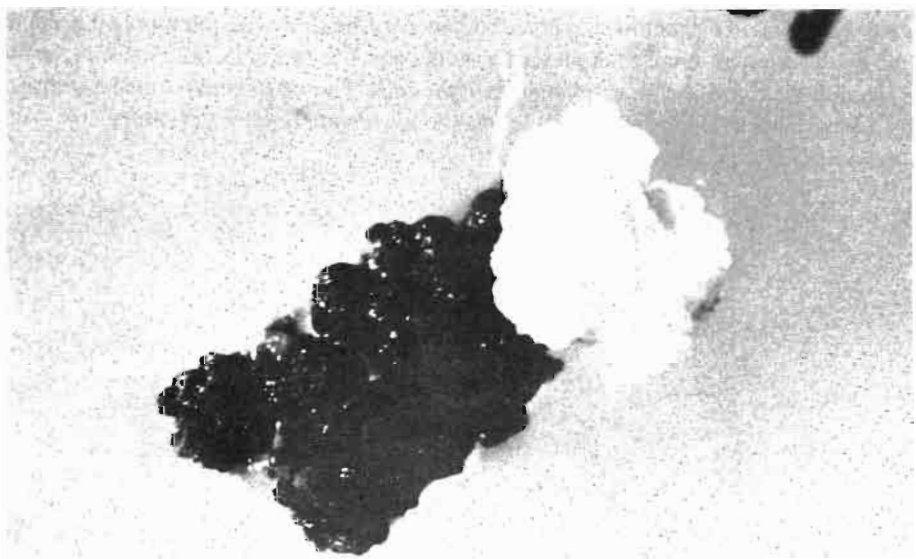

**Figure 4. Proliferation of a cold tolerant cell line on a callus cultivated for 6 months at low temperature.**

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## RESUMEN

Se demostró la naturaleza celular de la tolerancia al frío y a la sal a través de las diferentes reacciones que presentaron a estos factores abióticos líneas celulares derivadas de variedades de arroz con distintos niveles de tolerancia. Se indujo la formación de callos a partir de semillas naturales de diversos genotipos; se observó una gran variación en cuanto a su crecimiento y a la eficacia de la regeneración mediante la embriogénesis somática. Se empleó la variación somaclonal para efectuar la selección de las líneas celulares tolerantes y se observaron las diferencias genotípicas en cuanto a la temperatura necesaria para impedir por completo el crecimiento de los callos. Después de tres a seis meses de mantenerlas a una temperatura subletal (13-15°C), algunas líneas celulares reiniciaron el crecimiento y generaron callos y plántulas saludables. De manera similar, los callos se mantuvieron de tres a seis meses en medios de cultivo que contenían concentraciones subletales de NaCl (1.65-1.75% dependiendo del genotipo). Después de seis meses, algunas líneas celulares habían sobrevivido y generaron plántulas una vez que fueron transferidas a un medio que no contenía NaCl. Algunas de las plantas regeneradas que se obtuvieron después de un período de selección de tres meses se sometieron a pruebas de cultivo en un medio que contenía 1.5% de NaCl.

# *Oryza minuta*, a source of blast and bacterial blight resistance for rice improvement

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Diseases and insects are the major threats to yield stability in rice and, hence, the incorporation of resistance is a major focus of many rice breeding programs. As the genetic variability for disease and insect resistance within cultivated rice is used up and overcome by new pathotypes and insect biotypes, the wild species will increase in importance as a germplasm source. *O. minuta*, a tetraploid species native to Asia, with a genomic composition of BBCC, is a potential source of resistance to two important rice diseases—bacterial blight and rice blast. This paper describes part of a crossing and disease evaluation program aimed at transferring bacterial blight and blast resistance from *O. minuta* into cultivated rice.

The wild *Oryza* species represent a rich, largely untapped source of resistance to biotic, as well as abiotic stresses. As the genetic variability for disease and insect resistance within cultivated rice (*O. sativa*; 2X; AA genome) is used up and overcome by new pathotypes and insect biotypes, the wild species will increase in importance as a germplasm source. *O. minuta*, a tetraploid species native to Asia, with a genomic composition of BBCC, is a potential source of resistance to two important rice diseases; bacterial blight caused by *Xanthomonas campestris* pv. *oryzae* and rice blast caused by *Pyricularia oryzae*.

## MATERIALS AND METHODS

### Hybrid production

Two accessions of *O. minuta* (IRGC accession numbers 101089, 101141), showing high levels of resistance against representative isolates of the six Philippine races of *Xanthomonas campestris* pv. *oryzae* (Xco) and the blast isolate PO-6-6, were hybrid-

ized to a susceptible *O. sativa* line, IR31917-45-3-2. All crosses were made using *O. sativa* as the female parent. Gibberellic acid (75 ppm) was applied to the pollinated panicles. To ensure hybrid survival, 14-day old embryos were extracted under sterile conditions, transferred to 1/4 strength MS (Murashige and Skoog 1962) medium, and incubated in the dark. Upon germination, the hybrid plants were transferred to the light and then to a nutrient solution.

**Disease evaluation**

Seeds of parental materials were sown in filter paper-lined petri dishes and later transplanted to soil in 4.5-cm diameter plastic cups. For the blast reaction test, five seedlings were planted to a cup while one seedling per cup was planted for the bacterial blight test. Ammonium sulfate at 0.3 g/cup was applied, 2/3 of the amount at transplanting and the rest 1 week before inoculation. Nodal sections from mature tillers of $F_1$ plants were incubated at 30 °C, roots and auxiliary shoots were allowed to grow, after which they were transplanted to soil. Fertilization was the same as for parent materials.

*Bacterial blight inoculation.* Plants were inoculated at maximum tillering stage (50-60 days after sowing in parent materials). Ten leaves from at least two seedlings or clones were tested against six Philippine Xco isolates. The bacterial cell suspension (1x109/ml) was applied using the clipping method. Percent lesion area was scored 14 days after inoculation.

*Blast inoculation.* Twenty seedlings of IR31917-45-3-2 and the *O. minuta* accessions were inoculated 21 and 36 days after sowing, respectively. Five clones of each $F_1$ hybrid were inoculated at the 3-4 leaf stage (approximately 1-2 months after establishment in soil). Plants were sprayed with a spore suspension of isolate PO-6-6 (1x105/ml), incubated in a dew chamber at 24 °C for 24 hours, transferred to an air-conditioned mist room (24-28 °C) with natural lighting for 5 days, after which the percent lesion area and reaction type were rated.

## RESULTS

**Hybrid production**

Crosses between IR31917-45-3-2 and accessions 101089 and 101141 gave seed sets of 3.0 and 4.2%, respectively. Embryo germination ranged from 48.3% for accession 101141 to 60.0% for accession 101089. The $F_1$ hybrids (3X; ABC) were intermediate between the parents in plant type, although the panicles were very similar to those of *O. minuta* (Fig. 1). An examination of meiosis in these hybrids revealed the presence of a low frequency of rod bivalents, with the occasional trivalent, as shown in Figure 2. A low frequency of chromosome pairing was also observed by other workers (for example, Li *et al.*, 1962) and indicates that gene transfer from the *O. minuta* genome into that of *O. sativa* may be possible by recombination.

**Disease reactions**

*Bacterial blight.* Table 1 shows the percent lesion area and reaction type of representative isolates of six Philippine Xco races on IR31917-45-3-2, *O. minuta* (101089, 101141), and their $F_1$ hybrids. The two *O. minuta* accessions showed high levels of

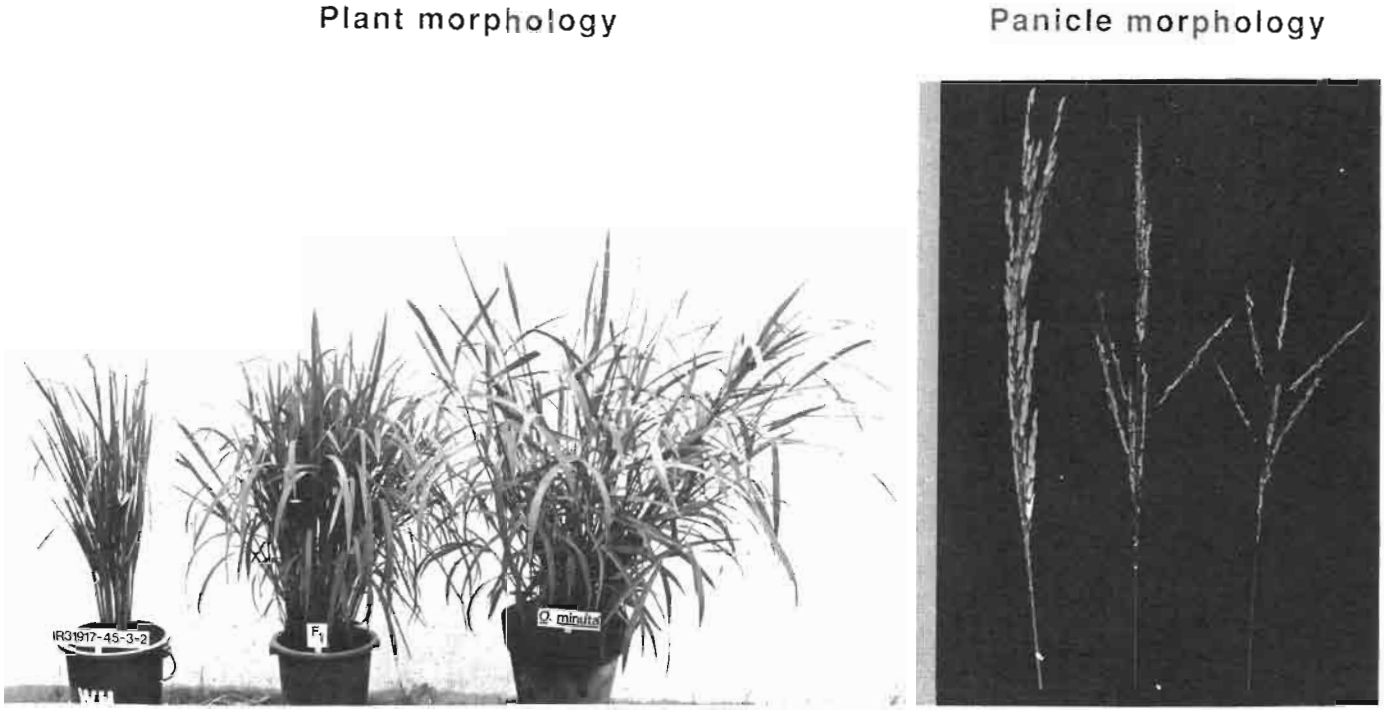


**Figure 1. Plant and panicle morphology (from left to right in each photo) of *O. sativa* (IR31917-45-3-2), the $F_1$ hybrid, and *O. minuta*.**

resistance to all representative isolates of Philippine Xco races while the *O. sativa* parent, IR31917-45-3-2, showed resistance to Xco race 5 and moderate susceptibility to all other races. Among the two *O. minuta* accessions, 101141 can still be regarded as the more resistant. In this accession, the bacteria could gain almost no entry into the leaves and lesions were already necrotic 14 days after inoculation. In contrast, lesion length was as long as 2.0 cm in *O. minuta* accession 101089 and some lesions were still water soaked up to day 14.

The difference in resistance to Xco between the two accessions is further reflected among the reactions of their $F_1$ progenies. The IR31917-45-3-2/Acc. 101141 $F_1$ hybrids were highly resistant to all Xco races, similar to the *O. minuta* parent, whereas the IR31917-45-3-2/Acc. 101089 $F_1$ hybrids generally showed moderate susceptibility to Xco races.

*Blast.* Table 2 shows the reactions of IR31917-45-3-2, *O. minuta* (101141, 101089) and their $F_1$ hybrids to the blast isolate PO-6-6. The *O. minuta* accessions 101089 and 101141 were highly resistant to the blast isolate PO-6-6. No susceptible lesions developed on the leaves of the two *O. minuta* accessions; instead, a hypersensitive reaction (brown specks) was observed. The same hypersensitive reaction was exhibited by the two accessions when exposed to natural inoculum at the IRRI Blast Nursery. The high levels of resistance observed in *O. minuta* would be typically conferred by one to a few major genes.

The $F_1$ hybrids of the two accessions with susceptible *O. sativa*, IR31917-45-3-2, both showed moderate resistance to PO-6-6. A few susceptible lesions averaging 0.89 and 0.64 per tiller were observed in the $F_1$ hybrids of 101141 and 101089, respectively.

**Table 1. Reactions of IR31917-45-3-2, *Oryza minuta* (101089, 101141), and their $F_1$ hybrids to representative isolates of six Philippine Xco races.**

| Plant | Percent lesion area and reaction type | | | | | |
|---|---|---|---|---|---|---|
| | Race 1 | Race 2 | Race 3 | Race 4 | Race 5 | Race 6 |
| | 61 | 86 | 79 | 71 | 111 | 99 |
| ***O. sativa*** | | | | | | |
| IR31917-45-3-2 | 11MS | 11MS | 19MS | 27MS | 6R | 22MS |
| ***O. minuta*** | | | | | | |
| 101141 | 1R | 1R | 1R | 1R | 1R | 1R |
| 101089 | 2R | 1R | 1R | 1R | 1R | 1R |
| ***O. sativa/O. minuta*** | | | | | | |
| IR31917-45-3-2/101141 | 1R | 1R | 1R | 1R | 1R | 1R |
| IR31917-45-3-2/101089* | a 10MS | 18MS | 5MR | 13MS | 12MS | 49S |
| | b 11MS | 25MS | 15MS | 36S | 21MS | 48S |

* Two $F_1$ hybrids tested with different reactions.

R = resistant, MR = moderately resistant, MS = moderately susceptible, S= susceptible.

IR31917-45-3-2, on the other hand, had an average of 15.5 susceptible lesions/tiller. The types of lesions typically observed on the parents and their hybrids are shown in Figure 3.

## DISCUSSION

All hybrids with accession 101141 were resistant to all Xco isolates, whereas those with accession 101089 were moderately susceptible. These results suggest that 101141 may have nonallelic genes conferring resistance to specific Xco races or one gene conferring nonspecific resistance to Xco.

All hybrids showed intermediate resistance to blast. While this $F_1$ reaction may be due to incomplete dominance of resistance gene(s) in *O. minuta*, it may also be possible that the resistance in *O. minuta* is controlled by a number of quantitative genes. In the latter case, transfer of blast resistance from *O. minuta* will be a difficult process because of the number of genes that have to be simultaneously transferred.

The presence of bivalent and trivalent chromosome associations at meiosis indicates that the transfer of disease resistance genes from *O. minuta* into cultivated rice may be possible through recombination. A backcrossing program is underway to

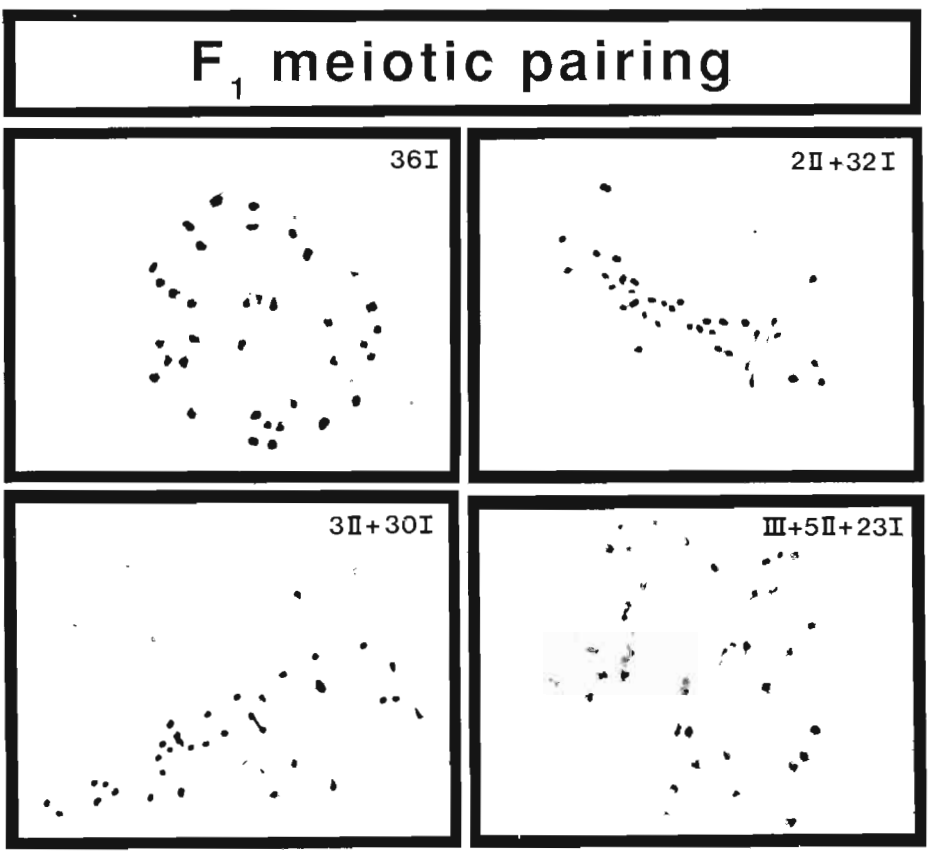


**Figure 2. Meiotic chromosome associations in *O. sativa/O. minuta* $F_1$ hybrids.**

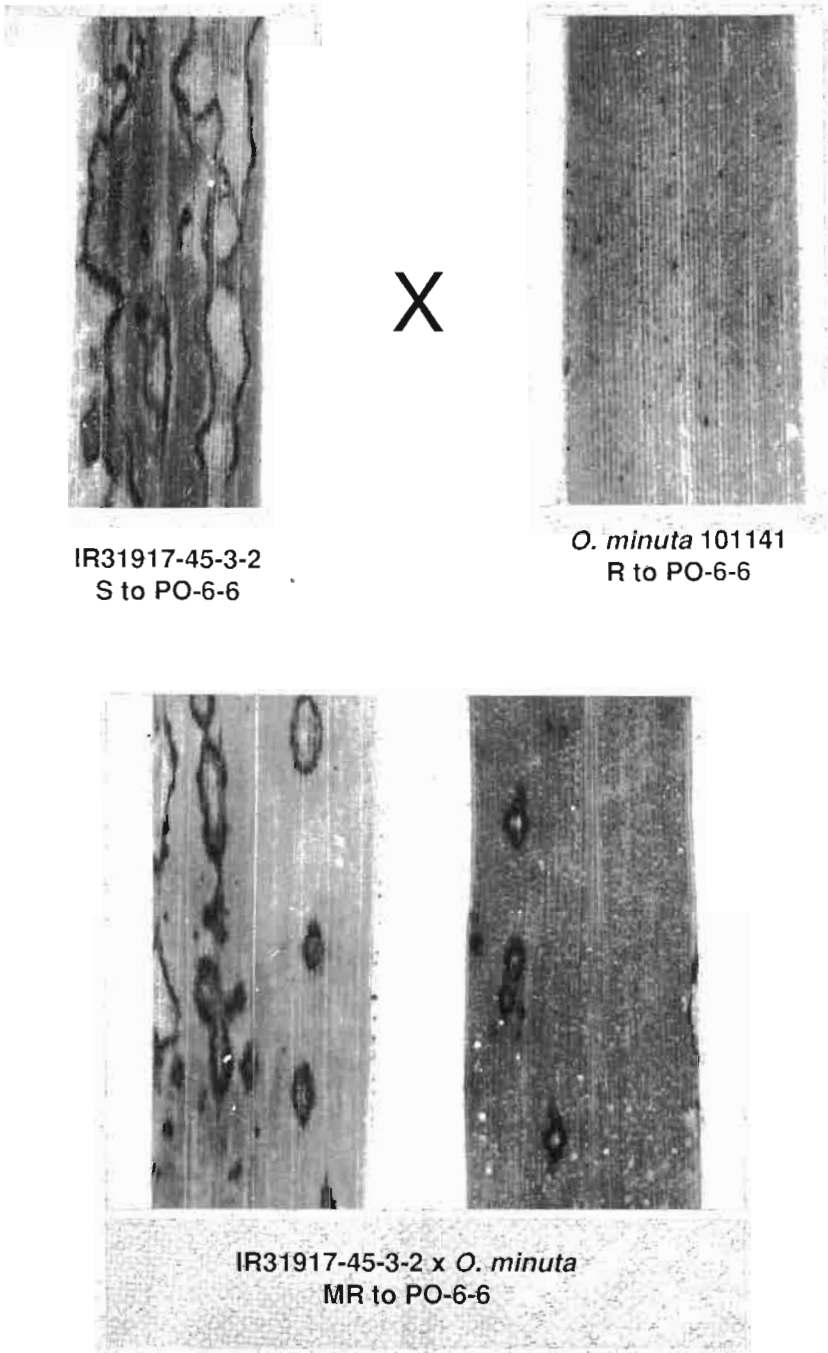


**Figure 3. Typical blast lesions on *O. sativa* (IR31917-45-3-2), *O. minuta* (Acc. 101141), and the $F_1$ hybrids.**

produce addition lines and ultimately to transfer the gene(s) concerned. If gene transfer by recombination proves ineffective, we hope to stimulate translocations by other methods, such as callus induction and irradiation.

**Table 2. Reactions of *Oryza sativa* IR31917-45-3-2, *O. minuta* (101141, 101089), and their $F_1$ hybrids to the blast isolate PO-6-6.**

| Plant | Disease score | No. of lesions /tiller Type 3 (1-2 mm) | Type 5 (3-7 mm) |
|---|---|---|---|
| ***O. sativa*** | | | |
| IR31917-45-3-2 | S | 13.0 | 2.5 |
| ***O. minuta*** | | | |
| 101141 | R | 0 | 0 |
| 101089 | R | 0 | 0 |
| ***O. sativa/O. minuta*** | | | |
| IR31917-45-3-2/101141 | MR | 0.88 | 0.01 |
| IR31917-45-3-2/101089 | MR | 0.64 | 0 |

R = resistant, few to many brown specks; MR = moderately resistant, brown margined, irregularly shaped lesions, 1-2 mm long; S = susceptible, several spindle-shaped lesions, 3-7 mm long, with grayish center.

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**RESUMEN**

Las enfermedades e insectos constituyen las principales amenazas a la estabilidad del rendimiento en el arroz y, por tanto, la incorporación de resistencia es uno de los objetivos primordiales de muchos programas de mejoramiento de arroz. Las especies silvestres de *Oryza* representan una fuente muy rica y prácticamente virgen de resistencia al estrés biótico y abiótico. A medida que los nuevos tipos patológicos y biotipos de insectos agoten y superen la variabilidad genética de la resistencia a enfermedades e insectos del arroz cultivado (*O. sativa*; 2X; genomio AA), aumentará la importancia de las especies silvestres como fuentes de germoplasma. *Oryza minuta*, una especie tetraploide originaria de Asia, con una composición genómica de BBCC, representa una posible fuente de resistencia a dos enfermedades importantes del arroz, el tizón bacteriano causado por *Xanthomas campestris* pv. *oryzae* y el añublo del arroz producido por *Pyricularia oryzae*. En este artículo se describe una parte de un programa de cruzamiento y evaluación de enfermedades cuyo objetivo consiste en transferir la resistencia al tizón bacteriano y al añublo de *O. minuta* al arroz cultivado (*O. sativa*).

# Haploid breeding and its cytogenetics in cotton (*Gossypium hirsutum*)

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Cotton (*Gossypium hirsutum*) hybrid lines were purified by using parthenogenesis, thereby reducing the number of selections needed and shortening the breeding period by half compared to conventional selection. Study of the chromosome behavior at the cell division stage of the haploids in this experiment suggested that *G. hirsutum* is a polyploid of homologous nature or a monophyletic polyploid, not a heterologous polyploid.

This study purified cotton hybrids by using a single step of parthenogenesis, thereby reducing the number of selections needed and shortening the breeding period. By studying cytogenetically parthenogenetic lines of cotton, we discovered that these lines were haploidy mixoploids, but at the same time they had normal reproductive capacity. To a certain extent these lines showed consistency in chromosome number and traits between generations. This implied that it would be possible to develop new types of cotton plants using parthenogenesis.

Over the years, there has been considerable argument about the true origin of the genus *Gossypium*. If haploids can be used as the experimental material to observe chromosome behavior at the cell division stage, perhaps some of the controversy can be alleviated. At the same time, this type of investigation can contribute to the development of the sciences of cytology and genetics.

## INDUCED PARTHENOGENESIS

The F1s from 15 cotton hybrid combinations with various useful traits were utilized in the study. We treated 1673 flower buds (50,190 ovules) either chemically, physically, or biologically to induce the ovules to produce seed through artificial parthenogenesis on the maternal plant. A large quantity of parthenogenetic bolls (pods) were produced. Among the ovules within these bolls, 105 fully developed and viable parthenogenetic seeds were produced.

High temperature was the most effective physical treatment, as well as the most effective treatment overall, providing a 2.66% frequency of seed formation. The most effective biological method was pollinating with *Hibiscus cannabinus*, which produced a 2.33% seed formation frequency. The most effective chemical treatment used was an application of 0.2% dimethyl sulfoxide plus 0.2% colchicine, which produced a 1.22% frequency of seed formation in the parthenogenetic boll.

A common characteristic of the parthenogenetic bolls was their unfilled appearance. In some, the style and stigma grew in size as the boll developed and did not shed from the ovary until August or September. The parthenogenetic seeds were more spherical than those from untreated bolls. Some of these seeds were so weak that they had to be dehulled before they could germinate. However, after one generation, dehulling was no longer necessary. In all, 82 parthenogenetic lines were produced.

## COMPARISON OF PARTHENOGENETIC AND INBRED LINES

We compared the purification efficiency between the progeny of parthenogenetic lines and inbred lines and found that 40.9% of the parthenogenetic lines (Pa2) reached standard stock varietal purity with stability through a single step of parthenogenesis. Although the vigor in the Pa1s and Pa2s of the parthenogenetic lines was less than in the $F_2$s and $F_3$s of the inbred lines, this became less noticeable in the third and fourth generations. Most importantly, the parthenogenetic method reduced by half the time required to purify the lines compared with conventional selection.

## CYTOLOGY OF PARTHENOGENETIC LINES

Five of the parthenogenetic lines were determined to be mixoploid. There was an obvious difference in chromosome number in the root tip cells, ranging from 12 to 52. Four of the lines were monads and the other was an aneuploid. The average chromosome number of root tip cells between the Pa1 and Pa2 appeared to be stable.

The number of chromosomes in the pollen mother cells was irregular. The frequency of pairing chromosomes at methaphase I was 90.38%, in which AA pairing was 23.15%, DD pairing 18.69%, and AD pairing 18.69%. Under a high resolution microscope, we could see that chromosome A had a loose structure, while chromosome D had a compact structure. The remaining chromosomes were trivalents plus multivalents (29.85%) and univalents (9.62%). The chromosomes in the pollen mother cells separated unequally at anaphase I.

This chromosome behavior at metaphase I and anaphase I suggests that *G. hirsutum* is a polyploid of homologous nature or a monophyletic polyploid, not a heterologous polyploid.

## RESUMEN

Se purificaron líneas híbridas de algodón (*Gossypium hirsutum*) mediante la partenogénesis, con lo cual se redujo el número de selecciones necesarias y disminuyó a la mitad el período de mejoramiento en comparación con el de la selección convencional. El estudio del comportamiento de los cromosomas en la fase de división celular de los haploides en este ensayo indicó que *G. hirsutum* es un poliploide homólogo o monofilético, pero no heterólogo.

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Second International Symposium on
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August 29-31, 1988

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Participants of the 2nd International Symposium on Genetic Manipulation in plants take a break from paper presentations to visit nearby wheat plots at CIMMYT's El Batán, Mexico station. (photos: G. Hettel)

ISBN 968–6127–34–8