EUROPEAN COOPERATIVE PROGRAMME FOR CROP GENETIC RESOURCES NETWORKS (ECP/GR)

Challenges in rye germplasm conservation

Proceedings of an International Conference on Crop Germplasm Conservation with Special Emphasis on Rye, and an ECP/GR Workshop
2-6 July 1996
Warsaw/Konstancin-Jeziorna, Poland

ECP/GR

T. Gass, W. Podyma, J. Puchalski
and **S.A. Eberhart,** *compilers*

HORTUS BOTANICUS ACADEMIAE SCIENTIARUM POLONAE · VARSOVIA ·

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International Plant Genetic Resources Institute
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IPGRI is an institute of the Consultative Group on International Agricultural Research (CGIAR)

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The International Plant Genetic Resources Institute (IPGRI) is an autonomous international scientific organization, supported by the Consultative Group on International Agricultural Research (CGIAR). IPGRI's mandate is to advance the conservation and use of plant genetic resources for the benefit of present and future generations. IPGRI's headquarters is based in Rome, Italy, with offices in another 14 countries worldwide. It operates through three programmes: (1) the Plant Genetic Resources Programme, (2) the CGIAR Genetic Resources Support Programme, and (3) the International Network for the Improvement of Banana and Plantain (INIBAP). The international status of IPGRI is conferred under an Establishment Agreement which, by January 1998, had been signed and ratified by the Governments of Algeria, Australia, Belgium, Benin, Bolivia, Brazil, Burkina Faso, Cameroon, Chile, China, Congo, Costa Rica, Côte d'Ivoire, Cyprus, Czech Republic, Denmark, Ecuador, Egypt, Greece, Guinea, Hungary, India, Indonesia, Iran, Israel, Italy, Jordan, Kenya, Malaysia, Mauritania, Morocco, Pakistan, Panama, Peru, Poland, Portugal, Romania, Russia, Senegal, Slovak Republic, Sudan, Switzerland, Syria, Tunisia, Turkey, Uganda and Ukraine.

The European Cooperative Programme for Crop Genetic Resources Networks (ECP/GR) is a collaborative programme among most European countries aimed at ensuring the long-term conservation and facilitating the increased utilization of plant genetic resources in Europe. The Programme, which is entirely financed by the participating countries and is coordinated by IPGRI, is overseen by a Steering Committee (previously Technical Consultative Committee, TCC) composed of National Coordinators nominated by the participating countries and a number of relevant international bodies. The Programme operates through ten broadly focused networks in which activities are carried out through a number of permanent working groups or through *ad hoc* actions. The ECP/GR networks deal with either groups of crops (cereals, forages, vegetables, grain legumes, fruit, minor crops, industrial crops and potato) or general themes related to plant genetic resources (documentation and information, *in situ* and on-farm conservation, technical cooperation). Members of the working groups and other scientists from participating countries carry out an agreed workplan with their own resources as inputs in kind to the Programme.

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International Conference: Crop Germplasm Conservation with Special Emphasis on Rye

Session I: Plant genetic resources conservation and rye breeding in Poland (Chair: Prof. Zbigniew Gertych)

Introduction

Dr Jerzy Puchalski, the chairman of the Organizing Committee of the conference welcomed cordially all participants and guests. He stressed his acknowledgements to the United States Department of Agriculture, Agricultural Research Service, for the financial support of the conference. The expenses were covered partly from two PL.-480 USDA projects (PL.-ARS-140A&B) conducted by the Botanical Garden. Dr Puchalski devoted special thanks to Dr Steve A. Eberhart, director of the USDA-ARS National Seed Storage Laboratory in Fort Collins, Colorado, who was the cooperating scientist of both projects and co-chairman of the Organizing Committee. Special acknowledgements were addressed to Dr Thomas Gass, Coordinator of the European Cooperative Programme for Crop Genetic Resources Networks, who suggested and supported financially the joint meeting of American and European scientists working on rye germplasm. The cooperation of the Botanical Garden of the Polish Academy of Sciences with the USDA-ARS research institutions started in 1979 and six research projects were carried out on rye germplasm. The Plant Breeding and Acclimatization Institute, coordinator of plant genetic resources activities in Poland, is an important partner of ECP/GR and contributes to different international projects initiated in the framework of the programme. Results obtained in the above-mentioned projects will be presented during the conference.

Welcome addresses were received from the honorary guests:

- Stanley Phillips - Agricultural Attaché of the United States Embassy in Poland
- Benicjusz Kramski - Deputy Director of the Department of Science, Education and Extension, Ministry of Agriculture and Food Economy in Poland
- Tadeusz Chojnacki - Secretary of the Division of Biological Sciences, Polish Academy of Sciences.

Rye breeding in Poland – past and present achievements

Mr Andrzej Szołkowski, leading breeder of the DANKO Company, introduced the history and present achievements of rye breeding in Poland. Rye has been the main cereal crop for centuries in Poland and still occupies the greatest area among cereals and occupies about 28% of the total grain acreage. However, in recent times rye has been replaced by winter wheat in total grain production. A gradual decrease of rye growing to 2.4 millions hectares is being observed. Future reduction of the rye growing area is expected in favour of winter wheat and Triticale on better soils, and because of the abandonment of agricultural production on the poorest sandy soils, which were traditionally used for rye cultivation. The major part of rye grain production (60-65%) is used for animal

feeding and only 30-35% for bread-making. 'Dańkowskie Złote' is the most commonly grown variety in Poland. The share of other more recent varieties, namely 'Dańkowskie Nowe', 'Moytto', 'Amilo' and 'Warko' is gradually increasing. These five leading varieties of the DANKO Company occupied about 95% of the area of rye certified seed production. The Polish rye population varieties are ranked among the best in the world. These varieties contribute greatly to the worldwide rye production as well as to the progress of rye breeding. Prof. Tadeusz Wolski is the creator of the success of Polish varieties. 'Dańkowskie Złote' is the most widely grown variety in the world. 'Dańkowskie Nowe' (Danko) is registered in all European rye-growing countries and in USA, Canada and Korea. 'Motto' is on the national lists of Germany, Austria, Denmark, Sweden, The Netherlands and New Zealand. The Smolice Plant Breeding Station of the Plant Breeding and Acclimatization Institute is also engaged in population variety breeding. Two new varieties – 'Wibro' and 'Zduno' – have been registered.

Strong hybrid breeding programmes are conducted by the Plant Breeding and Acclimatization Institute and the DANKO Company together with Poznańska Hodowla. The first Polish hybrids have been included in official trials.

Conservation programme of genetic resources for food and agriculture in Poland

Wiesław Podyma
Gene Bank Laboratory, Plant Breeding and Acclimatization Institute, Radzików, Poland

Summary

The increased appreciation of the importance of genetic resources for agricultural production in Poland has led to the establishment of the National Crop Plant Genetic Resources Conservation Programme. One of the aims of the programme is the conservation of the indigenous variability of agricultural plants. During past expeditions, regions rich in local races of agricultural and horticultural crops (north-eastern and southern Poland) were visited. More than 1500 seed samples have been collected during the missions performed throughout Poland. Seed samples were obtained from farmers or in local markets. Over the past 20 years, replacement of old cultivated forms with new breeding materials has been observed in all regions visited. This trend is most rapid in field crops, in which nearly all landraces and weeds associated with old agricultural communities have become extinct. The replacement of vegetables and other crops grown in home gardens has slowed down. However, in the last few years, this process has accelerated. Grassland ecotypes have systematically been collected in all regions of Poland. Presently, about 75% of the country's total area has been sampled.

Introduction

Collecting and conservation of plant genetic resources were initiated in Poland by Prof. Kaznowski in the Research Institute of Agronomy (PINGW) at Puławy in 1922, and at the Agricultural Academy at Dublany. Since the establishment of the Plant Breeding and Acclimatization Institute in 1951, particular attention has been given to collecting local Polish cultivars and ecotypes. The National Crop Plant Genetic Resources Conservation Programme, established in 1979, continued earlier research activities in this area. The main goal is to conserve genetic material of major crop plants and their wild and weedy relatives for breeding and research. The objectives of the programme are realized through:

- collecting of genotypes endangered with extinction
- evaluation of collected materials
- conservation of collected materials in viable form and provision for breeders
- documentation of collected materials.

The National Crop Plant Genetic Resources Conservation Programme has long experience in *ex situ* conservation of crops plants, possesses genebank facilities, and has developed standards of storage, documentation and evaluation (Bulińska-Radomska and Górski 1991). The National Crop Genetic Resources Conservation Programme in Poland is based on multi-institutional input (Bulińska-Radomska *et al.* 1990). Three universities, nine branch institutes, seven experimental stations, and the Botanical Garden of the Polish Academy of Science carry the responsibilities for crop collections. The programme is financed by the Ministry of Agriculture. About 60 000 accessions have been collected. They represent all

economically important plant groups: cereals, fodder plants, root crops, vegetables, fruit crops, herbage and industrial plants. Seed samples collected under the auspices of the National Crop Plant Genetic Resources Conservation Programme are stored, since 1981, in central long-term storage located at the Plant Breeding and Acclimatization Institute (Fig. 1). In total, 44 883 accessions of plants are currently in long-term storage. The seeds are kept in temperature-controlled chambers at -18°C and 0°C. The collections of hop, garlic, asparagus and fruit plants are maintained in the form of plantations. At the Institute of Potato Breeding in Bonin the potato strains are stored *in vitro*. The samples in the collections are recognized as a part of the national heritage. The structure of collections is breeder-oriented; they prefer to work with advanced materials and breeding lines. However, collecting missions provided important amounts of unique material.

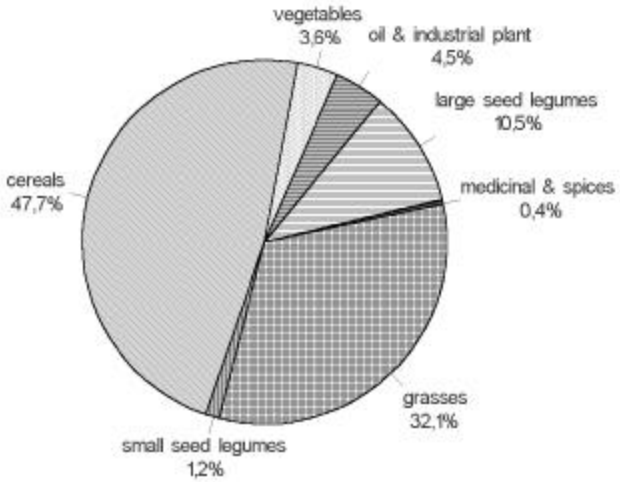

Fig. 1. Conservation of seed samples in long-term storage.

Collecting missions

Systematic collecting and conservation of indigenous plant genetic resources in Poland started in 1971. The expeditions are carried out almost every year. The collecting missions have several aims:

- collecting old cultivars, local landraces of agricultural and horticultural crops and their weedy and wild counterparts
- collecting ecotypes of grasses
- collecting plant material for research - special purpose collection
- monitoring the progress of genetic erosion.

The priorities of the missions have changed over time. During the period 1976-79, expeditions mainly focused on collecting old cultivars and landraces of field crops in the main regions of their occurrence. New tasks, made necessary by evidence of erosion of genetic resources in other cultivated groups of plant, have been added to the collecting activities. Systematic collecting of vegetables began in the early 1990s, while the recording of old gardens, fruit trees and collecting of medicinal and ornamental plants found in home gardens are tasks recently assigned to the expeditions.

The expeditions are organized jointly by the Gene Bank Laboratory of the Plant Breeding and Acclimatization Institute (agricultural crops and other species), the Botanical Garden of the Plant Breeding and Acclimatization Institute (grasses) and the Department of Germplasm Collection of the Institute of Vegetable Crops (vegetable).

Collecting landraces of field crops

Poland is a unique example in Central Europe of a country where the old local forms of crop plants survived owing to the 'crumbled' structure of farming. Since 1976, 14 trips have been made to northeastern, eastern and southeastern parts of Poland to collect cultivated, wild and weedy germplasm. These regions have been traditionally regarded as the least advanced agriculturally and, therefore, most likely to provide old varieties and landraces of crop plants. Most of the indigenous germplasm was collected in the mountainous regions of southeast Poland. During the aforementioned 14 missions, 1512 samples were collected of which 346 were landraces of cereals.

The main occurrence areas of landraces and old varieties were defined during missions conducted between 1976 and 1979 (Hammer and Hanelt 1979; Hanelt and Hammer 1977; Kulpa and Jastrzębski 1986; Kulpa and Górski 1986). They are situated in the southern part of the country and include the mountain regions of Beskidy, the Tatra and their foothills. Minor refugial regions have been discovered in eastern and southeastern Poland in Polesie, Wyżyna Lubelska and in the basin of Sandomierz. Because of climatic, ecogeographic and edaphic conditions as well as fairly primitive agricultural practices, those areas for many years served as refugia for primitive forms of cultivated plants. It should be emphasized that local races competed successfully with new varieties in these regions. Well adapted to the specific environmental conditions, they guaranteed not high, but stable yields, even in unfavourable years. The expeditions also resulted in documented examples of active breeding activities of farmers, e.g. on *Vicia dasycarpa*, which was selected for fodder purposes from weedy populations of the species (Kulpa and Hanelt 1981). The regions mentioned were characterized by the cultivation of some relic crops, e.g. *Camelina sativa*, *Raphanus sativus* var. *oleiformis*, *Panicum miliaceum* (Kulpa and Hanelt 1981), and were refugia for typical weeds related to cultivation such as *Agrostemma githago* and *Bromus secalinus* or archaeophytes like *Avena strigosa*.

During collecting missions organized in the period 1985-90 a systematic decrease in the number of samples of field crops was observed. In 1995 we decided to return to some places that were visited during the 1978 mission. Nearly all local field crops have disappeared. Fifteen samples of cereals were collected, in comparison with the previous mission when 111 samples of cereals were gathered. Only one sample of wheat was found whereas in 1978, 33 variable accessions were collected (Fig. 2). Local forms of *Hordeum vulgare* and *Avena sativa* are still grown on fields located above 1000 m asl, while at lower elevation the cultivation of local spring rye populations was also recorded. Landraces well adapted to the specific environ-mental conditions could successfully compete with modern cultivars here. The observations made during the last expedition, in contrast to those of earlier years, indicate almost complete eradication of old cultivars and landraces. The modernization of Polish

agriculture, the exclusion of marginal areas from cultivation and wide access to seeds of new varieties are threatening the local populations of all crops.

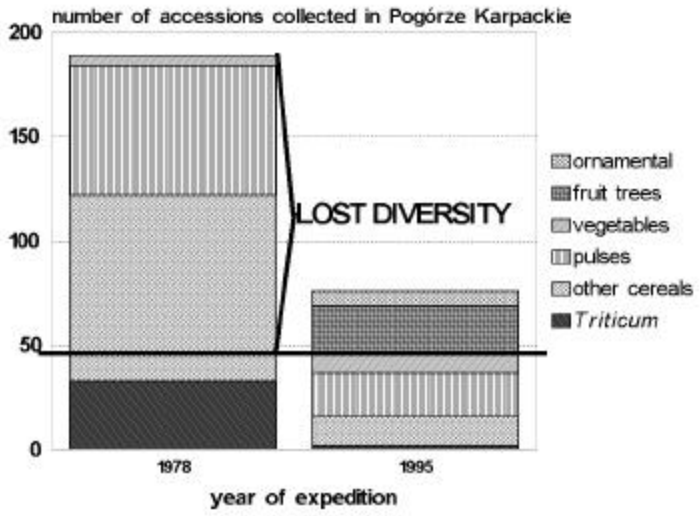

Fig. 2. Samples collected during missions in Pogórze Karpackie in 1978 and 1995.

The accessions collected during missions in the period 1976-79 constitute the 'core' of the maintained landraces of field crops of Polish origin. Presently, the local crop cultivars are available mainly as material stored in genebanks. According to our evaluations in the last decade, the local populations of crop plants disappeared almost completely. However, there are still regions where traditional vegetable varieties are being grown.

Collecting vegetables

Expeditions are carried out in regions which have a long tradition of vegetable growing. Nowe Miasto nad Pilicą and Przybyszewo are well known for the cultivation of old ecotypes of onion of the type Żytawska-Przybyszewska and cucumber of the type Przybyszewski. The seeds of these vegetables are still available on the market. The areas around Jędrzejów, Pińczów, Skalbmierz and Kazimierza Wielka are very rich in garlic ecotypes.

In the Pogórze region different types of common beans, differentiated by morphological and agronomic characters, are still grown. Some of them were cultivated there in the 19th century. Different types of shallot and garlic, *Brassica napus* var. *napobrassica*, still exist. Landraces of *B. napus* var. *napobrassica* are used here for human consumption as well as for fodder.

Old vegetable varieties also persist in the northeast region of Poland. Those areas are especially interesting because emigrants from former Eastern Poland live there. They still grow a lot of vegetables brought from their native regions, such as pumpkin, common bean and tomatoes. In eastern regions every small garden contained ecotypes of different vegetables. In areas inhabited by fairly large populations of Byelorussians, who preserve ethnic traditions, many local races of beans and pumpkin grown as fodder crop have been conserved.

The Lublin district is well known for vegetable production. Mainly the areas near Lubartów, Szczebrzeszyn and Frampol are famous for local varieties of onions (Lubartowska and Szczebrzeszyńska).

The surveys support the opinion that vegetable landraces are in danger of extinction, as a result of ongoing cultural changes in the countryside. The most interesting materials were collected from old people strongly attached to tradition. With these people, knowledge about methods of home multiplication of crops is dying.

Collecting grass ecotypes

Since 1971 several independent collecting missions were conducted mainly to northern regions of Poland and they resulted in rich and varied collections of grass ecotypes. Presently, about 75% of the country's total area has been sampled. More than 17 000 seed samples of grasses have been collected during the expeditions. The grass collection predominantly consists of ecotypes collected over the last 20 years in Poland. Ecotypes comprise 94% of the total number of accessions. Species of seven basic forage grasses make up most of the collection: *Dactylis glomerata, Festuca arundinacea, Festuca pratensis, Festuca rubra, Lolium perenne, Phleum pratense, Poa pratensis.* Taking into consideration changes of preference of potential users of grass material, with new direction in breeding and new implementation of bred varieties, such as the reclamation of devastated lands, a wider set of species is being conserved.

Future prospects

Throughout the years of activities in the framework of the National Crop Plant Resources Conservation Programme, technology and expertise of *ex situ* conservation of plant genetic resources have been developed. However, an important task to be resolved is the conservation of landraces and wild relatives of crops which can be done by *in situ* conservation. A new philosophy tends to replace the traditional understanding of nature conservation. Conservation of the biodiversity needs new, durable solutions. Genebanks should therefore make use of every opportunity to join new environmental programmes, which means that *ex situ* conservation in genebanks will support complex environmental protection.

Acknowledgements

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Polish botanical gardens as genebanks

Jerzy Puchalski
Botanical Garden of the Polish Academy of Sciences, Warsaw, Poland

Introduction

Botanical gardens always played an important part in the collecting of plants for scientific purposes as well as for their utilization. Thanks to the development of botanical exploration of new colonies in the 18th and 19th centuries, botanical gardens were able to introduce many new crops like rubber, nutmeg, African oil palm and cinchona (Plucknett *et al.* 1987).

At the end of the 20th century the role of botanical gardens, as institutions collecting plants, has further increased. Owing to increasing threats to many plant species, the active conservation of endangered plants by botanical gardens in so-called *ex situ* conditions became a necessity (Ashton 1988). Many botanical gardens have enriched their living collections with indigenous species threatened by extinc-tion. Also in numerous botanical gardens, seedbanks or *in vitro* culture banks were established for the conservation of endangered plants (Anonymous 1989).

Botanical gardens in Poland

Poland currently has 23 botanical gardens (Fig. 1). Ten of these are typical botanical gardens holding collections of different kinds of plants, one palm house with tropical and subtropical plants, nine arboreta or dendrological gardens collecting the woody plants, and three special botanical gardens devoted to collections of medicinal plants or spices (Fig. 1). Fifteen of them have actively participated in the *ex situ* conservation programme on Polish endangered plants (Lankosz-Mróz and Zarzycki 1993). They collected about 150 species of legally protected plants in Poland and over 100 species of threatened plants listed on the Polish Red List (Zarzycki and Szeląg 1992). This Red List consists of 418 species of vascular plants, covering about 19% of the Polish vascular flora (Table 1).

Some botanical gardens specialize in particular groups of plants. The richest collections of plant species from the Tatra Mountains Region are kept as living plants in the Mountainous Botanical Garden in Zakopane. The Arboretum at Bolestraszyce has many achievements in *ex situ* conservation of rare and endangered species from southeastern Poland.

The Botanical Garden of the Polish Academy of Sciences in Warsaw-Powsin from the beginning of its research has put a lot of effort into *ex situ* conservation of Polish endangered plants. Presently the living collections in Powsin include 90 plant species representing groups of rare, endangered or protected plants in Poland, among them *Cochlearia polonica*, an endemic plant, which became extinct on primary sites, but grows well in the botanical garden. The Botanical Garden has organized in recent years a special seedbank for long-term seed storage of endangered Polish plants in cryogenic conditions. Seeds are collected from natural sites, especially in the national parks. For each species, studies on seed germination procedure and safe seed ultrafreezing technique in liquid N_2 are conducted. Also preliminary experiments were carried out on cryopreservation of *in vitro* cultures, e.g. for *Gentiana* species and *Leucoium vernum*. The collections are presented to visitors as special displays: protected plants in Poland; plant

communities in Poland – woodland communities, aquatic and marsh communities, peat bog and meadow communities or steppe plants.

Fig. 1. Location of botanical gardens in Poland.

● **Botanical gardens**

1. Botanical Garden of the Polish Academy of Sciences in Warsaw-Powsin
2. Botanical Garden of Warsaw University
3. Botanical Garden of Jagiellonian University in Cracow
4. Botanical Garden of A. Mickiewicz University in Pozna⌧
5. Garden of M. Sk⍺odowska-Curie University in Lublin
6. Botanical Garden of Wroc⍺aw University
7. Botanical Garden of the City of ꝏód✓
8. Botanical Garden of the Plant Breeding and Acclimatization Institute in Bydgoszcz
9. Botanical Garden of the Culture and Recreation Park in Bydgoszcz
10. Mountainous Botanical Garden of the Institute of Nature Protection in Zakopane
11. Pozna⌧ Palm House

■ **Arboreta and dendrological gardens**

12. Arboretum of the Dendrology Institute of the Polish Academy of Sciences in Kórnik (near Pozna⌧)
13. Forest Arboretum of Warsaw Agricultural University in Rogów
14. Arboretum Bolestraszyce near Przemy◩l
15. Arboretum of Wroc⍺aw Botanical Garden in Wojs⍺awice
16. Dendrological Garden in Przelewice (near Szczecin)
17. Dendrological Garden in Glinna (near Szczecin)
18. Forest Arboretum in Syców
19. Forest Arboretum in Zielonka
20. Dendrological Park of State Forests Administration in Go⍺uchów (near Kalisz)

s **Botanical gardens of medicinal plants**

21. Medicinal Plants Botanical Garden of Medical College in Wroc⍺aw
22. Medicinal Plants Botanical Garden of the Institute of Herbal Products in Pozna⌧
23. Medicinal Plants Botanical Garden of Medical College in Gda⌧sk.

Table 1. Endangered vascular plant species in Poland according to Zarzycki and Szel⏲g 1992

IUCN category		Number of species 1986	1992
Extinct	Ex	31	40
Endangered	E	32	54
Vulnerable	V	90	142
Rare	R	130	146
Intermediate threat	I	56	36
Total		339	418
% of Polish flora (2300 species)		15%	19%

Polish endemic and subendemic plants	59 species
Relict plants	17 species
Vascular plants protected by law in Poland	200 species

Plant collections of the Botanical Garden of the Polish Academy of Sciences

Priority is given to indigenous Polish vascular plants and in total as many as 540 species were collected, which represent about 23% of the whole vascular flora in Poland.

Besides indigenous plants many economic plants and crops used in horticulture and agriculture were collected. The most valuable is the collection of the *Secale* genus represented by more than 1500 accessions. Other agricultural crop germplasm collections include wild species of the *Triticeae* tribe (319 taxa), cultivars of triticale (100 taxa) and lupins (100 taxa). Horticultural plants are represented by ornamental plants (1514 taxa), vegetables (295 taxa), medicinal plants and spices (229 taxa), a pomological collection (573 taxa), dendrological collections (1500 taxa) and tropical or subtropical plants (1412 taxa) cultivated in the greenhouses. High emphasis is given to the collections of old apple cultivars (144 taxa) and wild species of edible fruits (207 taxa). Among ornamental plants the richest is the collection of the genus *Iris,* with 500 wild or botanical species and horticultural cultivars. The total number of the plant collections in the Botanical Garden of the Polish Academy of Sciences has reached 8140 taxa, including 540 wild species of the Polish flora and 7600 taxa of economic and cultivated plants.

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Session II: Long-term storage practices for orthodox seeds (Chair: Dr Jerzy Puchalski)

An analysis of seed storage practices

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Summary

Breeders and genebank operators require seed storage systems which maintain highly vigorous seeds for several years to several decades. Seed lifespans are dependent on the seed moisture content and the storage temperature. There is an optimum moisture level at which seeds should be stored. This level is a function of a number of factors including the effects of seed moisture content on ageing kinetics, the energy costs of drying seeds, and the number of seeds that must be processed. The effect of seed moisture content on ageing kinetics is, in turn, a function of the seed species and the temperature. The energy cost of drying seeds is a function of building specifications, drying conditions and final water content. The volume of seeds that can be processed is a function of building specifications and drying rate. On the basis of theoretical and experimental considerations, we have found that ageing rates are minimized in seeds from most agronomic crops if they are in equilibrium with a relative humidity of about 22%. The determination of this "physiological" optimum has allowed us to predict optimum drying and storage procedures for seeds from diverse species. We present a model which relates the energy costs for drying and storage of seeds to the predicted longevity under various storage conditions. Analyses indicate how storage regimes can be optimized to provide the required seed longevity at the lowest energy cost.

Introduction

Seeds are stored for many reasons and the required longevity is dependent on the application. When seeds are used for production purposes, survival for only a few years is necessary. When seeds are stored as genetic resources, viability must be maintained for several decades or even centuries. The stringency of the conditions for seed storage increases as the required longevity increases. For orthodox seed species, very long storage lives can be achieved by adjusting the water content of the seeds to an optimal level and by reducing the storage temperature.

Current standards for seed storage recommend that seeds be dried to 5 ± 2% water and then maintained at 0 to –20ºC (Justice and Bass 1978; FAO/IBPGR 1992, 1994). Recent experiments using a number of different species have shown that longevity may be further improved in some species if seeds are dried to moisture contents less than the recommended mean value (Cheng *et al.* 1990; Ellis *et al.* 1988, 1989, 1990; IBPGR 1992, 1993; Vertucci 1993; Vertucci and Roos 1990, 1993b; Walters-Vertucci *et al.* 1996). It has thus been recognized that the range of water contents recommended by IPGRI is a guideline and the properties of the seed must be considered to achieve the appropriate moisture content for storage. Experiments also have shown that there are limits to the beneficial effects of drying such that drying below a critical moisture content will not improve

longevity (Ellis *et al.* 1988, 1989, 1990; Vertucci 1993; Vertucci and Roos 1990, 1993b; Vertucci *et al.* 1994) and may even have detrimental effects on seed survival in storage (Vertucci and Roos 1990, 1993b; Vertucci *et al.* 1994 and references therein). Knowing the value of this critical moisture level is important since insufficient drying results in less than maximum longevity and overdrying expends energy unnecessarily, may reduce vigour because of longer drying periods, and may even have an adverse effect on ageing rates. It is well established that the critical moisture level varies with species and even with cultivars of the same species (Cheng *et al.* 1990; Ellis *et al.* 1988, 1989, 1990; IBPGR 1992, 1993; Vertucci 1993; Vertucci and Roos 1990, 1993b; Walters-Vertucci *et al.* 1996).

Lowering the temperature to increase the storage life is a basic paradigm of seed storage practices (Stanwood 1985). Unfortunately, the cost of refrigeration is substantial (IBPGR 1976). There have been suggestions that there are limits to the beneficial effects of refrigeration or that comparable longevities can be achieved if refrigeration is replaced by drying (Dickie *et al.* 1990; FAO/IBPGR 1992, 1994). "Ultra-dry" technology, which involves drying seeds to moisture contents close to the critical level, was developed to enhance longevity when refrigerated systems were inefficient (FAO/IBPGR 1992, 1994; IBPGR 1992, 1993). Recent research has shown that the value of the critical moisture content varies with temperature (Vertucci 1993; Vertucci and Roos 1993b; Vertucci *et al.* 1994). This further complicates the job of the germplasm bank operator who must know the critical moisture content for each accession to maximize longevity and minimize costs.

The effect of moisture on seed ageing

The primary problem with determining the optimum conditions to store seeds is that we have a poor understanding of the effect of water on seed ageing. Determining how water content influences seed longevity is logistically difficult because seed ageing experiments are long term, and it can take years to see a discernible change in the vigour or viability of the seeds. Because direct evidence is not available, we must rely on extrapolation. The basis of extrapolation can be divided into two approaches: an empirical approach which is represented by the classic viability equations (Roberts 1960; Ellis and Roberts 1980) and a more theoretical one based on principles of physical chemistry (Vertucci and Roos 1990; Vertucci 1993).

In the theoretical approach, we use the concept of "hydration levels." These hydration levels are identified by changes in the water properties and are associated with various levels of physiological activities (reviewed in Leopold and Vertucci 1989; Vertucci 1993). There are five hydration levels and the first three are relevant to seed ageing. "Accelerated ageing" occurs at the third level where respiration is enabled. In genebanks, seeds are usually stored at water contents within hydration level 1 or 2. In the second hydration region, water behaves like a glass: it has solvent properties, but it is extremely viscous. As seeds are hydrated within this region, the viscosity decreases, allowing more molecular motions, more chemical reactions and consequently faster ageing. The glassy properties of water are disrupted when seeds are dried within the first hydration level. Although the nature of reactions in this hydration level is not known, it is believed that they include structural changes in proteins and membrane bilayers brought about by a loss of the stabilizing effect of water (Vertucci 1993). In addition, when the protective effects of water molecules are removed, free radicals can attack

macromolecular surfaces and initiate autocatalytic reactions (Khan *et al.* 1996). The effects of water content on the kinetics of these types of reactions are not known. This information is critical for understanding whether drying to very low moisture contents will have a detrimental effect on the stability of biological structures and consequently an adverse effect on seed viability.

We have hypothesized that ageing results from a series of degradative reactions and that maximum longevity is achieved at the water content in which the sum of all the deteriorating effects is minimized (Vertuccci 1993; Vertucci and Roos 1993b). At the boundary between hydration levels 1 and 2, viscosity is minimal, but water is available to protect cellular components. We therefore expect the moisture content corresponding to the boundary of hydration levels 1 and 2 to be a critical value, and drying below this amount will not improve longevity. Further, if the kinetics of some deteriorative reactions are enhanced by removal of water within hydration level 1, then drying below the critical moisture content will probably result in more rapid ageing. The existence of a critical moisture content has been established experimentally (Ellis *et al.* 1988, 1989, 1990, 1995; Vertucci and Leopold 1987a; Vertucci and Roos 1990 and references therein). In addition to theoretical predictions, there is also much evidence to suggest that the critical moisture content is really an optimum, and drying below this value has an adverse effect on longevity (Carpenter and Boucher 1992, 1992a; Carpenter *et al.* 1993; Ellis *et al.* 1995; Vertucci and Leopold 1987a; Vertucci and Roos 1990; Vertucci *et al.* 1994 and references therein).

Water contents that define the hydration regions vary among species. However, if hydration level is defined using the thermodynamic terms of water activity and / or water potential, the values defining boundaries are fairly constant among species (Leopold and Vertucci 1989; Roberts and Ellis 1989; Vertucci and Roos 1990, 1993b; Vertucci 1993). Similarly, the water content at which maximum longevity is achieved varies among species, but if expressed in terms of water activity, the critical value is fairly similar among species (Ellis *et al.* 1988, 1989, 1990; Vertucci and Roos 1990). For example, when seeds of different species were stored at 35°C, the water content for maximum longevity ranged from about 7% for pea to less than 2% for yew seeds, and this relationship is correlated with lipid content (Fig. 1). When ageing rates are expressed on a RH (RH ≈ water activity x 100) scale, the optimum level is 22±2% (Fig. 2) (Walters-Vertucci and Roos 1996).

The observation that the critical water content differs among species but the critical relative humidity is similar among species can be explained using water sorption isotherms. Water sorption isotherms describe the relationship between water activity and water content. The amount of water absorbed by seeds is a function of the lipid content such that seeds with higher lipid contents absorb less water (Fig. 3) (Vertucci and Leopold 1987b; Vertucci and Roos 1990). If seeds with different lipid contents are held at a single RH, the water contents of the seeds will vary. Thus, if the critical RH is constant among species, we deduce that the critical water content must vary among species. This provides a tremendous tool for the genebank operator who, now, does not need to determine the critical moisture content for each accession. Rather, the optimum level for storage can be achieved by merely equilibrating seeds to a specific RH. This understanding has led to adjustments in seed-drying procedures traditionally used (Justice and Bass 1978; FAO / IBPGR 1992).

Several procedures have been proposed: (1) equilibrate seeds to about 15% RH and 15°C (Wieland 1995), (2) equilibrate seeds to 10% RH and 20°C (FAO/IPGRI 1994), and (3) equilibrate seeds to a moisture level that gives 20-25% RH at the storage temperature (NPGS 1996).

The existence of a variety of new drying protocols underscores a controversy that has developed over the value of the critical moisture level (Ellis *et al.* 1991, 1995; Smith 1992; Vertucci and Roos 1991, 1993a). This controversy stems from the different bases of extrapolation in the empirical and theoretical models of seed deterioration. In the empirical model, water content and temperature are viewed as independent variables. Thus, critical water contents are constant with temperature. In the theoretical model, water properties are crucial, and these are defined in terms of water activity. Thus, water content and temperature are viewed as interdependent variables, and the critical water content is a function of the storage temperature.

Water sorption isotherms can be used to explain how water content, temperature and water activity (relative humidity ÷ 100) interact (Fig. 4) (Vertucci and Roos 1993b). As with chemical composition, the amount of water absorbed by seeds is a function of temperature. Seeds absorb more water at lower temperatures. Thus, if there is a single critical RH (as the theoretical model proposes), we deduce that the critical water content must vary with temperature. In fact, it must increase as temperature is reduced (Vertucci and Roos 1993b). If there is a single critical water content (as the empirical model proposes), then we must deduce that the critical relative humidity increases with increasing temperature. The deduction made using the empirical approach is difficult to justify in thermodynamic terms, and so we have concluded that it is based on false premises.

The previous discussion leads us to ask why the empirical and theoretical models arrived at similar conclusions of a single critical RH when different species were involved, and divergent conclusions when temperature was the variable. This is an important question because it relates directly to how genebank operators should dry their seeds. Conclusions from both models were based on storage experiments conducted at elevated temperatures (>50°C) and the results from these experiments were essentially identical (Ellis *et al.* 1988, 1989, 1990; Vertucci *et al.* 1994). The major difference in the two approaches is an acknowledgment that the relative humidity at which seeds are dried is **not** equal to the RH at which seeds are stored if the drying and storage temperatures are different (Vertucci and Roos 1993b). Using the seeds described in Figure 4 as an example, when pea seeds are dried to equilibrium at 20°C and 10% RH, the water content is about 6%, and this was determined as the critical water content for storage at 65°C (Ellis *et al.* 1989). But the RH surrounding the seeds stored at 65°C is not 10% (as it was at the drying temperature of 20°C); it is now about 50% (Fig. 4). In fact, the moisture level achieved by drying seeds at 20°C and 10% RH is somewhat greater than the optimum moisture level at 65°C proposed by the theoretical model. According to the theoretical model the optimum moisture content at 65°C occurs at the moisture content corresponding to 65°C and 22% RH or about 2.5% water (Fig. 4). The theoretical model was supported by analyses of critical moisture levels at 65°C and at lower temperatures: the critical water content varied, but the critical RH was almost constant at about 25% (Vertucci *et al.* 1994). Thus, the theoretical model predicts that the moisture levels achieved by equilibrating seeds at 15°C and 15% RH (Wieland 1995) or 20°C and 10% RH

(FAO/IPGRI 1994) will give optimum water contents at about 30 and 45°C, respectively, but will dry seeds to below the critical level if seeds are stored under ambient or refrigerated conditions. Thus, the drying procedures recommended by some genebanks may be an unnecessary expense if seeds are stored at ambient temperatures or below. In addition, if overdrying has a detrimental effect on longevity, extreme drying may be counterproductive.

Seed-drying procedures

Using the theoretical model, we have concluded that the moisture content which provides maximum longevity is a function of the storage temperature. Genebank operators may view this discovery as a burden since the complexity of an additional variable – the temperature at which seeds are stored – must now be considered. On the contrary, we believe that the theoretical approach to seed ageing can be used to achieve optimum storage conditions efficiently and with flexibility. The target moisture

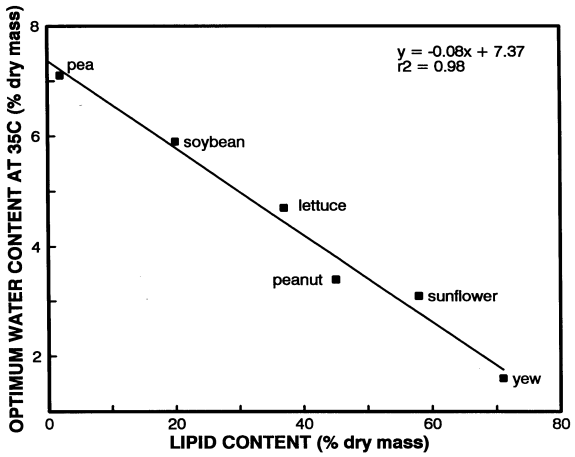

Fig. 1. The relationship between the lipid content of different seed species and the water content which gives maximum longevity when seeds are stored at 35°C. The curve represents the linear regression of lipid content versus critical water content points. Data are from Vertucci and Roos (1990) and Walters-Vertucci *et al*. (1996).

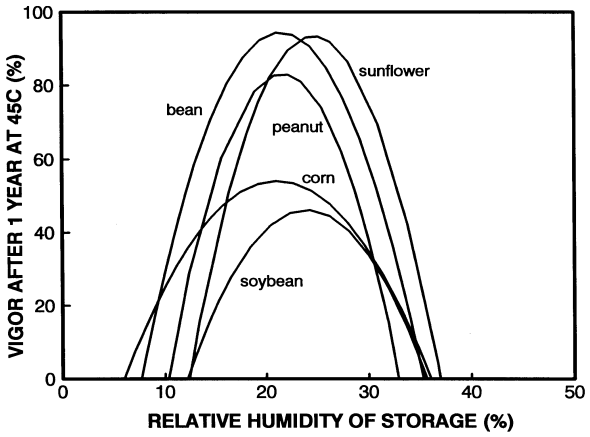

Fig. 2. The relationship between the relative humidity of storage and the vigour of different species of seeds following 1 year storage at 45°C. Curves represent quadratic equations fit to RH versus aging rate data. The RH at which vigour is maximum is 22±2%. Vigour is defined as the growth of the radicle after a specified germination period.

level for storage can now be predicted even if there is little information about the seed. Further, numerous drying strategies can be used to achieve the optimum moisture level. For example, if pea seeds are to be stored at 5°C, the optimum moisture level at 22% RH corresponds to about 11% water (Fig. 4) and can be achieved by equilibrating at 35°C and 50% RH, 25°C and 45% RH, or 15°C and 35% RH (Fig. 4). The optimum moisture level at –20°C (12.5% water) can be achieved by equilibrating at temperature/RH combinations of 35/65, 25/55, 15/45, or 5/35 (Fig. 4). It should be emphasized that the above temperature/RH combinations are given as an illustration. Before exact recommendations can be given, the hypothesis that the optimum exists at about 22% RH must be tested more rigorously and a more detailed study of isotherms is necessary.

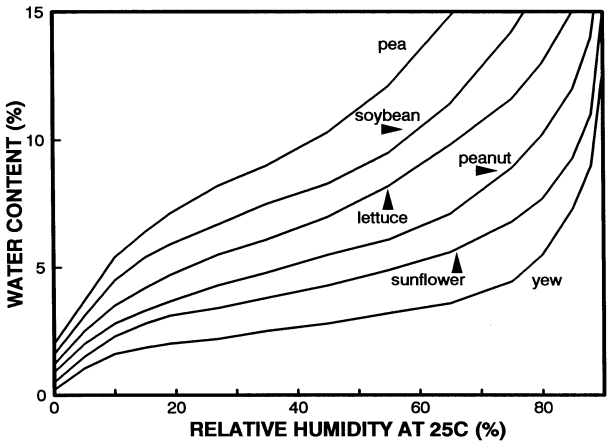

Fig. 3. Water sorption isotherms of different species of seeds drawn at 25°C. Data are from Vertucci and Roos (1990) and Walters-Vertucci *et al*. (1996).

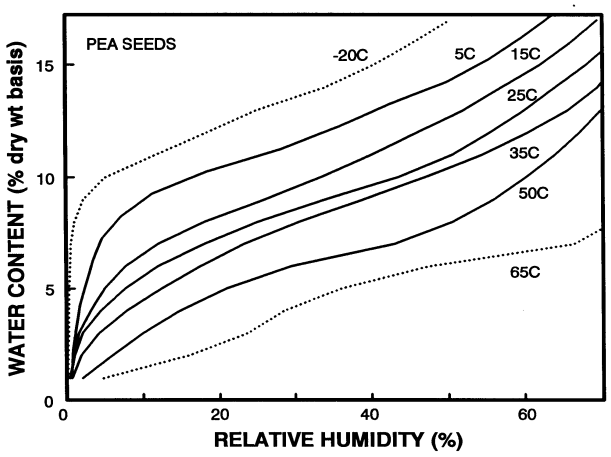

Fig. 4. Water sorption isotherms of pea seeds at different temperatures. Solid lines represent temperatures where isotherms were measured directly and dashed lines represent curves calculated from extrapolating van't Hoff analyses. Data are from Vertucci and Roos (1993b).

Genebank operators may need some guidelines for deciding which drying procedure works best for them. This decision should be based on the available resources, the number of accessions to be processed, the energy and labour costs for processing seeds, and the acceptable level of deterioration that occurs during drying. We have developed a cost-benefit model that describes how different processing procedures affect energy costs (the lower the drying temperature, the more expensive), the processing time (the higher the RH, the longer it takes), and the initial seed quality (the longer the drying time and/or the higher the temperature, the lower the quality) (Walters-Vertucci and Roos 1996).

Darcy's Law was used to describe drying rates:

$$J_w = D \cdot \Delta\psi$$

where J_w is the rate of drying, D is the diffusion coefficient, and $\Delta\psi$ is the water potential difference between the seed and the drying room. The diffusion

coefficient is a function of temperature and package dimensions. Thus, drying rate can be predicted given the initial water content, the drying-room temperature and RH, and the way seeds are packaged. Drying time courses given in Figure 5 illustrate how these variables interact. In the scenario, pea seeds with an initial water content of 20% are dried at 15°C and 20% RH. The package depth varies from a monolayer to 20 cm. Seeds in a monolayer dry faster than seeds in bulk, with the time required to reach equilibrium varying from 12 days to about 70 days. The equilibrium water content is about 8.5% regardless of the packaging or the initial water content. The RH of the drying room also affects drying rate with faster rates achieved at lower relative humidities. For example, samples packaged in 20 cm bags and dried at 15°C and 5% RH reach 8.5% water in only 18 days (Fig. 5). The equilibrium water content under this drying regime is 6.5%, and it is achieved in 52 days. Drying rates can also be accelerated by increasing the temperature. If the same package of seeds is dried at 30°C and 20% RH, a water content of 8.5% can be achieved in 10 days, and the equilibrium water content of 7.5% is achieved in 21 days (Fig. 5). However, when seeds are dried to equilibrium at these lower RHs, seed moisture will be below optimum for storage at 5°C and –20°C.

Cost-benefit analysis of seed-drying practices

The effects of different drying procedures can now be evaluated in terms of the number of seeds that can be processed, the energy required to process seeds, and the quality of seeds after they have been processed. The drying rate is a central component of this analysis since it provides the time by which power requirements and ageing rates are integrated.

The volume of seeds processed is a function of the drying space, the efficiency of packing and the number of turnovers in the drying room. Seeds dried in bulk take longer to dry (so fewer turnovers), but are far more efficiently packed than seeds in a monolayer. In a drying room that is 3.5 x 2.75 x 2.5 m and set at 15°C and 20% RH, between 20 000 and 35 000 kg can be processed each year depending on whether seeds are dried in a monolayer or in packages 20 cm deep (Fig. 6). If the RH were increased or the temperature decreased, fewer accessions could be processed (Fig. 6).

There are four major factors contributing to the energy costs of seed drying: (1) the amount of water to be removed from the seed, (2) the packaging efficiency, (3) the difference between drying room environment and external environment, and (4) the time required to maintain the drying room conditions (i.e. the integral of the drying rate). The drying procedure (final moisture content, room RH, temperature) and package characteristics influence energy costs at many levels. An analysis of the different components of heat and moisture loads shows that the energy required to remove water from seeds and to refrigerate seeds during drying is greater than the energy required to maintain relative humidity (Fig. 7).

We can now evaluate the energy costs of different drying protocols (Fig. 8). In the analysis we assume room dimensions of 2.5 x 2.75 x 3.5 m, outside conditions of 25°C and 75% RH, initial moisture content of the seed of 20%, and package size as 14 x 9 cm and 9 cm deep (Fig. 8). Drying at 20°C / 10% RH (FAO / IPGRI 1994) is energy efficient because the cost of refrigeration is slight. Drying at 15°C / 15% RH (Wieland 1995) or at 10°C / 30% RH (NPGS 1996) is more expensive because of the increased refrigeration costs (Fig. 8). The final moisture content achievable with each regime is the equilibrium water content for the particular

temperature/RH combination of the drying regime (Fig. 4). Energy costs are high when the equilibrium water content is sought, and declines if seeds are dried to moisture levels greater than the equilibrium value. The high cost of drying to equilibrium is perhaps the most important point of this analysis. A two-step drying procedure may prove more economical. In the first

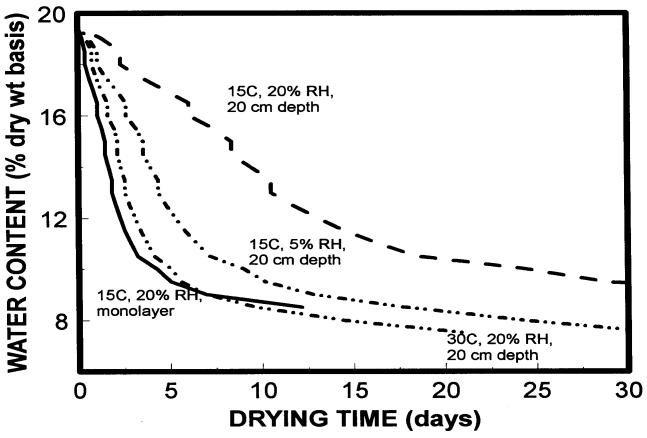

Fig. 5. The effect of drying room conditions and packaging on pea seed drying rates. Curves are calculated using Darcy's Law. Drying room temperature and RH are as indicated. Monolayer refers to seeds spread out on a drying tray and 20 cm refers to seeds packaged in 9x14 cm paper bags to a depth of 20 cm.

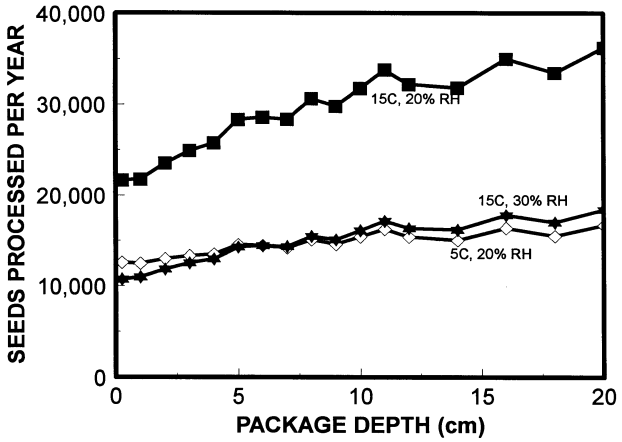

Fig. 6. The effect of the depth to which seeds are packaged in 9x14 cm bags on the amount of seeds that can be processed each year. The calculations consider the effect of package dimensions on drying rates as well as the volume of seeds that can be accommodated in a room 3.5x2.75x2.5 m. Temperature and RH of the drying room are as noted.

step, seeds may be dried rapidly at ambient temperatures and low RH to near-optimum seed moisture. In the second step, the final equilibration can be done at an appropriate temperature/RH combination to achieve the optimum seed moisture, which is dependent on the temperature at which the seeds will be stored. The final component of an analysis of various drying procedures is an evaluation of the effect of these procedures on seed quality. In our model, seed quality is measured by radicle growth and the rate of change of growth with storage time at different moisture content/temperature combinations (Vertucci *et al.* 1994) is used to quantify seed deterioration during drying. If pea seeds are dried to equilibrium at 15% RH and temperatures between 5 and 35°C, we predict a decrease in seed quality of 0.5 to 2.5% (Fig. 9). Such small changes are not measurable with traditional vigour assays, but they may have significant impact on the overall longevity of seed. The sharp decrease in seed quality observed

when pea seeds are dried to less than about 8% at 15 to 35°C is a result of the prolonged exposure to these elevated temperatures.

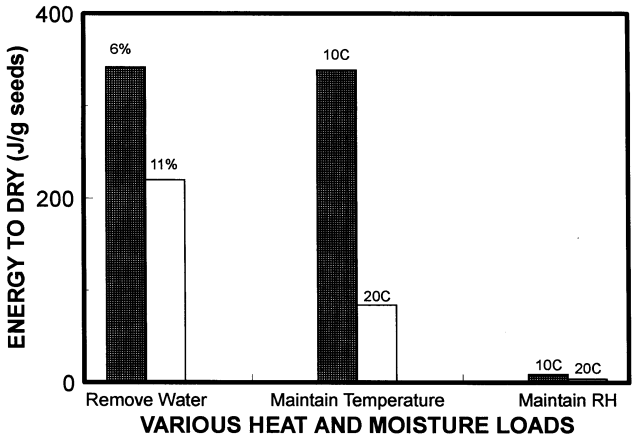

Fig. 7. The relative contribution of various heat and moisture loads to the cost of drying pea seeds. Outside conditions are assumed as 25°C and 75% RH and NSSL drying room building specifications are used. The relative humidity of the drying room is less than 5%. The energy required to maintain temperature and RH is calculated based on seeds packaged in paper bags 9x14x9 cm and dried from 20 to 6% water content.

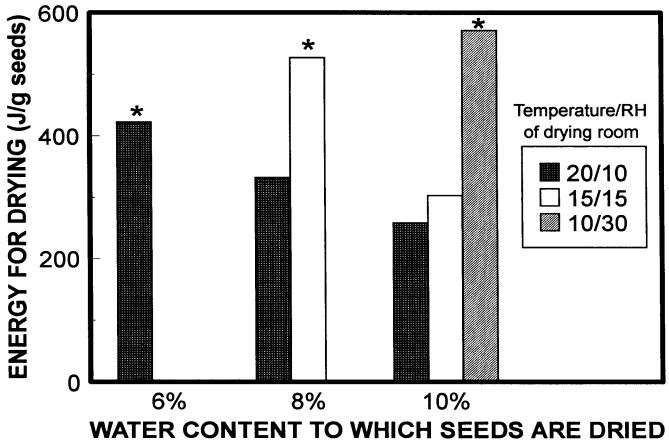

Fig. 8. The energy required to dry seeds to different moisture contents using different drying room environments as indicated in the legend. Assumptions used in the model are as described in Figure 7. The asterisks represent the equilibrium (i.e. minimum) moisture contents achievable using the different drying regimes.

This decline emphasizes the costs of bringing seeds to equilibrium. If seeds were dried to the same levels, but at a lower RH (i.e. they were not brought to equilibrium), the rate of drying would be faster (Fig. 5), and the moisture content at which there was a serious decline in quality would be considerably lower (data not shown).

The impact of packaging on seed quality is rarely considered in seed-drying recommendations. Generally, dense packaging increases the volume of seeds processed and decreases the energy costs of drying because the increased packaging efficiency out-factors the slower turnover times. In a scenario where pea seeds are dried at 25°C and 15% RH, the equilibrium water content of 6.5% is achieved within 7 to 75 days, depending on whether seeds are spread in a

monolayer or packaged to a depth of 20 cm. The slower drying rates have an adverse effect on seed quality with a reduction of up to 2.5% of the original value (Fig. 10). The extent of the detrimental

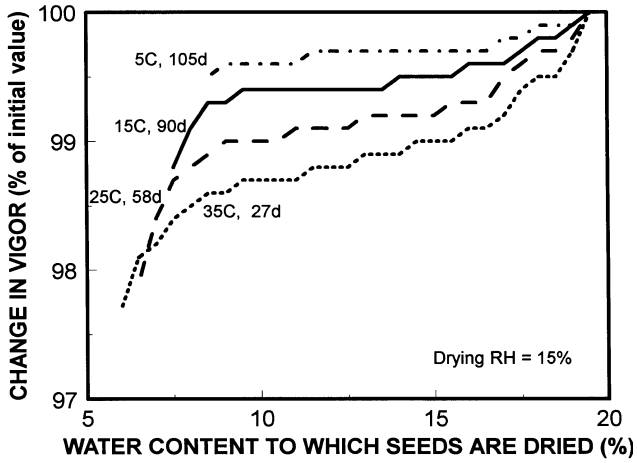

Fig. 9. The effect of drying temperature on changes in pea seed quality during drying. Initial seed water content is set at 20% and seeds are packaged in 9x14x9 cm bags. The room RH is set at 15% which gives minimum water contents (i.e. at equilibrium) of 8.5, 7.5, 6.5, and 6% at 5, 15, 25, and 35°C, respectively. Equilibria were achieved in 105, 90, 58, and 27d, respectively. Vigour calculations were done using quadratic curves similar to those in Fig. 2, but determined for pea seeds at different temperatures (Vertucci *et al.* 1994).

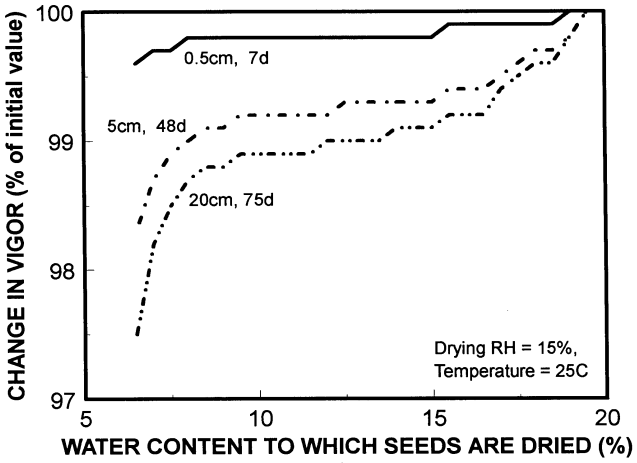

Fig. 10. The effect of packaging depth on changes in pea seed quality during drying. Initial seed water content is set at 20% and seeds are packaged in 9x14 cm paper bags to the indicated depth. The room is set at 25°C and 15%RH. The minimum water content for each treatment is 6.5% which was achieved in 7 to 75 days depending on the depth of the packaging. Vigour calculations were done using quadratic curves similar to those in Fig. 2, but determined for pea seeds at different temperatures (Vertucci *et al.* 1994).

effect is a function of the drying temperature, with lower temperatures preserving quality longer. This means that the major benefit of refrigerated drying is that seeds can be packaged in bulk and dried without a loss in quality. The greater packaging efficiency and fewer turnover times (because of the slower drying rate) will reduce the amount of seasonal labour. The major detriments of refrigerated drying are the increased energy costs (Fig. 7) and the reduced number of samples that can be processed each year (Fig. 6). Genebank operators may find that

energy costs can be reduced without a loss in seed quality by balancing the drying temperature and RH with the type of packaging.

Conclusions

An analysis of seed storage practices demonstrates that both the moisture content to which seeds should be dried and the method by which seeds are dried must be considered in order to achieve maximum longevity at minimum costs. We have used thermodynamic principles to predict optimum moisture contents for storage and to estimate the costs and benefits of various drying protocols. Our analyses show that accurate predictions can be made on a variety of species for which there is little existing information. This approach allows genebank operators to evaluate different drying options based on the equipment and labour available to them as well as the energy costs, processing time and final seed quality.

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The effect of different gaseous atmosphere and seed moisture content on viability of rye seeds in long-term storage experiment

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Summary

Rye seed samples (cv. Dańkowskie Złote) were dried to 3.7 or 5.5% moisture content (fwb) and stored in different gaseous environments: air, vacuum, CO_2 and N_2. Seed samples were sealed in hermetic glass containers and stored in a chamber at ambient temperature (about 10ºC). Seed viability was checked periodically. Data were recorded after 2, 3, 10, 15 and 19 years of storage. The first visible differences in seed viability were observed after 3 years of storage. After 10 years of storage seeds kept in vacuum or neutral gases preserved their viability at a level close to 80% of germinability, whereas in seeds stored with air, viability decreased to about 30%. Investigation of two levels of seed moisture content did not show significant effect on seed viability during the first 10 years of storage, either in vacuum or in air. However, after 15 years of storage, in the case of seed kept in vacuum, samples with lower seed moisture content showed higher viability.

Introduction

Seed moisture content and storage temperature are known as the major factors affecting seed longevity during storage (Roberts 1972, 1975). Additionally, the surrounding atmosphere, in particular oxygen pressure, is the other factor influencing seed storability. However the role of oxygen in seed deterioration during storage versus seed moisture content and temperature is still discussed (Wilson and McDonald 1986; Benson 1990) and the experimental results obtained by different authors on various crops are to some extent controversial (Justice and Bass 1978; Tao 1990). Therefore our experiment was conducted to evaluate the effects of seed moisture content and storage atmosphere on seed viability during long-term storage at constant temperature.

Materials and methods

Results of long-term experiment on the storage of seed samples of rye (*Secale cereale* L.) are presented. Seed samples of the cultivar Dańkowskie Złote were obtained from the Breeding Station Choryń. The experiment started in 1977. The seed samples were dried in a forced-air oven (at a temperature of 38ºC) to 5.5 or 3.7% moisture content (fwb) and packed in glass airtight containers. Before sealing in the case of samples stored in vacuum, CO_2 and N_2, air was evacuated with the aid of a vacuum pump to less than 1 mm Hg inner pressure; containers with seed stored under CO_2 and N_2 were refilled with the appropriate gas. Samples were stored at ambient temperature (about 10ºC).

Periodically two samples of each storage and moisture content variants were opened and seed viability (germinability) was evaluated. Data on seed samples viability were recorded after 2, 3, 10, 15 and 19 years of storage. Germination tests were carried out in wet sand or rolled filter paper in germinators at 20ºC, in

the darkness. After 7 days, normal seedlings were counted. For each germination test, 4 x 100 seeds were taken. The results presented are the mean values of two samples.

Results and discussion

Data on the viability of seed samples presented in this paper are part of long-term experiments concerning the effects of storage in gaseous environments on rye seed storability. The comparison of rye seed viability after different lengths of storage period is presented in Table 1. Data for samples of moisture content 3.7 and 5.5% (fwb) stored in air or vacuums are presented. Results showed clearly the advantageous effect of vacuum storage on the longevity of rye seed samples after 10 years of storage. After 15 years of storage it was also revealed, in the case of vacuum storage, that the seed moisture content did influence seed viability during storage. Storability of seed with lower (3.5%) moisture content was better than for the sample with 5.5% moisture content. This corresponds with the generally accepted theory of long-term seed storage (Roberts 1972; Ellis and Roberts 1980). A lack of similar impact of different moisture content on the longevity of seed samples stored in air might be a result of a higher rate of seed viability decrease and might not be detected owing to the long time between viability evaluations at the third and tenth years of storage. A positive effect of vacuum and other neutral gasses used in this experiment could also be observed (Table 2). Such evident superiority of vacuum storage, however, was not observed in all studies on controlled atmosphere storage of seed (Justice and Bass 1978; Tao 1990; Roberts 1991). It is very difficult to explain the exact reason of such controversy. It might be supposed that in some cases storage parameters were not properly controlled (Justice and Bass 1978) and/or that the role of oxygen in seed physiology and biochemistry at different moisture content might differ (Roberts 1983).

Table 1. Viability of rye (*S. cereale*) seed stored in vacuum or air at ambient temperature

	Air		Vacuum	
Years of storage	**3.7%**[†]	**5.5%**[†]	**3.7%**[†]	**5.5%**[†]
0	90	94	90	94
2	75	84	76	83
3	78	76	89	88
10	31	20	81	81
15	23	20	80	58
19	17	11	67	35

[†] Seed moisture content.

Table 2. Viability of rye seed stored in different gaseous environments

	Storage gaseous environment			
Years of storage	**Air**	**Vacuum**	**CO_2**	**N_2**
0	90	90	90	90
2	75	76	80	75
3	78	76	83	81
10	31	81	81	72
15	23	80	–	–
19	17	67	49	60

In spite of the lack of a consensus about advantages of vacuum storage for long-term conservation of genetic resources (Tao 1990), it seems useful and profitable in the case of rye seeds. Vacuum storage of rye seed samples almost

doubled their lifespan under seedbank regimes as compared with air. As a cross-pollinating species, cultivated rye requires special care and additional work during regeneration, to avoid contamination. A prolongation of the storage time would reduce the frequency of regeneration and might minimize the costs of maintaining genetic resources and reduce the risk of possible genetic changes due to frequent seed sample regeneration (Breese 1989).

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Long-term storage of rye at the National Seed Storage Laboratory

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Summary

The National Seed Storage Laboratory (NSSL), US Department of Agriculture, located in Fort Collins, Colorado has been in operation since 1958 to provide long-term storage of genetic resources. Physical facilities were modernized and expanded four-fold in 1992. High-security vaults have the capacity to provide protection from natural disasters including floods, tornadoes, fires and earthquakes for about one and a half million samples. Seed samples are dried to equilibrium at about 10°C and 30% relative humidity to obtain the optimum seed moisture for long-term storage and packaged in moisture-resistant containers for conventional storage at –18°C or in plastic tubes for cryostorage over liquid nitrogen at about –160°C. The US National Plant Germplasm System collections include 1924 accessions of rye. High-quality seed samples of 1628 of these accessions were obtained from the cooperative Rye Seed Regeneration for Long-Term Storage project with the Botanical Garden, Warsaw, Poland. Of the 1881 accessions in the base collection at NSSL, 83% are in cryostorage and 17% are in conventional storage. Seed quality is excellent with 92% of the samples having above 84% germination, and 99% having >64% germination. Seed numbers are adequate with 98% of the samples having more than 1500 seeds.

Introduction

Landraces and wild relatives of crops from centres of diversity have been rich sources of resistance to new pathogens, insect pests and other stresses as well as for traits to improve food and fiber quality, animal feed and industrial products. But, as farmers in centres of diversity switch to new stress-tolerant, higher-yielding cultivars, these valuable sources of useful genes will be lost forever unless they have been collected and conserved *ex situ* in genebanks.

No country has all of the plant genetic resources required to develop and maintain a high level of agricultural productivity. The USA has an extremely limited number of native species of economic importance. As with many countries, our exceptionally productive agricultural systems were founded on introduced plant genetic resources.

Immigrants from Europe, Asia and Africa brought seed with them. In 1819 American consuls overseas were asked to collect seeds of useful plants. The US Patent Commissioner administered the introduction of plants from 1836 to 1862. The continuing need to acquire and introduce plant germplasm into the USA was one of the reasons for establishing the US Department of Agriculture (USDA). The Organic Act, of 1862, establishing the Department of Agriculture, directed the first Commissioner of Agriculture, Isaac Newton, "to collect, as he may be able, new and valuable seeds and plants; to test, by cultivation the value of such of them as may require such tests; to propagate such as may be worthy of propagation, and to distribute them among agriculturists."

Before the late 1940s, introductions were sent directly to interested scientists without any requirement that they be maintained. Adequate preservation methodologies and facilities were not available, and many accessions were lost.

The National Plant Germplasm System

The Research and Marketing Act of 1946 (Public Law 733) authorized the creation of four Regional Plant Introduction Stations (Ames, Iowa; Pullman, Washington; Geneva, New York; and Griffin, Georgia) with the mission to acquire, maintain, evaluate and distribute germplasm to scientists to be used for crop improvement. The National Small Grains Collection (NSGC), now in Aberdeen, Idaho, began in 1894 as a breeder's collection in Beltsville, Maryland. The Inter-Regional Potato Introduction Station, Sturgeon Bay, Wisconsin was established in 1947. National Clonal Germplasm Repositories were established in the mid-1980s to provide more systematic maintenance of vegetatively propagated germplasm. These repositories grow and maintain the active collections and distribute samples to scientists worldwide. The National Seed Storage Laboratory (NSSL), Fort Collins, Colorado was dedicated in 1958 as a long-term storage facility to conserve the base collection for back-up of the active collections.

These units have been integrated into a National Plant Germplasm System (NPGS) (Shands *et al.* 1989; ARS Information Service 1990). The NPGS is a network of cooperating institutions, agencies and research units in the Federal, State and private sectors. In order to increase crop productivity, improve quality, and ensure a safe and secure food and fiber system in the United States and the world, it is the mission of the NPGS to acquire, characterize, document, conserve, evaluate and make readily accessible the broadest possible range of plant genetic resources needed by research scientists. In addition to the active and base collections in NPGS, plant breeders maintain working collections of plant materials used in their programmes.

New acquisitions must be increased, characterized and conserved as part of the active collections. Each repository conducts a systematic evaluation programme to obtain specific information on disease and insect resistance, nutritional quality, agronomic and physiological attributes, and other traits of interest. Information on each accession, including passport, characterization and evaluation data, is entered in the Germplasm Resources Information Network (GRIN) database. When requested, samples are distributed to scientists worldwide at no cost for use in crop improvement and basic research. Research relating to improved methods of collecting, regeneration, propagation, conservation, evaluation and distribution is conducted, and the results are published.

The National Germplasm Resources Laboratory (NGRL) located at the Beltsville Agricultural Research Center (BARC), Beltsville, Maryland, is responsible for a number of activities that support the entire NPGS. The Plant Exchange Office, the Germplasm Resources Information Network (GRIN) Database Management Unit, and the Plant Germplasm Quarantine Office are components of the NGRL.

The Plant Exchange Office coordinates the acquisition and exchange of plant germplasm, documents passport data and descriptive information for newly acquired material and assigns unique Plant Introduction (PI) numbers, publishes an annual USDA Plant Inventory of newly received accessions, and serves as a liaison on quarantine matters. Strategies are developed for increasing the genetic diversity of US collections. Based on these strategies, gaps in current germplasm collections are identified and communicated to the appropriate Crop Germplasm Committee (CGC) or to other crop specialists for their concurrence. The NGRL facilitates the activities of the CGCs. The public and private scientists on these committees represent the germplasm user community for a particular crop or a group of crops. These committees provide crop-specific expert guidance on

germplasm needs, collection gaps, descriptors, documentation, regeneration, evaluation and research goals to various components of the NPGS. The GRIN is the official database of the NPGS. Information in GRIN is available to any plant scientist or researcher worldwide, either through direct connection to the database, through PC GRIN, through World Wide Web (http://www.ars-grin.gov), or through contact with the curator for the active collection of the crop of interest. GRIN contains data on taxonomy, origin, evaluation and characterization for plant germplasm conserved in the NPGS.

All plant germplasm entering the NPGS from outside the USA must comply with federal quarantine regulations, which are designed to facilitate the exchange of plant germplasm while limiting/preventing the movement of pathogens. Regulations are written, interpreted and enforced by the USDA Animal and Plant Health Inspection Service (APHIS).

Although the ARS components of the NPGS are administered by the Area Director for the geographic location of that component, the Associate Deputy Administrator for Genetic Resources and the National Program Leader for Plant Germplasm on the National Program Staff provide leadership for the NPGS and coordinate activities. They also provide administrative support to the various advisory councils and committees for plant genetic resources.

The NPGS maintains one of the largest *ex situ* collections of plant genetic resources in the world. A detailed report of the NPGS history, policies, and architecture is given in *Plant Breeding Reviews* (1989, ed. by J. Janick). Since 1898, about 575 000 accessions with real or potential economic importance to US agriculture have been acquired through the former Plant Introduction Office. Many of these are among the more than 433 000 accessions, representing over 9000 plant species, that are now conserved in the NPGS. Between 1986 and 1995, the NPGS distributed an average of 161 358 samples each year to US public scientists (64%), US private industry scientists (13%), foreign public scientists (8%), foreign private industry scientists (13%), and international centres and USAID (2%).

The principal mission of NSSL is to conserve the base collection of the NPGS and to conduct research to develop new and improved technologies for the conservation of seed and other plant propagules. NSSL also provides long-term storage for plant materials not in the NPGS that are not to be distributed: (1) voucher samples of cultivars and parental lines licensed by the US Plant Variety Protection Office, (2) accessions of endangered species maintained by botanical gardens, (3) quarantined samples queued for regeneration under APHIS inspection, and (4) security back-up materials from international centres and other genebanks.

Physical facilities of NSSL were modernized and expanded fourfold in 1992 (Fig. 1). High-security storage vaults have the capacity to provide protection from natural disasters, including floods, tornadoes, fires and earthquakes for nearly one and a half million samples. The insulated walls, ceiling and floor of the cold vault environmental chambers are 15.2 cm (6") thick, and moveable shelves increase capacity. The main seed vault is 1420 m^3 (14.5 x 32.6 x 3). Walters *et al.* (unpublished) emphasize that energy requirements are much less with 15.2 cm insulation and moveable shelves than with 10.2 cm of insulation and nonmovable shelves.

Minimizing genetic change during *ex situ* conservation is paramount to retain as much genetic variation as possible for future use (Crossa *et al.* 1994). A key

first step to minimize genetic change is to conserve the initial regenerated sample in the base collection. This regeneration should be done with an appropriate number of plants with the required pollen control under optimum growing conditions to produce high-quality seed. Careful processing and drying are required to maintain high viability. Storage of dry, high-quality seed at subzero temperatures can extend viability for many years before a second regeneration of the base collection is necessary. When continuing demand on the active collection occurs, seed from the base collection should be used for every second or third regeneration.

Scientists in the Plant Germplasm Preservation Research Unit (PGPRU) at NSSL focus on the development of new and improved technologies for the long-term conservation of all forms of plant germplasm. This research is expected to increase: (1) the number of species that can be stored at NSSL, (2) the longevity of the various accessions, and (3) the efficiency of viability testing of accessions. Longer storage periods and reduced number of field and/or greenhouse regeneration cycles will result in lower costs and greater genetic integrity of the germplasm.

Fig. 1. The National Seed Storage Laboratory, Fort Collins, Colorado, USA (Agricultural Research magazine, USDA).

Conservation of orthodox seeds

The technologies for conserving orthodox seeds are well understood. Seeds should be dried and stored at a low temperature (Justice and Bass 1978; Roos 1986, 1989). Research by Justice and Bass (1978), Bass (1980), and Bass and Stanwood (1978) showed that reducing the storage temperature from 5ºC to

subzero temperatures increased seed longevity from less than 10 years for some species to several decades for most species.

The ultra-low temperature of liquid nitrogen used in cryogenic storage should extend seed longevity (Stanwood 1980, 1985; Stanwood and Bass 1981). After 10 years of storage, Stanwood and Sowa (1995) reported that oxygen uptake rates and average seedling root lengths were greater for onion samples stored in the vapour phase above LN (approximately –160ºC) compared with samples stored at –18ºC. Germination percent did not change during 10 years of storage at either of these temperatures. However, major differences in germination were observed between 5ºC and the subzero temperatures.

Although seed drying extends longevity, there are limits to the beneficial effects, and the optimum moisture content varies with the chemical composition of the seed (Vertucci and Roos 1990, 1993; Vertucci *et al.* 1994; Walters-Vertucci and Roos 1996; Ellis *et al.* 1989, 1990). Drying seeds beyond a critical moisture content can result in accelerated deterioration at above-zero temperatures. Using basic thermodynamic principles, scientists at the NSSL (Vertucci 1989; Vertucci and Roos 1990, 1993; Vertucci *et al.* 1994) have established that, contrary to the viability equations (Ellis and Roberts 1980; Ellis *et al.* 1989), the effects of storage temperature and water content of seeds are not independent. Consequently, the optimum water content for seed storage varies both with the species and with the storage temperature. The thermodynamic principles used by Vertucci and Roos (1990, 1993) and Vertucci *et al.* (1994) can be used to predict optimum moisture levels for all orthodox seeds at all storage temperatures. Equilibration at about 22% RH at a specified temperature provides the optimum seed moisture for storage at that temperature for all orthodox seeds studied. Seed moisture at equilibrium will be less in seed with a greater lipid content (Walters-Vertucci and Roos 1996). The procedure of drying to equilibrium at an appropriate RH and temperature eliminates the requirement of determining the moisture content of each accession and this saves processing time.

Long-term storage of rye at the NSSL

The active collection of *Secale* is maintained and distributed by staff of the National Small Grains Collection (NSGC), Aberdeen, Idaho. As accessions are regenerated by the NSGC, seed samples are divided with part staying in the active collection and the other part deposited in the NSSL base collection.

At NSSL, seed samples are dried initially to equilibrium at about 10°C and 30% RH to obtain optimum seed moisture for long-term storage. At this temperature, the dehumidifier seldom runs to achieve 30% RH, whereas at 15°C the RH dropped below the desired 35% for this temperature because of the naturally low RH of ambient air at Fort Collins. Either of these combinations gives the same seed moisture as 5°C and 25% RH (Vertucci and Roos 1993).

Seed quality is evaluated by germination tests with two replications of 50 seeds each using standard Association of Official Seed Analysts procedures for rye. In addition, two replications are stored for 24 hours over LN before the germination tests are conducted. Results of these tests are used to determine if the seed sample should be stored in cryotanks. The seed quality data for accessions that are in the NPGS active and base collections are entered in GRIN. As the seed counts and germination tests are being conducted, a final equilibration is done at about 5°C and 25% RH (seed moisture will range from 5 to 8% depending on chemical composition). Viability is monitored periodically at NSSL depending on initial

viability (about every 15 years except for accessions with poorer initial quality that are tested more often).

After seed counts have been made, samples are packaged in moisture-resistant aluminium foil envelopes and stored in the cold vault at about –18ºC (Fig. 2) or placed in polyolefin tubes and stored in the vapour phase above LN at about –160ºC in cryotanks (Fig. 3). Samples that are substandard for germination (below 65% or if the LN-treated sample deviates from the control by 10% or more) or are substandard for seed number (below 1000 seeds) are stored in cold vaults while the accessions are queued for regeneration. Operating costs at NSSL to maintain samples at –18ºC are estimated to be about $0.04 per sample per year and about $0.14 in cryotanks.

When high-quality seed is dried to the optimum moisture content and stored at subzero temperatures, longevity of several decades can be expected (Walters *et al.* unpublished). Table 1 shows results from NSSL for rye seed received from 1958 through 1986. These results emphasize the importance of ensuring that high-quality seed is available for storage when accessions are regenerated.

The NPGS collections include 1924 accessions of rye. High-quality seed samples of 1628 of these accessions were obtained from the cooperative Rye Seed Regeneration for Long Term Storage project with the Botanical Garden of the Polish Academy of Sciences. Of the 1881 accessions now in the base collection at NSSL (98% of the total rye accessions), 83% are in cryostorage and 17% are in conventional storage. The status of the NSSL rye samples is shown in Table 2. Seed quality is excellent with 92% of the accessions having samples with >84% germination, and 99% having >64% germination. Seed numbers are adequate for most accessions with 98% having samples with more than 1500 seeds each.

Fig. 2. Retrieving a seed sample from a movable carriage in the NSSL –18°C vault

Fig. 3. Manually filling a cryotank with liquid nitrogen in the NSSL cryostorage

(Agricultural Research magazine, USDA).

vault (Agricultural Research magazine, USDA).

Core subsets

When a scientist determines that there is inadequate genetic variation in available germplasm for a desired attribute, new accessions are needed that will provide the highest probability of identifying useful source materials with minimum screening. Sometimes this can be achieved by obtaining accessions from an area where the problem has been endemic for many years, e.g. low soil pH. A list of candidate accessions can often be generated when appropriate information is in the database.

Table 1. Viability of rye accessions deposited in long-term storage[†] at NSSL from 1958 through 1996

Initial % germination	Last % germination	No. of accessions
85 – 100	***85 – 100***	***55***
	65 – 84	15
	45 – 64	8
	25 – 44	0
	1 – 24	1
	0	0
65 – 84	85 – 100	0
	65 – 84	***7***
	45 – 64	9
	25 – 44	4
	1 – 24	2
	0	0
45 – 64	85 – 100	0
	65 – 84	0
	45 – 64	***1***
	25 – 44	3
	1 – 24	4
	0	0
25 – 44	85 – 100	0
	65 – 84	0
	45 – 64	0
	25 – 44	***1***
	1 – 24	2
	0	1
1 – 24	85 – 100	0
	65 – 84	0
	45 – 64	0
	25 – 44	0
	1 – 24	***2***
	0	0

[†] Stored at about 5°C and 35% RH from 1958 through 1977; at about -18°C in moisture-resistant containers from 1978 through to present.

In other cases, especially for new pathogen strains or insect biotypes, searching database information is of little or no value. When the scientist must search within the crop collection for the desired trait, an initial screening of a diverse but smaller subset may reduce time and costs. The idea of developing such a subset was proposed by Frankel (1984) and further developed by Brown (1989a, 1989b, 1995). They suggest that "A core collection consists of a limited set of accessions derived from an existing germplasm collection, chosen to represent the genetic spectrum of the whole collection. The core should include as much as possible of its genetic diversity." The core subset is suggested to be about 10% of the crop collection, but may vary from 5% for very large collections to 50% or more for very small collections, with about 3000 suggested as a maximum number.

Brown (1989a) recommended stratified sampling methods when establishing core collections. Grouping begins with taxonomic affinity (species, subspecies, cytological races). Accessions within each taxon then can be assigned to strata based on ecogeo-graphic zones and genetic characteristics (ploidy level, photoperiod response, races, etc.). Groups such as races of maize (based primarily on ear morphology) may be preferable to country of origin for defining groups

because geopolitical boundaries often are incongruent with ecogeographic niches. In other crops, country of origin (or region of adjacent countries) may be the only available means for developing preliminary groups.

Table 2. Status of rye accessions in NSSL

			Number	%
Rye accessions in NPGS			1924	
Rye accessions in NSSL base collection			1881	98
Not tested for germination				
Low seed number			230	–
Not processed			12	–
Tested for germination			1639	
85-100%	≥1500 seeds		1482	91
	550 – 1499 seeds		19	1
	1 – 549 seeds		2	–
Subtotal			**1503**	**92**
65 to 84%	≥1500 seeds		94	6
	550 – 1499 seeds		14	1
	1 – 549 seeds		1	–
Subtotal			**109**	**7**
45 to 64%	≥1500 seeds		7	–
	550 – 1499 seeds		1	–
	1 – 549 seeds		1	–
Subtotal			**9**	**–**
1 to 44%	≥1500 seeds		16	1
	550 – 1499 seeds		0	–
	1 – 549 seeds		1	–
Subtotal			**17**	**1**
0%	≥1500 seeds		0	–
	550 – 1499 seeds		0	–
	1 – 549 seeds		1	–
Subtotal			**1**	**–**
Subtotal	≥1500 seeds		**1599**	**98**
Subtotal	550 – 1499 seeds		**34**	**2**
Subtotal	1 – 549 seeds		**6**	**–**

Development of a useful core subset may involve the following steps: (1) assembling and reviewing passport data and other information to be used in establishing nonoverlapping groups, (2) assigning accessions to appropriate groups, (3) choosing accessions for the preliminary core subset from each group, and (4) collecting data on phenotypic and genetic traits for accessions in the preliminary core and using multivariate analytical methods to construct clusters and dendrograms to elucidate systematic and statistical genetic relations for further refinement of the core subset.

When funding is available to characterize and statistically analyze the entire crop collection for several descriptors, steps 2, 3 and 4 can be conducted simultaneously. Assigning heavier weights to genetic markers and highly heritable phenotypic traits may improve clustering. Groups generated as clusters from statistical analyses of the data will usually be the most robust. If only a few descriptors were analyzed initially, additional descriptors may be measured for the preliminary core, and then step 4 repeated with data from all available descriptors. When financial resources are limiting or very large numbers of accessions must be characterized, steps 2, 3 and 4 will need to be completed sequentially.

Proportional sampling within each group may provide a more representative sample of the total genetic diversity in the core subset than would a completely random sampling from the crop collection. Once the number needed from each group has been determined, accessions for the core subset are usually chosen randomly within each group. However, some curators are choosing accessions with more desirable agronomic traits within each group.

Clusters generated by multivariate analyses may provide a better understanding of patterns of genetic divergence and diversity and will often identify ecogeographic regions that have not been adequately sampled, especially when the origin of each accession in the core is plotted geographically. This information may be valuable in planning future acquisitions.

The core collection concept has gained wide acceptance and core collections are being developed in many countries (Hodgkin *et al.* 1995; Knüpffer and van Hintum 1995). The NPGS is developing a core subset for each of the major crop collections (Erskine and Muehlbauer 1991; Holbrook *et al.* 1993; Diwan *et al.* 1994). Characterization data obtained by the Botanical Garden of the Polish Academy of Sciences during regeneration will be valuable information as the NSGC develops the rye core subset.

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Session III: Long-term storage and seed deterioration (Chair: Dr Steve A. Eberhart)

Biochemical aspects of seed deterioration during storage

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Generally ageing is manifested by the decrease of anabolic activity and the increase of catabolic processes. Several hypotheses have been formulated to explain the mechanism of ageing. One group of so-called deterministic hypotheses is based on the assumption that termination of ontogenesis is located in the genome. More recently, a stochastic hypothesis of ageing has been developed. According to that hypothesis, ageing is the consequence of the accumulation of damage in DNA visible in mutation effects and DNA dysfunction. Ageing seems to be a genetically programmed event that is finally expressed with the contribution of different external factors.

Viability and vigour of seeds during storage

Generally, maximum viability and seed vigour are reached after the final drying stage of maturation (mass maturity) and from this point gradually deteriorate until death. Usually loss of seed vigour precedes the loss of seed viability. A number of distinct but interacting determinants are known to influence seed vigour during storage. These are: genetic factors, maternal and preharvest effects, mechanical damage at harvest and handling, storage environment (particularly water content, temperature and atmosphere, intrinsic factors (changes to essential metabolites and macromolecules) and microbial infection.

Changes associated with seed ageing

Membrane changes and permeability

Release of solutes during the first hours of imbibition can be broadly correlated with ageing (Grzesiuk and Tuczkiewicz 1982). Koostra and Harrington (1969) were among the first scientists to analyze phospholipid changes and to raise the possibility that membrane peroxidative changes were associated with ageing. This subject will be discussed later. These changes were associated with viability loss from 99 to 2% in cucumber seeds subjected to accelerated ageing at 100% and 38°C over 4 weeks. Since then, many studies were carried out seeking evidence for changes in membrane phospholipids, but the picture that emerges is far from clear, especially in the case of seeds stored under ambient conditions. This is owing to the different ageing condition used by researchers, species investigated, analytical techniques and seed parts used.

Ultrastructure changes (cell destruction)

Ultrastructural examination of embryo tissues by electron microscopy confirms that deteriorative changes of membranes occur during both dry and humid storage. These changes can be summarized as follows:

- fragmentation of plasmalemma and its withdrawal from the cell
- fusion of lipid droplets to form larger bodies or irregular pools
- disintegration of mitochondrial and plastid outer and inner membranes
- deformation of nuclear envelope
- fragmentation or loss of endoplasmic reticulum and Golgi bodies
- dissolution of boundary membrane of vacuole and protein bodies
- degradation of polysomes
- occasional appearance of floccular material in the extraprotoplasmic space.

Biochemical changes

Lipid degradation

Lipids were most often studied among biochemical analyses in aged seeds. Degradation of lipids may be catalyzed by their own enzymes or by microbial enzymes. Lipase and lipoxygenase are two principal enzymes involved in the degradation of lipids in seeds. Lipase catalyzes the hydrolysis of triacylglycerols to glycerol and fatty acids. Lipoxygenase is found in oil seeds and reacts only with cis, cis-1,4-pentadiene structures, such as linoleic, linolenic or arachidonic acids that are degraded to either free acids or triacylglycerols. Lipoxygenase oxidizes polyunsaturated fatty acids to hydroperoxydes, which degrade to ketons, aldehydes, acids and other low-molecular compounds. Several of these compounds cause off-flavours and odours. These compounds can also react with proteins and amino acids, further lowering seed quality.

Changes in lipid content

A decrease in total lipid content has been sometimes reported in oily seeds during prolonged storage. Storage lipids presumably decrease as a consequence of slow metabolism under relatively humid storage conditions. The extent of this slow metabolic depletion is unlikely to threaten seed viability.

In contrast, loss of membrane lipids is probably of much greater significance than a decrease in storage lipids. During storage at high humidity, phospholipidic content (mainly phosphatidylcholine) has been noted to decline (about 50% or more) in seeds of cucumber. Decline in phospholipids was also reported in soyabean, pea, sunflower and tomato seeds (Priestley 1986; Francis and Coolbear 1987). Other reports suggest that changes in phospholipids are much milder during dry storage than with artificial ageing. Based on these observations it is believed that metabolic changes associated with artificial ageing differ from those during natural ageing.

Two suggestions have been offered to explain the decline in phospholipid level in aged seeds: the lipids may have been subjected to peroxidation or they may have been degraded by lipolytic enzymes, especially phospholipase D, an enzyme that cleaves the polar head from phospholipid to leave phosphatidic acid.

Fat acidity

Hydrolysis of triacylglicerols catalyzed by lipase liberates free fatty acids. As a consequence, the pH of aqueous seed extract is lowered. The accumulation of free fatty acids in seed tissues could be to some extent responsible for their loss of

viability. Free fatty acids have particularly deleterious effects on membranes, probably because of their ability to act as detergents.

Lipid peroxidation

In the presence of oxygen, polyunsaturated fatty acids are spontaneously oxidized, producing highly reactive free radical intermediates, a class of compounds called hydroperoxides. Initiation of the reaction involves abstraction of hydrogen (H^-) from the methylene group ($-CH_2-$) adjacent to the double bond. Following hydrogen abstraction, and after diene conjugation and addition of oxygen, a peroxy radical (LOO^-) is obtained. The latter, by reaction with another unsaturated fatty acid (LH), forms a lipid hydroperoxide (ROOH) as the primary oxidation product. Superoxide anion radicals (O_2-) have been implicated in lipid peroxidation occurring within hydrated, metabolically active systems, where they are formed as a consequence of electron transport chain. Reaction of superoxide with H_2O_2 yields the hydroxyl radical (OH^-) and singled oxygen, i.e. highly potentate oxidant. These can induce considerable destruction, particularly to large polymers and to membrane lipids. The hydroxyl radical readily abstracts hydrogen from a lipid methylene group, beginning the chain of lipid peroxidative degradation.

In stored seeds peroxidation may occur either through atmospheric oxidation or through the action of lipoxygenase. The activity of lipoxygenase is accompanied by hydroperoxide lyase that breaks down oxygenated fatty acids. It is very likely that the action of lipoxygenase is favoured at elevated seed moisture content (accelerated ageing). Parallel changes in lipoxygenase (LOX) and superoxide radical (O^-_2) production have been observed in senescing tissues. Linoleic (18:2) and linolenic (18:3) acids, released from the membrane by the action of phospholipase D and phosphatidic acid phytase, serve as substrates for LOX.

The lipid hydroperoxides may be reduced to hydroxy fatty acids, partially degraded, or undergo further peroxidation cycles. This results in the production of a variety of smaller secondary products. Oxygenated fatty acids (primarily hydroperoxide, hydroxy and epoxy fatty acids) have been reported in stored seeds. These fatty acids are likely to accumulate as nonvolatile products. When triunsaturated fatty acids (e.g. linolenic acid) are subject to lipid peroxidation the malondialdehyde is released ($OHC-CH_2-CHO$). This aldehyde may further react with proteins and nucleic acids. By GC-MS studies it was found that peroxidation of linoleic acid may result in the release of a variety of volatile products. Harman *et al.* (1978, 1982) found that deteriorated soyabean and pea seeds produced more than 20 times of volatile carbonyl products compared with nonaged seeds.

Harman and Mattick (1976) studied the accelerated ageing of pea seeds and found that decrease in germination rate was paralleled by a pronounced decline in linoleic (18:2) and linolenic (18:3) acids, whereas the saturated and monoeic fatty acids remained unchanged. This suggests that the decline in dienoic and trienoic fatty acids is consistent with oxidation of unsaturated lipids and free radical formation. However, Priestley and Leopold (1979, 1983) found evidence for such mechanism in naturally aged soyabean seeds, not in artificially aged ones. In contrast, Buchvarov and Gantcheff (1984) detected large increases in free radicals in axes of naturally and artificially aged soyabeans in an advanced stage of deterioration.

Studies of dried seeds and foods have demonstrated the important influence of water on lipid peroxidation mediated by lipid radicals (Wilson and McDonald 1986; Smith and Berjak 1995). Peroxidation (auto-oxidation) is generally facilitated at low water content. With increasing moisture, the reaction is

attenuated for several reasons: water slows the access of oxygen to the sensitive sites; by hydrating metal ions, it lowers their catalytic effectiveness; it renders chelators and antioxidants more efficient by increasing their rate of diffusion; and by hydrogen bonding it interferes with the decomposition of hydroperoxides (Kappus 1991).

Several defence mechanisms operate in seed tissues to protect them from lipid peroxidation and the production of free radicals. The major role seems to be that played by natural antioxidants such as tocopherols (vitamin E), ascorbate, glutathione and β-carotene. Tocopherols seem to be very effective in quenching both superoxide (O^-_2) and lipid peroxy radicals.

Less is known about enzymatic defence mechanisms in preventing lipid peroxidation in seeds. Superoxide dismutase is the key enzyme in many tissues, protecting against high level of superoxide radicals (O^-_2). It is absent in dry soyabeans. Its activity develops during the first hours of imbibition (Stewart and Bewley 1980). The enzyme superoxide dismutase converts O^-_2 to H_2O_2, and this in turn can be removed by catalase:

$$O^-_2 + O^-_2 + 2\ H^+ \rightarrow\ H_2O_2 + O_2$$

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

On the basis of the existing evidence, the suggestion that the longevity of stored seeds may be directly linked to superoxide dismutase levels seems rather impossible.

Lipid peroxidation may affect cellular function in several ways. First of all peroxidation of membrane lipids leads to their massive dysfunction. DNA, for example, is a subject of denaturation and translation of mRNA to proteins is hindered. Proteins are exposed to several forms of perturbation, depending upon hydration levels. The interaction of lipid hydroperoxides and proteins at relatively high moisture content leads to protein-protein crosslinking. Lipid-protein adducts are frequently formed via both hydrogen bonding and covalent linkages. Aldehydes, as lipid breakdown products, tend to form covalent links with proteins in "browning" reactions.

In spite of much work, it is still difficult to define the role of lipid peroxidation in seed ageing, and satisfactory understanding of the importance of free radicals in deterioration is even more elusive. Seed lipids do tend to undergo peroxidative degradation in long-term storage, but not always before viability is lost. Furthermore, when seeds age under conditions of high humidity, they may or not exhibit signs of lipid peroxidation.

Changes in enzymes and reserve substances

Loss of viability is not usually accompanied by any dramatic changes in storage reserves. Often hydrolysis of these compounds under unfavourable storage conditions is a consequence of fungal attack secreting egzoenzymes. In some cases, however, activation of certain endogenous enzymes might occur and limited breakdown of reserves occur (Bewley and Black 1982).

Seed deterioration during storage can result in marked changes in the content and activity of enzymes capable of degrading the stored reserves. In nonviable seeds such enzymes are not synthesized *de novo*, although activity of preformed enzymes can remain for many years after germinability has been lost. For

example the activity of proteinase, β-amylase, phosphatase and catalase may decrease slowly, whereas peroxidase and dehydrogenases usually decline rapidly and correlate to the loss of viability.

Proteolysis has not been widely linked to seed ageing, although a few studies showed that declining levels of protein in embryo and endosperm were related to loss of viability in long-term storage (Grzesiuk and Kulka 1971; Kulka 1971; Górecki 1986). Some authors revealed changes in the electrophoretic patterns and solubility of proteins extracted from aged seed tissues (Priestley 1986; Dell'Aquilla 1994). Changes in protein structure during storage may arise from interaction with lipid peroxidation products, polymerization, etc.

There are many deteriorative reactions in biological systems that involve carbonyl-amine reactions of which Maillard reaction is the best known. This is a nonenzymatic browning reaction occurring at low water content between carbonyl groups of carbohydrates and the amino groups of aminoacids and proteins. Recent data have suggested a role for such sugar-protein interaction in soyabean seeds during accelerated ageing (Wettlaufer and Leopold 1991). Maillard reactions have potentially devastating effects on proteins.

It is reasonable to assume that the chemical composition of seed determines its storability in dry storage. Lately attention has been paid to the seed oligosaccharides, i.e. sucrose, raffinose, stachyose and verbascose. Raffinose content declines during deteriorative changes in some seeds (Bernal-Lugo and Leopold 1992). Recent data have led to the assumption that oligosaccharides (mainly raffinose) induce the formation of glassy state in seeds as the water content is decreased (Blackman and Leopold 1993; Bernal-Lugo and Leopold 1995). It is argued that a glassy state contributes to desiccation tolerance in seeds and provides protection against deterioration (Blackman and Leopold 1993). Sugar alcohols (cyclitols and galactosyl cyclitols) may play a similar role. It is suggested that seed storability is a reflection not of the total soluble sugar content, but of the ratio of sucrose to oligosaccharides. Orthodox seeds of species with a sucrose-to-oligosaccharide ratio of <1.0 have half-viability periods >10 years, while those >1.0 have a storability half-viability period <10 years (Horbowicz and Obendorf 1994).

Respiration and ATP production

Age-induced alterations in stored seeds are ultimately revealed as metabolic deficiencies during germination. The respiratory characteristics of deteriorated seeds during imbibition have received particular attention. The activity of mitochondria in viable embryos and axes increases with time after the start of imbibition. Unaged dry seeds contain very low levels of ATP, but during the early hours of imbibition there is a rapid increase in ATP. The accumulation of ATP and development of energy charge in seeds during imbibition is a function of deterioration. Low rates of oxygen uptake are usually observed in aged embryo and axis tissues (Priestley 1986). Depressed rates of oxygen consumption have usually been ascribed to mitochondrial deterioration. The rate of oxygen consumption during imbibition of aged seeds may be depressed by two factors: direct lesions in mitochondrial structure and sluggish mitochondrial development.

Our studies indicate an imbalance between glycolysis, TCA and respiratory chain during the imbibition of aged seeds (Górecki *et al.* 1985, 1992). As a consequence anaerobic respiration is enhanced, resulting in high production of ethanol and acetaldehyde.

Chromosome aberrations and damage to the DNA

Chromosome aberrations are the evidence of the major genetic damage in deteriorated seeds. Usually there is a good correlation between viability loss and chromosome damage in seeds, over a fairly wide range of storage conditions. Such studies, made on radicle tips, involve the number of abnormal mitotic figures as a percentage of all those occurring. Aberrant cells may be eliminated during seedling growth, but minor genetic damage in the form of recessive mutation may persist. These may be manifested in pollen abortion, chlorophyll mutants, anomalous leaf shapes, chlorotic spots and abnormal branching. Many observations indicated that cleavage of DNA by DNAse inhibitor appeared to have taken place during prolonged seed storage. Repair enzymes such as DNA ligases may also be inactivated during seed storage (Bewley and Black 1982; Smith and Berjak 1995).

Changes in RNA and protein synthesis

Protein synthesis is reduced in imbibing aged seeds. In embryos limited breakdown of 18S and 25S subunits with seed age have been well documented. Structural deficiencies in rRNA has been attributed to RNAse activity during storage. In addition to modifications in rRNA, there are reports that the electrophoretic properties of ribosomal proteins from embryos are altered with age. However, the ribosomes are still partially functional even in severely aged seeds, although their synthetic capacity may be reduced. Analysis of the postribosomal supernatant obtained from dry nonviable embryos showed that elongation factor 1 was almost without activity. Additionally, there is a decrease in rRNA transport out of the nucleus to the cytoplasm. There are indications that long-lived mRNA is lost to some extent during extended storage. Besides loss of activity of the long-lived mRNA, some authors reported a decline in all classes of newly synthesized RNA that occurred in parallel with a decline in protein synthesis during imbibition (Priestley 1986; Smith and Berjak 1995).

Recent hypothesis for deterioration of orthodox seeds

From the data presented above we can conclude that structural, compositional and functional changes accompany seed deterioration. They occur with and without the involvement of enzymes. Taking this into consideration we should realize that ageing is a multicausal phenomenon (Smith and Berjak 1995). In other words, ageing of orthodox seeds is the sum of all deteriorative events. Several hypotheses have been proposed to elucidate the mechanism of seed ageing. Among them, the theory of lipid peroxidation and the involvement of free radicals has been widely accepted. However, there is some evidence that such an explanation of seed deterioration cannot be universal owing to the variety in seed ontogeny, structure, chemical composition, storage conditions and different experimental approaches of researchers.

Smith and Berjak (1995) proposed an interesting hypothesis for the deterioration of orthodox seeds. Their idea is borrowed from food chemists and is based on the realization that both enzymatic and nonenzymatic reactions are substantially influenced by the extent and the nature of water binding in seeds (Fig. 1). It is proposed that certain molecular events that can be associated with hydration level give information about thermodynamic, structural and motional aspects of enzymatic and nonenzymatic components which collectively contribute to loss of viability. Smith and Berjak's proposal takes into account three zones of

water content and activity: I (0-20% RH, 0-8% water content in seeds), II (20-85% RH, 8-22% water content in seeds, and III (85-99% RH, 24-34% moisture content). With the increase of moisture content the water becomes less tightly bound to protein molecules and this induces hydrogen exchange rate to reach a maximum. The onset of enzyme activity in zone II occurs when there is sufficient water to enhance catalytic activity. Figure 1 also shows the level of lipid peroxidation in lettuce seed as a function of time and humidity. There is evidence that Maillard reactions are active in dry systems, resulting in highly devastating potential to seed proteins.

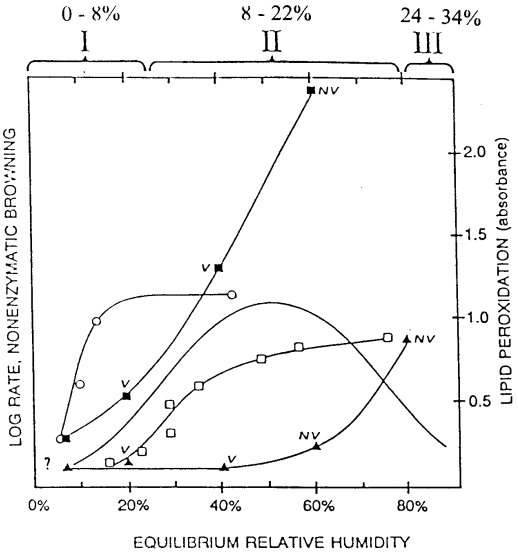

Fig. 1. The model describing interrelationship among storage conditions, water-binding properties, enzyme activity, the Maillard reaction, and lipid peroxidation, which collectively contribute to the deterioration of orthodox seeds (lettuce seeds, after Smith and Berjak 1995).

○—○	effect of hydration on the log rate of peptide hydrogen exchange for lysozyme
□—□	enzymic activity (log v_0) and rotational relaxation of an electron spin resonance (log t^{-1})
S — S	Maillard reactions
■—■	lipid peroxidation in seeds stored at 30°C for 17 and 30 months
V	viable seed
NV	nonviable seed
I, II, III	zones of water content (water activity)

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Observations of chosen morphological traits in rye accessions in relation to long-term seed storage and regeneration

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Summary

Selected morphological characters were observed for plants of nine rye (*Secale cereale* L.) cultivars. Each cultivar was represented by seed samples of different viability (5-95%) due to long-term storage in a seedbank. During the experiment, seed samples were regenerated under field conditions for 3 years consecutively, during which morphological observations were collected. Observations were carried out on at least 60 individual plants for each population on open experimental plots. Data on ear emergence, underflag leaf length, plant height, ear length and 1000-kernel weight were recorded. Significant differences among samples of different initial viability were noticed only for plant height, but not for all investigated cultivars. The observed differences were preserved in spite of three regeneration cycles. It seems that reduction of seed viability during storage is accompanied by genetic changes, reflected in differences in plant height. It might be assumed that such phenomena could be related to a genetic shift in heterozygous rye population.

Introduction

Despite optimal storage conditions, long-term seed storage in a seedbank is accompanied by natural seed ageing processes. This could induce undesirable genetic changes in the conserved germplasm, especially mutations (Roberts 1973, 1991; Dourado and Roberts 1984; Murata *et al.* 1981). In heterogenous rye populations, as with allogamous rye, other genetic changes might be induced. Different longevity of individual genotypes might cause a selection effect during field regeneration of stored accessions (Roberts and Ellis 1983; Roos 1984a, 1984c, 1988). Also the genetic structure of regenerated populations might be altered due to natural selection, gene erosion and too small a population size which causes genetic drift (Roos 1984b, 1988; Breese 1989).

Regeneration might create more difficulties for cross-pollinated species with poor storability such as cultivated rye (*Secale cereale* L.). It seems to be very important for long-term storage practices to estimate the optimal viability level assuring the lowest level of possible genetic changes which is required for conservation of the primary genetic diversity of germplasm populations stored in genebanks.

The aim of the studies presented here was to compare chosen morphological traits of the populations representing progenies of seed samples of variable viability. Results were evaluated to study the effect of natural ageing and multiple regeneration on genetic stability of rye accessions stored in a seedbank. Four characters selected from a list of rye descriptors were used for observations. Observations were made on plants growing from seed of initial samples, as well as on three subsequent generations.

Materials and methods

The Botanical Garden of the Polish Academy of Sciences, owing to its large collection of rye germplasm, possesses a set of seed samples representing extremely different levels of viability due to natural ageing occurring during long-term storage in the seedbank. For this study the seed samples of nine rye cultivars originating from different geographical regions were chosen. Each accession was represented by a number of samples with viability varying from 5 to 97% germinability. These samples (listed in Table 1) were investigated as initial samples. For three consecutive years the seed sample was regenerated under field conditions on experimental plots of 1.5 m^2 area. In total, 350 seeds were sown at a sowing density of 2.5 x 10 cm (Molski *et al.* 1982). During flowering the whole plot was isolated with a cotton/linen cage. At full maturity ears were cut off manually, threshed in the laboratory and cleaned manually. When necessary seed samples were dried and stored in cotton bags above silica gel in glass desiccators.

Table 1. Rye samples used in the experiment

Cultivar name	Origin of sample†	Sample code	Seed viability as germinability (%) 1988	1993
Bonel	USA	1.0	48	21
		2.0	56	36
		3.0	90	78
Castelo Branco	PRT	1.0	28	–
		2.0	73	55
		3.0	85	77
		4.0	97	85
Ceske Normalni	CSK	1.0	28	12
		2.0	48	24
		3.0	76	64
		4.0	91	72
Da⊠kowskie Selekcyjne	POL	1.0	13	–
		2.0	51	34
		3.0	61	41
		4.0	96	79
Da⊠kowskie Z⌀ote	POL	1.0	5	1
		2.0	45	37
		3.0	68	42
		4.0	97	78
Kastoria	GRC	1.0	40	23
		2.0	54	40
		3.0	79	47
		4.0	97	78
Kharkovskaja	SUN	1.0	38	25
		2.0	57	28
		3.0	75	66
		4.0	90	76
Lungauer Tauern	AUT	1.0	18	–
		2.0	26	–
		3.0	77	63
		4.0	94	81
Lvovskaja	SUN	1.0	7	–
		2.0	53	26

3.0	79	70
4.0	87	73

† AUT=Austria, CSK=Czechoslovakia, GRC=Greece, POL=Poland, PRT=Portugal, SUN=Union of Soviet Socialist Republics, USA=United States of America.

Morphological observations were carried out on individual plants, growing on open 4-m^2 experimental fields. Seeds were sown at a density of 25 x 25 cm, resulting in 75 plants per plot. If necessary, in the case of seed samples with a low viability, to maintain equal density of the plants on the plot, young seedlings germinated in a greenhouse were planted in the field. During the vegetation period the following morphological traits were recorded:

- date of ear emergence (when 75% of plants were heading)
- length of underflag leaf (at heading time)
- plant height (at full maturity stage)
- length of ear (at full maturity stage)
- 1000-kernel weight.

Results were statistically tested with the ANOVA procedure using the STATGRAF programme for PC.

Results and discussion

Field observations of the chosen morphological characters revealed visible intrapopulation variability of the rye populations studied. It was related to the heterozygous, cross-pollinated character of rye (Wojcieska 1983). A significant influence of climatic conditions during the vegetation period on the measured traits was also observed. Among the observed morphological traits, characters such as the spike length, length of underflag leaf, and 1000-kernel weight did not reflect any significant effect of decreased seed sample viability or multiple regeneration on the genetic stability of the rye population. Only one observed character (plant height) showed clear differences between investigated populations of rye cultivars. Similar observations were made concerning the usefulness of observation of morphological characters of rye cultivars for identification purposes (Tinnman and Stewart 1979).

In the presented experiment, differences in mean values of plant height were not observed for all investigated cultivars, but only in four of nine cultivars. Results obtained for the initial samples are presented in Table 2. Decrease of seed viability resulted in the lowering of derived plant height, if observed. It concerned especially the samples with the lowest viability. This may be assumed to be an effect of decrease in seed and/or plant vigour. However, it was not noticed for all studied cultivars, in spite of similar viability levels. What seems more important is that significant differences among plants growing from seed samples with varying initial viability were retained after three reproduction cycles (Table 3). In the case of the cultivar Dańkowskie Selekcyjne, selection toward taller plants was observed.

It seems that the reduction of seed viability is accompanied by genetic changes reflecting differences of plant height. These changes remained throughout three cycles of regeneration. Observed changes in plant height were observed for the lowest level of seed sample viability. It might be assumed that this effect was related to genetic shift in heterozygous rye populations. Genetic shift in the same samples of rye was observed for isozyme loci (Puchalski 1993).

Table 2. Plant height for initial rye samples

Cultivar name	Sample code	Average plant height (cm)	
Bonel	1.0	182.2	*
	2.0	182.1	*
	3.0	179.1	*
Castelo	1.0	166.6	*
branco	2.0	188.2	**
	3.0	186.1	*
	4.0	191.6	*
Ceske	1.0	167.7	*
normalni	2.0	169.7	*
	3.0	175.8	*
	4.0	179.0	*
Da⊠kowskie	1.0	171.3	*
Selekcyjne	2.0	180.1	*
	3.0	177.7	*
	4.0	180.0	*
Da⊠kowskie	1.0	156.3	*
Z⌀ote	2.0	164.4	*
	3.0	–	
	4.0	167.0	*
Kastoria	1.0	191.7	*
	2.0	197.9	*
	3.0	189.0	*
	4.0	198.5	*
Kharkovskaja	1.0	171.4	*
	2.0	177.0	**
	3.0	182.3	*
	4.0	180.5	**
Lungauer	1.0	175.2	*
tauern	2.0	179.4	**
	3.0	179.6	***
	4.0	185.0	***
Lvovskaja	1.0	182.1	*
	2.0	186.3	*
	3.0	185.9	*
	4.0	184.1	*

* Homologous group.

Table 3. Plant height after three regeneration cycles of rye samples

Cultivar name	Sample code	Average plant height (cm)	
Bonel	1.3	202.9	*
	2.3	195.9	*
	3.3	194.7	*
Castelo	1.3	180.5	*
branco	2.3	203.3	*
	3.3	195.6	*
	4.3	200.7	*
Ceske	1.3	192.9	*
normalni	2.3	194.6	*
	3.3	197.2	*
	4.3	202.3	*
Da⊠kowskie	1.3	195.3	*
Selekcyjne	2.3	181.8	*
	3.3	186.4	*
	4.3	175.0	*
Da⊠kowskie	1.3	164.4	*
Z⌀ote	2.3	175.4	**
	3.3	170.8	*
	4.3	196.4	*
Kastoria	1.3	201.5	*
	2.3	196.2	*
	3.3	202.3	*
	4.3	202.3	*
Kharkovskaja	1.3	158.7	*
	2.3	190.5	*
	3.3	193.2	*
	4.3	194.4	*
Lungauer	1.3	188.9	*
tauern	2.3	187.3	*
	3.3	182.3	*
	4.3	186.0	*
Lvovskaja	1.3	191.8	*
	2.3	184.3	**
	3.3	186.9	**
	4.3	192.5	*

* Homologous group.

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Isozyme loci as markers of genetic changes in rye cultivars in relation to long-term seed storage and regeneration

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Introduction

The caryopses of rye in comparison with other crops exhibit relatively poor storability. Cross-pollination of cultivated rye also creates extra difficulties for its germplasm conservation. Therefore rye genebanks are interested in the maximal prolongation of the storage period of rye seeds. It is necessary to estimate the minimal level of seed viability which could secure the primary genetic diversity of the conserved rye germplasm.

It is known that seed ageing is accompanied by genetic deterioration such as chromosomal mutations (Abdalla and Roberts 1968; Murata *et al.* 1981; Sawicka and Sadowska 1990) or phenotypic mutations (Abdalla and Roberts 1969; Dourad and Roberts 1984). Mutation was the most often observed effect in aged orthodox seeds. However, it was noted that after seed regeneration the high percentage of plants with chromosomal mutations was removed (Sawicka and Sadowska 1990). Genetic shift as a selection effect was also noted in aged seeds (Roos 1984; Stoyanova 1991).

The aim of the present studies was to analyze the genetic changes in rye seeds induced by natural ageing during long-term storage in a seedbank and after regeneration in field conditions, by means of isozyme markers. Different isozymes were used for the genetic studies on rye populations in the years 1972-90. They were the most commonly used genetic markers in various genetical laboratories (Jaaska 1972; Ramiréz and Pisabarro 1985; Benito *et al.* 1990).

Material

For these studies a set of seed samples of cultivated rye (*Secale cereale* L.) represented by two cultivars – Dańkowskie Złote and Českе Normalni, originating from the seedbank of the Botanical Garden of Polish Academy of Sciences – was used.

The analyses were carried out on the plants from the first generation of reproduced seeds derived from three series of samples: high viability (series 4.1), intermediate viability (series 2.1) and low viability (series 1.1). They were compared with initial samples with a germinability higher than 97%. The primary seed samples were stored at ambient temperature (5-18°C) over silica gel and seed moisture content was about 7-8%. After 4 or 7 years of such storage the germination ability was still 97%, but after 12 or 14 years of storage natural ageing caused evident decrease of viability (Table 1). Later they were regenerated in the field.

Methods

The genetic deterioration in such regenerated seed samples was estimated in the populations of young seedlings by means of isozyme loci. In each population 100-120 single plants were analyzed. In total, nine enzyme systems were used for the studies. Their activity was detected in the leaves of young green seedlings.

Isozymes were separated by means of starch/slab technique in two buffer systems. More details on the applied technique are given in Puchalski (1993).

Table 1. Analyzed samples of rye seeds

Cultivar	Sample code	Storage period of initial seed sample (years)	Germination ability (%)	
			Initial sample after storage	Regenerated sample
↓eske Normalni	CN 4.0	7	97	–
	CN 4.1	7	97	98
	CN 2.1	12	48	96
	CN 1.1	14	28	97
Da⌧kowskie Z⌀ote	DZ 4.0	4	97	–
	DZ 4.1	4	97	98
	DZ 2.1	12	45	97
	DZ 1.1	14	5	96

The genetic structure of populations was estimated on the basis of allele frequencies in six chosen polymorphic isozyme loci: Dia-3, Pgi-2, Aat-3, Cpx-4, Mdh-2 and 6-Pgd-2. Later the indices of allozyme and genotype polymorphism, gene diversity and heterozygosity were calculated.

Results and discussion

The results indicated that the reduction of the viability of rye seeds was reflected in the regenerated samples as various genetic changes. Generally, rye plants originated from aged seed of very low viability showed decreasing frequencies of isozymes and allozymes in the plant populations. It was also observed that in the progenies of seed lots with low viability, higher gene diversity and loci heterozygosity indices were estimated (Puchalski 1993). It was especially visible in two loci: Pgi-2 and Mdh-2, where overdominance, calculated according to Hardy-Weinberg's law was discovered (Table 2).

Table 2. Heterozygosity (H) in various enzyme loci in populations of analyzed samples of two rye cultivars according to Hubby and Lewontin (1996) (The expected heterozygosity values from Hardy-Weinberg's law are given between brackets)

	cv. ⑦eske Normalni			
Locus	**CN 4.0**	**CN 4.1**	**CN 2.1**	**CN 1.1**
Dia-3	0.39 (0.41)	0.45 (0.37)	0.18 (0.26)	0.39 (0.37)
Aat-2	0.08 (0.13)	0.10 (0.26)	0.06 (0.08)	0.14 (0.24)
Pgi-2	0.53 (0.50)	0.66 (0.50)	0.72 (0.49)	0.81 (0.50)
Mdh-2	0.35 (0.37)	0.45 (0.39)	0.35 (0.39)	0.42 (0.39)
6-Pgd-2	0.21 (0.34)	0.22 (0.44)	0.15 (0.37)	0.23 (0.42)
Cpx-4	0.17 (0.17)	0.27 (0.26)	0.13 (0.13)	0.06 (0.06)
Mean	0.288	0.358	0.265	0.342
	cv. Da⌧kowskie Z⌀ote			
Locus	**DZ 4.0**	**DZ 4.1**	**DZ 2.1**	**DZ 1.1**
Dia-3	0.23 (0.20)	0.18 (0.20)	0.17 (0.15)	0.10 (0.10)
Aat-2	0.09 (0.28)	0.16 (0.50)	0.22 (0.32)	0.19 (0.31)
Pgi-2	0.66 (0.50)	0.72 (0.50)	0.80 (0.50)	0.69 (0.49)
Mdh-2	0.39 (0.41)	0.33 (0.35)	0.44 (0.41)	0.56 (0.49)
6-Pgd-2	0.24 (0.43)	0.23 (0.42)	0.34 (0.31)	0.28 (0.41)
Cpx-4	0.08 (0.10)	0.11 (0.11)	0.19 (0.18)	0.11 (0.15)
Mean	0.282	0.288	0.360	0.322

These observations proved that natural selection was a primary reason of the genetic changes in rye caryopses originated during long-term storage due to seed ageing and regeneration. As a result of such actions the genetic shift effect was found. It was the first evidence for genetic shift effect in relation to natural seed ageing. Earlier the genetic shift was described due to accelerated ageing (Roos 1984; Stoyanova 1991, 1992). It is also possible that genetic drift was the other cause of such changes. But it should be concluded that natural selection occurring during rye seed storage and regeneration is the most significant factor promoting distinct changes of rye germplasm conserved in the seedbank. It was inherited by the multiplied samples in spite of the full regeneration of their viability.

This fact gives new indications for seedbank management in the case of regeneration of strongly aged seed lots. The reproduction is satisfactory only for the restoration of full viability, but the genetic structure of plant population originated from seed lots of low viability might be definitely changed. In the case of allogamous rye the increase of heterozygosity level due to selection acting on ageing seeds was the most significant effect.

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Preliminary molecular studies on genetic changes in rye seeds due to long-term storage and regeneration

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Summary

The use of the Randomly Amplified Polymorphic DNA technique for studies of the occurrence of genetic effects in populations of rye (*Secale cereale* L.) induced by natural seed ageing and regeneration was investigated. In the experiment only viable seeds of three initially genetically identical samples were used: (a) seeds after 14 years of storage with viability decreased to 5% due to natural ageing, followed by one reproduction cycle (DZ1.1 series); (b) as above but after three reproductions (DZ1.3 series); (c) seeds with initial viability circa 95% followed by one reproduction (DZ4.1 series). Genomic DNAs were isolated from 75 seeds of each sample according to the SDS procedure. Polymerase chain reactions (PCR) were accomplished by means of five primers of our own synthesis which were ten nucleotides long each. Amplification products were separated electrophoretically on agarose gels and stained with ethydium bromide. One hundred and ten RAPD markers shared among series were generated and analyzed with Pharmacia LKB The Discovery Series™ system. The significance of the total variability of the three populations DZ4.1, DZ1.1 and DZ1.3, as well as the significance of comparisons DZ4.1 vs. DZ1.1 and DZ1.1 vs. DZ1.3, were checked by the standard χ^2 test. Fifty markers were statistically significant among samples. The DZ1.1 in comparison with the control DZ4.1 sample displayed the same number of increased (14) and reduced (14) frequencies of RAPD markers, demonstrating inheritance of genetic changes by the DZ1.1 population and possibly reflecting the presence of genetic shift. However the nature of the changes was not the subject of our study. Their exposition by means of the RAPD technique suggests the existence of 'hot spots' which could result from their presence within the genome due simply to ageing or reproduction of aged genomes. Amplification of the DNAs isolated from the several times reproduced DZ1.3 sample, in comparison with DZ1.1, resulted in increased (17) and decreased (18) frequencies of the markers. These changes can be attributed to genetic shift and/or drift. Moreover genome instability also should be taken into account. Our data demonstrate that comparison of marker frequencies obtained from individual seeds of initially genetically identical populations could be useful for the investigation of effects connected with ageing and reproduction of outcrossing species, and that the standard χ^2 test allowed analysis of the results in the background of intrapopulation polymorphisms.

Introduction

Sociological and political changes in the last decade led to alterations in the structure of agricultural crops and resulted in "rapid erosion of genetic variability" (Roos 1988). Those were the main prerequisites for organizing seedbanks. However, long-term storage may itself introduce changes into the genetic pool.

There are three major effects faced during ageing and reproduction of plants. Roos (1982, 1984) distinguished induced genetic alterations due to loss of viability causing natural selection. De Pace *et al.* (1982) stressed the role of genetic drift, originated from the population's size. Generally the genetic changes increase, and on the other hand, genetic drift decreases the level of population variability. Some phenotypically detected chlorophyll mutations and chromosomal aberrations at different cell deviation stages have been described (Rao *et al.* 1987; Roberts 1988). Both were rarely inherited and usually eliminated during plant growth (Murata *et al.* 1984). On the other hand it is known that with ageing the total number of unrepaired DNA single-stranded breaks grows (Osborne *et al.* 1980). Potentially these could be the regions responsible for genome changes. However the methods used for the investigation of such events, based on protein markers, seem to be insufficient.

Possible resolution of the problem mentioned above might be the use of sophisticated polymerase chain reaction (PCR) procedures, especially those based on random primers. Randomly Amplified Polymorphic DNA (RAPD) technique is highly sensitive to changes within complement DNA-primer complex (Williams *et al.* 1990). If such alterations occur during ageing within predominant DNA sites, or even if they are statistically dispersed throughout the entire genome but some kind of evolutionary pressure is stressed, one can suspect the appearance of 'hot spots' which could be 'captured' by comparison analysis of DNA fragments derived from control and treated samples. Moreover, in the case of allogamous species, to eliminate the influence of intrapopulation polymorphisms and detect changes resulting from ageing and reproduction, the frequencies of markers derived from individuals of the populations under study should be compared.

We were interested to see whether RAPD markers obtained from individual plants of initially genetically identical sample populations could be applied to the investigation of effects amenable for genome alterations inherited by reproduction of long-term stored rye seeds.

Material and methods

Seed and seed treatment

Rye seeds (*Secale cereale* cv. Dańkowskie Złote (abbreviation DZ)) originated from the Seed Bank of the Botanical Garden of the Polish Academy of Science. Three kinds of initially genetically identical populations were used: (a) seeds after 14 years of storage under controlled conditions in desiccators over silica at 10°C with moisture content 6-8% and viability before reproduction as low as 5%, followed by one reproduction cycle (DZ1.1 series); (b) as above but reproduction was repeated for three consecutive years (DZ1.3 series); (c) seeds with initial viability circa 95% after one reproduction cycle (DZ4.1 series). Individual seeds were cut into two pieces with a scalpel. The halves with germ were used as viability controls in germination tests carried out on moistened filter paper in a germination cabinet at 20°C in the darkness for 7 days. The second halves of those which germinated were then employed for total DNA extraction.

DNA extraction

The total DNAs from 75 separate, but only viable, seed halves per set were isolated according to the SDS procedure[1] (McDonald *et al.* 1994) followed by RNase treatment.[2] DNA quantity and concentration were determined spectrophoto-metrically (GeneQuanta, Pharmacia LKB). DNA integrity and RNA impurities were analyzed on an agarose gel in the presence of the λDNA digested with restriction endonuclease *PstI*. For routine works standard dilutions of 10 μg / ml were used.

Oligodeoxyribonucleotide synthesis and purification

Oligodeoxyribonucleotides of arbitrary sequence (Table 1) were synthesized on a 0.2 μM scale according to standard phosphoramidite scheme by means of DNA / RNA Synthesizer Model 394 (ABI) and purified by the HPLC technique on Reversed Phase in linear (0-40%) acetonitrile gradient for 60 minutes (Pharmacia LKB) resulting in 95% purity products.

Table 1. Sequences of primers

Code	(5' ® 3' direction)
A14	CAG GCC CTT C
B7g4	GGT GAC GCA G
B7t	GGT GAC GCA T
C83	TGG ACC GGT G
2624	CGC CCC CAG T

RAPD optimization and protocol

Components of the RAPD reaction mixtures, namely concentrations of magnesium ions (1.5-2.5 mM $MgCl_2$), primers (0.1-0.4 μM), dNTPs (100-400 mM) and DNAs (10-80 ng) varied and were optimized according to a modified Taguchi's approach (Cobb and Clarkson 1994). Bulk mixes containing all except DNAs and *Taq* DNA polymerase were prepared. After the DNAs of each set were combined with the appropriate amount of bulk mixes, 0.5U of AmpliTaq DNA Polymerase (Perkin Elmer) was added to each reaction tube. Thermocycling was performed in a final volume of 25 μl in GeneAmp System 9600 Thermocycler (Perkin Elmer), using a profile described elsewhere (Cobb and Clarkson 1994). The amplification products were analyzed by means of electrophoresis in 1% agarose gels (Serva, electrophoresis grade) containing 0.5 μg ethydium bromide per ml in 1x TBE buffer (Sambrook *et al.* 1989).

Computer analysis of electrophoretic images

Photographs of electrophoretic images of the PCR products, obtained by means of the same primer and reflecting polymorphisms of the given seed population (DZ1.1, DZ1.3 or DZ4.1), were combined, and, after scanning with Pharmacia LKB system (The Discovery Series™) analyzed by means of the Diversity One™ for

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1 The procedure was scaled for 20 mg of initial material. This reflects the average weight of a single half seed.

2 Ten units of RNase were used. Hydrolysis was performed according to supported certificate.

3 Also available from Operon BioTechnology or University of British Columbia.

Polymorphic Systems software. After within- and among-population RAPD markers arrangements, digital data were transferred to a calculation package using '0-1' (absence-presence) matrices forms. Frequencies of '1's for all markers (except null data markers) were calculated. Significance of the total variability of the three populations DZ4.1, DZ1.1 and DZ1.3, as well as significance of comparisons of DZ4.1 vs. DZ1.1 and DZ1.1 vs. DZ1.3, was checked by the standard χ^2 test (Table 2).

Results

SDS extraction from single seed halves of only viable rye seeds provided about 10 μg of nucleic acids. DNA integrity and RNA contaminations were checked electrophoretically. Samples were obtained by mixing about 0.15 μg of each extract prepared from 75 single seed halves. Weak bands of high molecular weight DNA products were detected. Any DNA deterioration was noticed after ethydium bromide gel staining. If probes were not degraded with RNase then smearing was seen along the lines and the lower molecular weight RNA band constituted the major part of the probe (not shown).

Total genomic DNAs were then successfully applied to polymerase chain reactions with ten nucleotide long primers of arbitrary sequences of our synthesis. Mathematical optimization of magnesium ion, dNTPs and primer concentrations and total DNA amount proved to be helpful in obtaining reproducible RAPD profiles. Developed and ethydium bromide stained agarose gels were registered with Polaroid film for data processing with The Discovery Series™ system.

Computer analysis of all RAPD profiles allowed detection of 110 markers for all five primers used. They were present in all investigated populations. Only in the case of 50 (39) of them[4] were changes of frequencies statistically significant.[5] Four of them being significant for all populations displayed no changes between the DZ4.1 – DZ1.1 and DZ1.1 – DZ1.3 pair simultaneously. In the case of DZ4.1 –DZ1.1 28 (22) and DZ1.1 – DZ1.3, 35 (26) markers were statistically significant. In the DZ1.1 population frequencies of 14 (12) markers increased and 14 (10) decreased in comparison with the DZ4.1 series. Comparison of the DZ1.1 and DZ1.3 populations showed that 17 (12) of the statistically significant genetic markers were more frequent in the DZ1.3 and frequencies of 18 (15) were reduced. Frequencies of 16 markers of the DZ1.1 series underwent simultaneous changes in opposite directions in comparison with other populations. Eleven (9) of them were bigger in the DZ1.1 than in the DZ4.1 and 6 (5) less than in the DZ1.3 series. Changes in the same direction were not noticed (see Table 2).

Discussion

Shatters *et al.* (1995) successfully used the RAPD technique for investigation of DNA changes induced by ambient and artificial seed ageing on total DNA probes from soyabean (*Glycine max* L.). It was found that seed ageing resulted in the appearance and disappearance of some markers. However the nature of genome alterations was not known; it was supposed that in DNA hot spots must have

[4] If two numbers follow one another then the first refers to significance level a=0.05 and the second (in parentheses) to a=0.01.

[5] Frequencies of markers which were significantly different in three populations are represented in Table 2; single arrows indicate changes significant at a=0.05, double arrows at a=0.01.

been present, which presumably underwent changes. It was also suggested that those hot spots could result from single- or double-stranded DNA breaks accommodated during ageing. Thus, alterations induced by ageing were not statistically

Table 2. Frequencies and alterations of the statistically significant RAPD markers

		DZ4.1		DZ1.1		DZ1.3
Primer	**N**	**frequencies**	**changes**[†]	**frequencies**	**changes**	**frequencies**
dC8	1	0.279	↓↓	0.222	↑↑	0.321
	2	0.488	↓↓	0.288	↓	0.125
	3	1.000	↓↓	0.800		0.732
	4	0.534		0.488	↓↓	0.017
	5	0.465		0.666		0.821
	6	0.093		0.111	↑	0.303
	7	0.209		0.355	↓↓	0.017
	8	0.162		0.066	↑↑	0.357
	9	0.186		0.333	↑	0.535
	10	0.767		0.666	↓	0.464
d262	11	0.145	↓	0.03	↑↑	0.262
	12	0.435		0.384	↑↑	0.623
	13	0.064		0.061	↑↑	0.344
	14	0.032		0.046	↑	0.180
	15	0.016		0.015	↑↑	0.262
	16	0.112	↓	0.015		0.016
	17	0.177	↑↑	0.476	↓↓	0.245
	18	0.322	↑↑	0.553		0.408
	19	0.258	↑↑	0.492	↓	0.295
	20	0.225	↓	0.092		0.032
	21	0.241	↑↑	0.523		0.524
	22	0.629	↑↑	0.923	↓↓	0.623
	23	0.790	↑↑	0.953	↓↓	0.721
	24	0.112	↓	0.015	↑↑	0.606
	25	0.500	↑↑	0.876	↓↓	0.606
B7t	26	0.049		0.036	↑↑	0.346
	27	0.491	↓↓	0.036		0.122
	28	0.147	↑↑	0.381		0.265
	29	0.508		0.381		0.224
	30	0.327		0.236		0.102
	31	0.196	↑↑	0.472	↓↓	0.102
	32	0.196		0.090	↑	0.285
	33	0.311	↑↑	0.690	↓↓	0.285
	34	0.377	↑↑	0.636	↓↓	0.285
	35	0.475	↑	0.672	↓↓	0.367
B7g	36	0.063	↑↑	0.333	↓↓	0.098
	37	0.235		0.267	↓	0.088
	38	0.204		0.117		0.040
	39	0.110	↓	0.017	↑↑	0.157
	40	0.204		0.267	↓↓	0.020
	41	0.204	↑	0.400	↓↓	0.138
A1	42	0.529		0.447	↑↑	0.720
	43	0.490		0.492	↑↑	0.780
	44	0.647	↓↓	0.373		0.420
	45	0.843	↓↓	0.626		0.560
	46	0.902	↓↓	0.626		0.440
	47	0.666	↓↓	0.283	↑↑	0.740
	48	0.941	↓↓	0.716		0.820
	49	0.843		0.850	↓↓	0.600
	50	0.843		0.731	↓	0.500

† Single arrows indicate changes at a=0.05 and double arrows at a=0.01 level of significance respectively. Upward arrows (↑) correspond to increase of markers' frequencies, downward arrows (↓) to their decrease.

distributed throughout the genome. To avoid intrapopulation polymorphisms our trials were performed on large viable rye seeds samples, which underwent one reproduction (DZ4.1 – DZ1.1 pair). They showed that changes of frequencies of some markers were statistically significant. This may indicate presence of ageing hot spots within genomic DNA, which correspond to those described by Shatters *et al.* (1995). However, on the basis of our experiments, we can also suppose that their detection could be related to both natural ageing and reproduction of the sample. In this context natural selection may play an important role and the presence of genetic shift is probable. This is in agreement with data presented formerly on isozyme systems (Puchalski 1993). Additionally our results may reflect a greater level of inherited genetic changes due to long-term storage than was suggested previously (Murata *et al.* 1984; Puchalski 1993). Because of indirect methods based on protein markers and their use for the investigation of such effects in ageing and seed reproduction on the one hand, and possibly predominant elimination of any 'unwanted' changes within coding regions of genome on the other, the contribution of such events to genetic variation might be underestimated.

Previously Roos (1984), Stoyanova (1991, 1992) and Puchalski (1993) have shown that genetic shift might have a great impact on alterations of aged seed population reproduced early. Stoyanova (1991, 1992) and Puchalski (1993) paid attention to genetic shift and drift. Investigations performed on wheat gliadin proteins and rye isozyme systems respectively showed shifts with ageing in favour of the prevailing biotypes. It was concluded that alterations connected with genetic shift were more significant than those induced by genetic drift. In the case of our data, frequency changes of the DZ1.3 markers in comparison with the DZ1.1 are not yet fully understandable. However, simultaneous alteration of 17 markers in all samples may indicate the presence of both genetic shift and drift. Changes of frequencies of remaining markers of the DZ1.3 sample seem to prove the above statement. However, this may also suggest instability of the aged genome even after several reproductions, or a genetically not representative sample size. Yet we believe that none of the above artefacts were the case and that analysis of 75 genomic DNAs per sample by 110 RAPD markers is sufficient for such investigation. Still, additional experiments are needed to select which effect influenced the population regenerated three times.

It should be mentioned that we cannot exclude that a presence of contaminating microorganisms such as bacteria or fungi (or their variation among samples), or template DNA deterioration connected with the extraction procedure, could influence our RAPD profiles. Previously Williams *et al.* (1993) demonstrated that even a 460-fold molar excess of bacterial genomic DNA over the template does not change the profile pattern. To 'avoid' fungal contaminations only seeds lacking in visible growth were chosen for our study. However we were not interested in the comparison of results due to DNA extraction procedures; research presented by Shatters *et al.* (1995) clearly ascertained that neither extraction procedure nor some lack of template DNA integrity affected their data. On the other hand there were no prerequisites for differences in genomic DNA complexity among individuals of samples because extractions were accomplished only on viable seeds of high-viability samples. Moreover DNA bands detected on an agarose gel and reflecting bulk probes (see Results) were of the same molecular weight and any deterioration could be seen. It should be stressed that the SDS procedure might be successfully used for

genomic DNA extraction from individual seeds and result in high-quality templates appropriate for reproducible RAPD procedure.

Our experiments performed on individual plants of allogamous species like rye belonging to initially identical samples treated differently, demonstrated the possible use of the RAPD technique for the investigation of DNA marker changes resulting from reproduction of aged seeds previously stored in seedbanks. It was proved that the analysis of marker frequencies shared among samples allowed the detection of effects connected with ageing and reproduction at the background of the intrapopulation polymorphisms. It was shown that changes connected with ageing were transferred to the next generation. Moreover, natural selection possibly influenced the DZ1.1 population, suggesting the presence of genetic shift. Rye seed population (DZ1.3) after several reproduction cycles underwent further alterations, probably due to both genetic shift and genetic drift. Instability of the aged genome even after several reproductions cannot be excluded. The approach proposed above proved to be useful for the investigation of rare genetic effects in populations induced by natural ageing and reproduction of seeds. Those effects could be captured by comparative analysis of RAPD markers derived from populations of allogamous species like rye. Our preliminary investigation raised many questions that should be solved. The most intriguing of them are: what is the nature of the discussed ageing DNA hot spots? do they appear within coding regions of genome or not? The answers may have great implications for genebanks, and biodiversity conservation generally.

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Session IV: Diversity analysis and rye germplasm evaluation (Chair: Prof. Stefan Malepszy)

Genetic assessment of strains, varieties and ecotypes

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Summary

The advent of DNA-based technology via the polymerase chain reaction (PCR) has revolutionized genetic analysis of populations of all organisms from viruses to metazoans. New issues arising from DNA analyses must be addressed before genetic techniques can be used to evaluate germplasm. To provide accurate and timely information for germplasm conservation, molecular markers should be abundant, dispersed throughout the genome, highly variable, relatively inexpensive and usable on large numbers of individuals. Mutation rates of different classes of molecular markers vary considerably, and care must be taken to use markers with mutation rates appropriate to the questions being asked. For instance, distinguishing varieties may not be possible using markers with mutation rates that are too low, such as allozymes or chloroplast DNA. Because of their relatively high mutation rates, molecular genetic markers based upon randomly amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) microsatellites can provide high-resolution measures of genetic variability. Finally, markers that will be useful in germplasm conservation should be inexpensive while still being representative of the whole nuclear genome. The genome of rye (*Secale cereale* L.) is well characterized on the molecular level, which should ease the development of genetic markers useful for assessing genetic resources in this crop species.

Introduction

The goal in germplasm conservation for both endangered plants and crops is to conserve and maintain existing genetic diversity. For many rare or endangered plants, and for almost all crop species, germplasm conservation involves *ex situ* strategies like botanic gardens or seedbanks. When assessing how to conserve genetic diversity within these species, including variation among known strains, varieties and ecotypes (i.e. different forms of a single species from distinct habitats), a number of questions naturally arise (Brown and Briggs 1991; Guerrant 1992): (1) How many accessions to collect and where to collect them? (2) How many individuals to collect per accession? (3) When does one know that the collection is representative? In part, these question can be answered through a population genetics approach, and the advent of DNA-based technology has provided a wide array of neutral molecular genetic markers that will be useful in germplasm assessment (Avise 1994; Hillis *et al.* 1996).

In this short review, I examine how genetic markers can be used to assess genetic diversity. I do not, however, attempt to answer the primary question of how to sample populations to create representative collections for conservation of genetic resources. Others have already discussed the topic in some detail (Brown and Briggs 1991; Templeton 1991; Guerrant 1992; Avise 1994). Instead, I address

which of the many molecular genetic markers currently available will be useful for judging germplasm conservation. I begin by assuming that those who work with each species have a fairly detailed knowledge of the species' geographic distributions, population structure (large and randomly mating or small and subdivided), and variation in important phenotypic (morphological or physiological) traits. In terms of vocabulary, I use "gene" to describe a particular DNA sequence found at a particular place (locus) in the genome and "marker" to describe the protein or DNA fragment that is detected to indicate genes within the genome. Alleles are different forms of a gene, however they are detected.

Meanings of genetic diversity in populations

Measures and meanings of genetic variability are central to the use of molecular markers in assessing genetic resources (Hamrick and Godt 1990; Hartl and Clark 1990; Brown and Briggs 1991; Hamrick *et al.* 1991). Three measures most often used in sexually reproducing organisms are the numbers of alleles per locus, the proportion of individuals in a population that are heterozygotes, and the proportion of loci that are polymorphic. For organelle DNA [i.e. mitochondria (mtDNA) and chloroplasts (cpDNA)] and for clonal or asexual organisms, genetic diversity is measured as the number of distinct genotypes within a population. All of these measures have been used in determining genetic divergence among populations of species (Hamrick and Godt 1990; Brown and Briggs 1991; Hamrick *et al.* 1991; Avise 1994).

However, not all of these measures will be useful for assessing genetic resources in crop species. For example, each of a large number of accessions for a species may be completely lacking in genetic variability, with no heterozygosity or polymorphic loci in each accession. However, each accession will harbour different alleles at several to many loci. Thus the best measure of genetic diversity within this collection as a whole would be the total number of alleles found at each locus (see Templeton 1991). For clonal or asexual species, the number of distinct genotypes will provide the best measure of genetic diversity because these species lack sexual reproduction and recombination to shuffle allelic diversity among genotypes.

Thus, without carrying out careful quantitative genetic experiments to measure levels of genetic variation in phenotypic traits of interest, allelic diversity of molecular markers is most likely to provide the best measure of genetic diversity in *ex situ* collections. Most molecular markers are selectively neutral and are only indicators of variation in closely linked genes that code for traits like growth rate, plant size or quality, insect or disease resistance, or even survival in long-term storage. Molecular markers are simply used to predict genetic variation of other loci that influence performance. Collections that include a large number of alleles at marker loci should also harbour the genetic diversity for a variety of phenotypic characteristics. It should also be pointed out that molecular markers will only be predictive of genetic variation within a species if they are representative of the genome as a whole. As a rule of thumb, molecular markers will be most useful when more than 50 are sampled within each species (see below).

Molecular markers for germplasm assessment

A bewildering variety of molecular markers for population genetic and systematic studies is available (Schaal *et al.* 1991; Avise 1994; Dowling *et al.* 1996). Avise (1994) and Dowling *et al.* (1996) provide especially clear summaries of the uses of

molecular markers in population genetics and evolutionary biology. In order for molecular markers to accurately sample genetic variation of any species, these markers should be abundant, dispersed throughout the genome and highly variable. To be useful for the assessment of variation of genetic resources within large collections of plants or seeds, the molecular markers should also be inexpensive and usable on large numbers of individuals or on small nondestructive samples of plant tissues. Finally, the markers should come from the nuclear genes to allow prediction of variation in the genes that code for traits important for plant performance.

A vital issue to consider in choosing markers to use is the rate of mutation, which is known to vary widely for different characteristics and among different parts of the genome (Grant 1975; Hartl and Clark 1990; Avise 1994). Table 1 shows mutation rates for plants. Mutation rates vary considerably, from morphological characteristics with fairly slow rates of mutation on the order of one visible mutant per 100 000 individuals, to the highly variable SSR microsatellites with as many as one mutant per 100 individuals. It is critical to use molecular markers with mutation rates that match the question being asked (Avise 1994; Dowling *et al.* 1996). For instance, because of their relatively low mutation rates, markers derived from organelles (mtDNA and cpDNA) are often useful for studies of evolutionary history and divergence at higher taxonomic levels. However, these markers are often not useful for detecting variation among populations of individual species because of lack of variation at that level. Microsatellites may be useful for determining parentage or for genome mapping, but because of convergence of allelic states via mutation, microsatellites may not be useful for population studies over broad geographic areas (Dowling *et al.* 1996).

Table 1. Mutation rates in plants for morphological traits, proteins detected through allozyme analysis, and for various parts of plant genomes

Mutation	Rate
Morphological, quantitative traits	$10^{-5} – 10^{-9}$
Proteins, enzymes, allozymes	$10^{-5} – 10^{-9}$
Nuclear DNA	
- coding: substitutions, deletions	$10^{-4} – 10^{-8}$
- repetitive DNA (microsatellites)	$<10^{-3}$ (?)
Chloroplast DNA	
- substitutions, deletions	10^{-9}
- rearrangements	10^{-4}
Mitochondrial DNA	
- substitutions, deletions	10^{-11}
- rearrangements	10^{-4}

Markers based upon protein variation (allozymes), which have a long history of use in population genetics (Hamrick and Godt 1990; Hamrick *et al.* 1991; Schaal *et al.* 1991), have mutation rates that are only ten times more rapid than the rate of visible mutations, are usually coded by nuclear genes and are dispersed throughout the genome. However, allozyme markers are currently losing favour because there is a limited number of loci that can be sampled per individual tissue sample and because they tend to have low allelic diversity. This is unfortunate because allozymes are among the least expensive of molecular techniques.

The advent of the polymerase chain reaction (PCR) has revolutionized the use of DNA-based markers (Avise 1994; Dowling *et al.* 1996; Palumbi 1996). In all cases, the most information is gained by determining the nucleotide sequences of all alleles at a genetic locus. However, this is also the most time-consuming and expensive approach, and a number of techniques are available for detecting nucleotide variation within PCR-amplified DNA fragments without sequencing. Knowledge and use of molecular markers is an incremental process. As more sequences are known from both repetitive and single-copy parts of genomes of many species, more primers become available for amplifying genes from less well-studied species (Dowling *et al.* 1996; Palumbi 1996). But the clearest advantage of markers based upon PCR is that PCR requires minute amounts of DNA. This is not true for detecting sequence variation through restriction fragment length polymorphisms (RFLP) of single copy genes or repetitive DNA (i.e. fingerprinting with minisatellite repeats), where large amounts of DNA are needed for detecting variation. A more recently used procedure called AFLP (amplified fragment length polymorphism) is essentially the same as RFLP, but restriction fragments are visualized with PCR rather than by hybridization with probes (Vos *et al.* 1995). With careful DNA extraction techniques, PCR can provide an unlimited number of markers even when as little as 10 mg of tissue are sampled (e.g. plant embryos). Further, previous to the invention of PCR, most methods of detecting variation required labeling and detection via radioactivity (RFLP, AFLP), while PCR amplifies sufficient quantities of DNA for samples to be directly stained and visualized with ethydium bromide or silver.

Clearly, markers based upon PCR amplification are most likely to meet the criteria for use in germplasm conservation. But which techniques to use? Currently, there appear to be two methods that will provide ample genetic markers. These are random amplified polymorphic DNA (RAPD) markers (Welsh and McClelland 1990; Williams *et al.* 1990), and SSR microsatellites (Quellar *et al.* 1993). The first has an advantage in that RAPD-PCR requires no previous knowledge of gene sequences, primer synthesis, or characterization of DNA probes because they amplify genes at arbitrary locations throughout the genome. Microsatellites are more difficult and costly to develop, but have the advantage of providing numerous alleles per locus that are codominant.

The use (and misuse) of RAPD markers has been questioned (e.g. Mitchell-Olds 1995), but the repeatability and dispersion of RAPD markers have been demonstrated in mapping studies of numerous plant species (Table 2). Even critics of RAPD-PCR agree that RAPDs can be made useful in organisms where laboratory methodology can be standardized and Mendelian inheritance of loci can be established by testing segregation of markers within families (Black 1993; Mitchell-Olds 1995; Dowling *et al.* 1996). RAPD markers are the least expensive methodology, and provide the highest number of polymorphic markers for each PCR reaction.

Table 2. Plants with RAPD-PCR maps already developed

Species name	Reference
Arabadopsis thaliana	Reiter *et al.* 1992
Eucalyptus spp.	Grattapaglia and Sederoff 1994; Byrne *et al.* 1995
Mimulus (monkey flowers)	Bradshaw *et al.* 1995
Pinus spp.	Tulsieram *et al.* 1992; Nelson and Doudrick 1993; Plomion *et al.* 1995
Oryza sativa (rice)	Kurata *et al.* 1995

Medicago sativa (alfalfa)	Echt *et al.* 1993
Hordeum vulgare (barley)	Giese *et al.* 1994
Lactuca sativa (lettuce)	Kesseli *et al.* 1994
Secale cereale (rye) †	Rogowsky *et al.* 1992.

† Arbitrary priming of known repetitive elements.

Recently we have improved the efficiency of RAPD-PCR by the application of single-strand conformation polymorphism (SSCP) analysis on large formal polyacrylamide gels that are silver-stained (Antolin *et al.* 1996). SSCP analysis combined with RAPD-PCR reveals polymorphisms that are not detected on agarose gels (Fig. 1). SSCP works because the electrophoretic mobilities of single-strand DNA molecules in nondenaturing gels depend upon both size and shape of the fragments. Several stable shapes or conformations are formed when secondary base-pairing occurs among nucleotides on a single DNA strand. The length, location and number of intrastrand base pairs determines secondary and tertiary structure of a conformation. Point mutations that affect intrastrand interactions will change the shapes of molecules and alter their mobility during electrophoresis. The SSCP technique detects 99-100% of point mutations in DNA molecules 100-300 base pairs (bp) in length and at least 89% of mutations in molecules 300-450 bp in length (Orita *et al.* 1989; Hayashi 1991; Hiss *et al.* 1994; Vidal-Puig and Moller 1995). More significant for germplasm assessment, SSCP analysis increases the number of codominant polymorphisms seen in RAPD-PCR products and reveals a larger number of alleles per locus (Antolin, unpublished). Finally, individual markers can be converted to sequence-tagged sites (STS) by cutting silver-stained bands from gels and either cloning them for sequencing or sequencing them directly (Antolin and Black, unpublished). Oligonucleotide primer pairs can then be designed for targeted PCR of the STS markers (e.g. see Hudson *et al.* 1995).

On the other hand, microsatellites have an advantage in measuring allelic diversity because of the large numbers of alleles that segregate at each locus (Quellar *et al.* 1993). In this way, microsatellites would be ideal for assessing genetic resources within collections. However, microsatellites require screening a genomic library for regions that contain simple sequence repeats (usually AT repeats in plants, Dowling *et al.* 1996), sequencing these regions to create primers for each end of the microsatellite, followed by PCR amplification of a larger sample to determine the range of variation within populations. However, it is being observed that microsatellites found within one species often will be conserved within closely related species, so that many of the initial steps can be saved in commonly studied groups of plants (Dowling *et al.* 1996). Additionally, we have recently developed a simple silver-staining procedure for microsatellite gels similar to that used for RAPD-SSCP by Antolin *et al.* (1996), which allows for visualizing results without the use of radioactivity.

Genomic analyses of *Secale cereale*

It is fortunate for those who wish to develop molecular markers specifically for assessing rye germplasm that the genomes of rye and other grasses have been extensively studied (Dean and Schmidt 1995; Paterson *et al.* 1995). Large regions of the chromosomes of wheat, rice, maize, sorghum, barley and rye are conserved, with the same genes in the same order within long stretches of chromosomes.

This suggests that individual genes identified from other grasses could provide variable molecular markers for rye germplasm via PCR amplification and SSCP analysis to search for sequence variation (alleles). The genome of rye is also characterized by a large fraction of highly variable repetitive DNA dispersed throughout the seven chromosomes. These include large-scale repeats located at the telomeric regions (Vershinin *et al.* 1995), smaller repeats dispersed throughout the genome (Rogowsky *et al.* 1992) and microsatellites (Roder *et al.* 1995). The microsatellites were originally found in wheat, but 9 of 15 wheat primer pairs also amplified microsatellites in rye (Roder *et al.* 1995). Finally, it is possible to characterize varieties of rye via the number of copies ribosomal RNA genes from the mitochondrion (Coulthart *et al.* 1994).

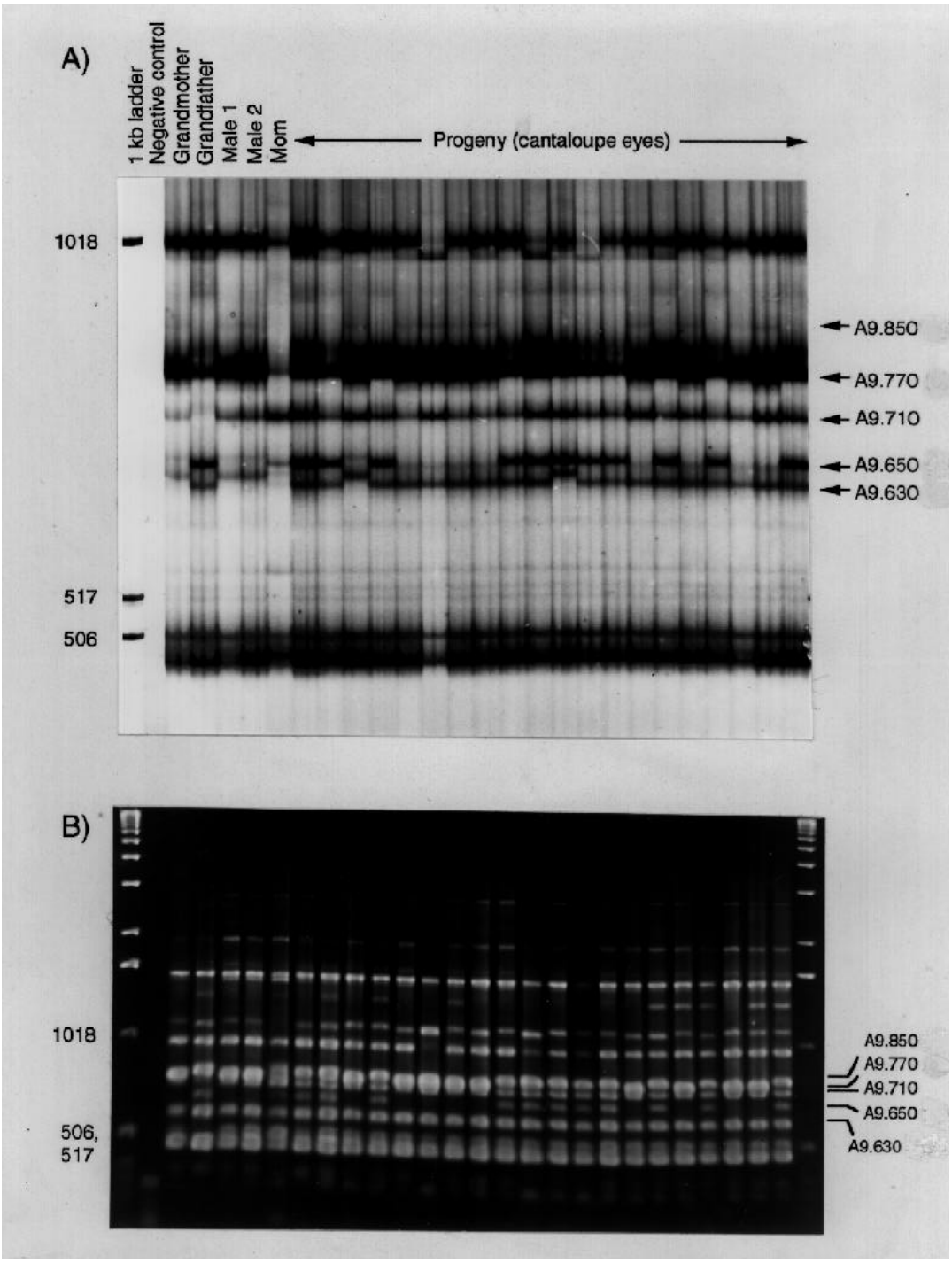

Fig. 1. Example of RAPD-PCR products amplified by primer A9 (Operon Technologies) from an extended family of a haplo-diploid parasitic wasp, *Bracon hebetor*, as seen on a silver-stained SSCP gel (A) and a standard 1.5% agarose gel stained with ethydium bromide (B) (see Antolin *et al.* 1996). The family includes a grandmother (diploid), grandfather (haploid), two haploid sons of the grandmother (Male 1, Male 2), the F_1 diploid female offspring of the grandparents (Mom), and 20 haploid progeny of the mother. Loci that were resolved as presence/absence polymorphisms can be seen on both gels (e.g. A9.650). However, loci that we did not resolve on agarose were resolved as codominant loci on the SSCP gel (e.g. A9.670), with the "slow" allele coming from the grandmother and the "fast allele" coming from the grandfather. Both alleles segregate among the haploid male progeny of the F_1 Mom.

Conclusions

For molecular markers to be useful for assessing genetic variation in *ex situ* collections, they must be highly variable, they must provide a broad coverage of the genome, and they must be usable on a large number of small individuals or samples. At the same time, they must be relatively inexpensive. RAPD markers, when visualized with SSCP, provide these kinds of markers, especially when patterns of inheritance of individual alleles are first verified within families. The power of RAPDs in plants has already been demonstrated (Table 2), with saturated linkage maps already available in numerous species. Because of the large number of alleles found at each locus, microsatellite markers will also be of use for assessing germplasm, especially for studies of genetic shifts through inadvertent selection during storage. But considering that molecular markers are neutral and simply provide information about levels of genetic variation likely to be found within regions of chromosomes, the real power of molecular markers is in combination with studies of the inheritance of traits that influence performance (Tanksley 1993; Mitchell-Olds 1995). Making the connection between variation of molecular markers and variation in important phenotypic traits will require extensive genome mapping and simultaneous linkage analysis of molecular markers and phenotypes. DNA-based technologies will make this more feasible, and the efforts may be quickly repaid through our ability to accurately assess genetic resources.

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Studies on application of protein and RAPD markers for identification of rye cultivars

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Summary

The results of genetic analysis of 29 rye taxa (*Secale cereale* L.) represented by agricultural varieties and local landraces originating from different parts of Europe and the USA are presented. This study was carried out on bulk samples consisting of a minimum of 10 to 25 seeds and based on both seed storage proteins (secalins) and randomly amplified polymorphic DNA markers (RAPDs). Secalins were extracted from all accessions with propanol and resolved electrophoretically on vertical polyacrylamide gels. For amplification with *Taq* DNA Polymerase, total genomic DNAs isolated by means of the SDS procedure and seven oligodeoxyribonucleotides of arbitrary sequences of our synthesis were used. Resulting DNA fragments were resolved on 1% agarose gels. Data were evaluated by means of The Discovery Series™ package from Pharmacia LKB. The number of protein bands changed from 9 to 18, while it varied from 13 to 26 in the case of RAPD depending upon the primer used. Seed storage proteins yielded 25 RAPD markers shared among accessions and genomic DNAs amplifications 120. Seventy-two and 92% of proteins and RAPD markers were monomorphic. Nei's coefficients were used for UPGMA analysis and results depicted in a dendrogram. Even data based on single RAPD profiles obtained with the separate primer were sufficient to distinguish among majority taxa. However better results were received when RAPDs were used in concert. Both types of markers grouped local landraces and cultivars within different clusters. However, various positioning of accessions within clusters was frequent. We demonstrated that those RAPDs and seed storage proteins obtained from bulk samples of both total genomic DNAs used as templates in Polymerase Chain Reactions, and gliadin fraction extract respectively, could be interchangeably applied for rye taxa identification (clustering). In general, within the group of local landraces the effect of geographic origin seems to be visible. Thus clustering may indicate possible sources of some accessions. RAPDs are more informative than seed storage protein markers but both could be used for taxa clustering. Yet, because of analysis costs, the latter one could still be used.

Introduction

For cultivar identification simple, quick and efficient methods are required. Phenotypic identification is commonly based on morphological traits recorded in the field. These traits are influenced by environmental conditions, making them unreliable for effectively distinguishing among cultivars (Faccioli *et al.* 1995). The development of isozyme or/and seed storage protein markers had great impact since they reflected some genome changes and were relatively inexpensive. The main drawbacks of these techniques were the limited amount of polymorphisms they could detect among closely related genotypes, and their indirect nature. Moreover, their application was restricted to a limited number of marker systems.

The development of the Polymerase Chain Reaction (PCR) techniques (Saiki *et al.* 1985; Mullis and Faloona 1987), offered a practically unlimited number of genetic markers and a possibility of more complex genome investigation. Progress in the PCR techniques resulted in approaches based on specific (Rogowsky *et al.* 1992), semi-random (Weining *et al.* 1991) and random (Welsh and McClelland 1990; Williams *et al.* 1990) genetic markers. The last type of markers – randomly amplified polymorphic DNAs (RAPDs) – can reflect changes appearing at arbitrarily chosen DNA fragments due to the nature of the oligonucleotides. Modern applications of RAPD markers include the identification of cultivated plants such as rice (*Oryza sativa* L.) (Yu and Nguyen 1994), tomato (*Lycopersicon esculentum*) (Klein-Lankhorst *et al.* 1991), grapevine (Collins and Symons 1993) and *Brassica oleracea* (Hu and Quiros 1991; Demek *et al.* 1992; Kresovich *et al.* 1992) or analysis of relationships among varieties, cultivars and lines (Klein-Lankhorst *et al.* 1991; Demek *et al.* 1992; Kresovich *et al.* 1992; Dweikat *et al.* 1993; Wilkie *et al.* 1993). However, to our knowledge such data concerning rye taxa have not been published.

Our goal was to investigate whether RAPD and seed storage protein markers obtained after amplification of genomic DNA and gliadin fraction of bulk samples respectively could be successfully applied for identification (clustering) of 29 randomly chosen taxa of cultivated rye (represented by registered varieties and local landraces), whether both types of markers would result in similar clustering patterns and whether geographic origins influenced the accessions' grouping.

Methods

Plant material

Twenty-nine modern cultivars and local landraces (Table 1) used in the experiment, with 95% viability, were provided by the Seed Bank of the Botanical Garden of the Polish Academy of Science, Warsaw.

Genomic DNA extraction

Bulk total genomic DNAs were isolated from 0.5 g of dry seeds (i.e. 20-25 seeds). DNA extractions were performed by standard SDS procedure (McDonald *et al.* 1994). Additionally, samples were treated by RNase to remove RNA impurities. The DNAs quantity and quality were determined spectrophotometrically (GeneQuanta, Pharmacia LKB). DNA integrity was proved by agarose gel electrophoresis in the presence of a molecular weight marker (λ DNA digested with PstI endonucleases). For PCR experiments freshly prepared standard dilutions (10 μg / ml) were used.

Extraction of seed storage proteins

Endosperm seed storage proteins as gliadin fraction (secalins) were extracted from bulk samples of each accession consisting of 10 seeds. For each seed of the bulk sample, 0.25 ml of 50% propanol was used at 4°C during one night. To ensure that the extract from 10 individuals was enough for data evaluation, extractions of protein samples from totals of 10, 25 and 50 seeds were performed, and the results were compared.

Primers

Seven 10-base random primers (Table 2) with 60-80% of G+C content were synthesized according to the standard phospoamidite scheme (Manual) on the DNA/RNA Synthesizer Model 394 (ABI) and purified on Reversed Phase column by means of High Performance Liquid Chromatography.

Table 1. Modern cultivars and local landraces of rye (*Secale cereale*) and their geographic origin

No.	Name	Origin
1	Local landrace 12403/67	Turkey
2	Local landrace 15542/67	Turkey
3	Local landrace 26619/68	Turkey
4	Local landrace 15560/67	Turkey
5	Local landrace 15579/67	Turkey
6	Local landrace 78 A-553	Portugal
7	Local landrace 77 A-24	Portugal
8	Local landrace 77 A-111	Portugal
9	Local landrace 77 A-83	Portugal
10	Local landrace 77 A-38	Portugal
11	Local landrace 30	Turkey
12	Local landrace 4	Turkey
13	Local landrace 3/72 b.	Yugoslavia
14	Local landrace 32	Turkey
15	Local landrace 29	Turkey
16	Lisitin	Romania
17	Charkovskaja (14924)	Ukraine
18	Chrysanth Hanserrogen	Austria
19	Hadmerslebener	Germany
20	Ludowe	Poland
21	Bonel	USA
22	Ceske Normalni	Czechoslovakia
23	Costelo Branco	Portugal
24	Lvovskaja	Ukraine
25	Local landrace Lungauer Tauern	Austria
26	Kastoria	Greece
27	Da⌧kowskie Z⁄ote	Poland
28	Charkovskaja	Ukraine
29	Da⌧kowskie Selekcyjne	Poland

Table 2. Sequences of the oligodeoxyribonucleotides

Sequences (direction 5'® 3')
ATG GAT CCG C
GTG TGC CCC A
CAG GCC CTT C
GGT GAC GCA T
GGT GAC GCA G
CGC CCC CAG T
TGG ACC GGT G

Polymerase chain reaction

The polymerase chain reactions were carried out in a total of 25 µl value containing 10-80 ng of genomic DNA template, 0.1-0.4 mM of separate primer, 100-400 mM of dNTPs, 1.5-2.5 mM $MgCl_2$ and 0.5 units of *Taq* polymerase (Perkin Elmer Cetus). Parameters affecting RAPD were optimized to obtain high resolution and reproducible banding patterns by modified Taguchi's method (Cobb and Clarkson 1994). Amplification was carried out in a GeneAmp System

9600 Thermocycler (Perkin Elmer) programmed for: [94°C-30 min; 32°C-30 min; 72°C-60 min]$_2$[94°C-5 min; 34°C-30 min; 72°C-60 min]$_{45}$[72°C-300 min][5°C-∞] (Cobb and Clarkson 1994).

Electrophoresis conditions

The 1% agarose gel containing 1^x TBE buffer and ethidinum bromide (0.5 µg/ml) were used to perform electrophoresis of amplification products consisting of 25 µl of the reaction mixture, which was conducted at a constant voltage of 20 V/cm for about 5 hours. Separated DNA fragments were viewed and photographed with Polaroid 52 film under UV light (Sambrook *et al.* 1989).

Seed storage proteins were resolved on vertical polyacryamide gels (PAGE) at pH=3.2, fixed in 12% trichloroacetic acid and stained with Coomassie Brilliant Blue.

Data analysis

Scanning and computer analyses of the electrophoretic images of single gels were performed by means of The Discovery Series™ package purchased from Pharmacia LKB and the RFLPrint software based on the UNIX operating system. The presence of a specific product was noted, whatever the intensity of the amplified product. The Nei's genetic identity coefficients (Nei and Li 1979**)** were used in pairwise comparisons between accessions to build dendrograms according to the unweighted pair group method with arithmetic mean (UPGMA) (Saitou and Nei 1989) independently of the markers' types used. If needed, original dendrograms were reclustered by software for better results presentations. In case of RAPD, data sets from single gels representing amplified products of only one or different arbitrary primers were analysed independently or combined to draw dendrograms based on any combination of 6 out of 7 primers, and finally using all genetic markers available.

Results and discussion

In total, the number of bands in the RAPD profiles varied from 13 to 26 bands ranging from 700 to 2500 bp depending upon the primer, while it changed from 9 to 18 bands for secalins. Each band in the gel with the same electrophoretic mobility (relative front) was scored as the same marker. The PCR amplifications of genomic DNA from 29 rye taxa yielded a total of 120 RAPDs and the electrophoresis of seed storage proteins 25 markers.

Among RAPD markers, 92% were polymorphic: 72% (18 bands) and 40% (10) markers were monomorphic among local landraces and cultivars respectively. Among protein markers 28% were monomorphic: 32% (8) and 28% (7) of markers were monomorphic for cultivars and local landraces respectively. As in the case of proteins, 18 RAPDs were found only in a few (usually in only one) accessions of local landraces and another 18 in cultivars.

Genetic similarity estimates (Nei's coefficient) varied from 61 to 97% and from 41.7 to 89.7% for RAPD and protein markers respectively (not presented) and were used for UPGMA analyses to display accessions clustering as dendrograms for both types of markers. Interestingly, the data based on single RAPD profiles obtained with the separate primer were sufficient to distinguish among majority taxa and could be divided into groups which:

- distinguished taxa among each other but did not cluster groups of similarity
- distinguished all taxa on clusters of similarity (local landrace and cultivars) but
  - distinguished poorly a local landrace within cluster
  - distinguished well a local landrace within cluster (data not presented).

For taxa grouping, combination of RAPD markers derived from any variation of six primers and finally any available RAPD markers were used. In the first case, major dendrograms (not shown), which were based on about 100 markers, reflected taxa grouping within local landrace and cultivar clusters. Some correlation with geographic origin could also be noted. Better results were obtained when all markers were used together. In general local landraces were found in one cluster that was also occupied by cultivar Lisitin. Local landraces obtained from Turkey, former Yugoslavia, and Portugal were often close to each other on the dendrogram (Fig. 1) forming subclusters (L.L.29, L.L. 3/72b, L.L. 32, L.L. 4; L.L. 15542/67, L.L. 12403/67, L.L. 15579/67; L.L. 78 A-553, L.L. 77 A-111, L.L. 77 A-24). In the case of cultivars two clusters were formed. However, explanation of taxa clustering without pedigree background seems difficult, but still those from Poland, Ukraine, Germany appeared to be more similar to each other than to those from Greece, Portugal, Austria or the USA, which may indicate various ancestors. Interestingly, the closely related cultivars Dańkowskie Złote[1] and Dańkowskie Selekcyjne[2] were grouped within the same cluster but in distinct subclusters. Moreover, based on the pedigree data, Dańkowskie Selekcyjne was the ancestor of Dańkowskie Złote. Anyhow this is not evident from the results depicted by the dendrogram. This could be explained by greater genetic influence of Petkuser Normalstroh as the ancestor of Dańkowskie Selekcyjne on the formation of cultivar Dańkowskie Złote.

Cluster analysis of the protein markers (Fig. 2) revealed similar grouping as for RAPDs. Practically all local landraces were grouped within the same cluster including cultivar Lisitin and forming subclusters that, in general, were geographically linked. Additionally, the cluster constituted of cultivars included the local landrace Lungauer Tauern. Moreover, the relationships of Dańkowskie Złote and Dańkowskie Selekcyjne correspond to those illustrated by RAPD markers. Despite that, in many cases dendrograms based on RAPD and protein markers displayed different positioning of accessions within clusters. Unfortunately, unavailability of the pedigree data made it impossible to check up which marker type is better suited. Moreover the results presented on both marker types may suggest that pedigree data reflect only formal relationships among related plants.

Thus 120 RAPD and 25 seed storage protein markers obtained from bulk samples of both total genomic DNAs used as templates in Polymerase Chain Reactions, and gliadin fraction respectively, are applicable for rye taxa identification and grouped accessions into cultivars and landraces equally well. However the first, because of its nature and availability in great number, seems to have greater analytical value. For the most part, within the group of local landraces the effect of geographic origin seems to be seen. Thus clustering may indicate possible sources of some poorly characterized accessions. Comparing RAPD and protein markers it should be noticed that the first were more polymorphic and informative, but both could distinguish among taxa. Regarding the analysis cost, their application might be preferred.

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[1] Pedigree of cultivar Dańkowskie Złote: (/Probasztajskie × Zelandzkie/ × Szampańskie) × Petkus.

[2] Pedigree of cultivar Dańkowskie Selekcyjne: Dańkowskie Złote × Petkuser Normalstroh.

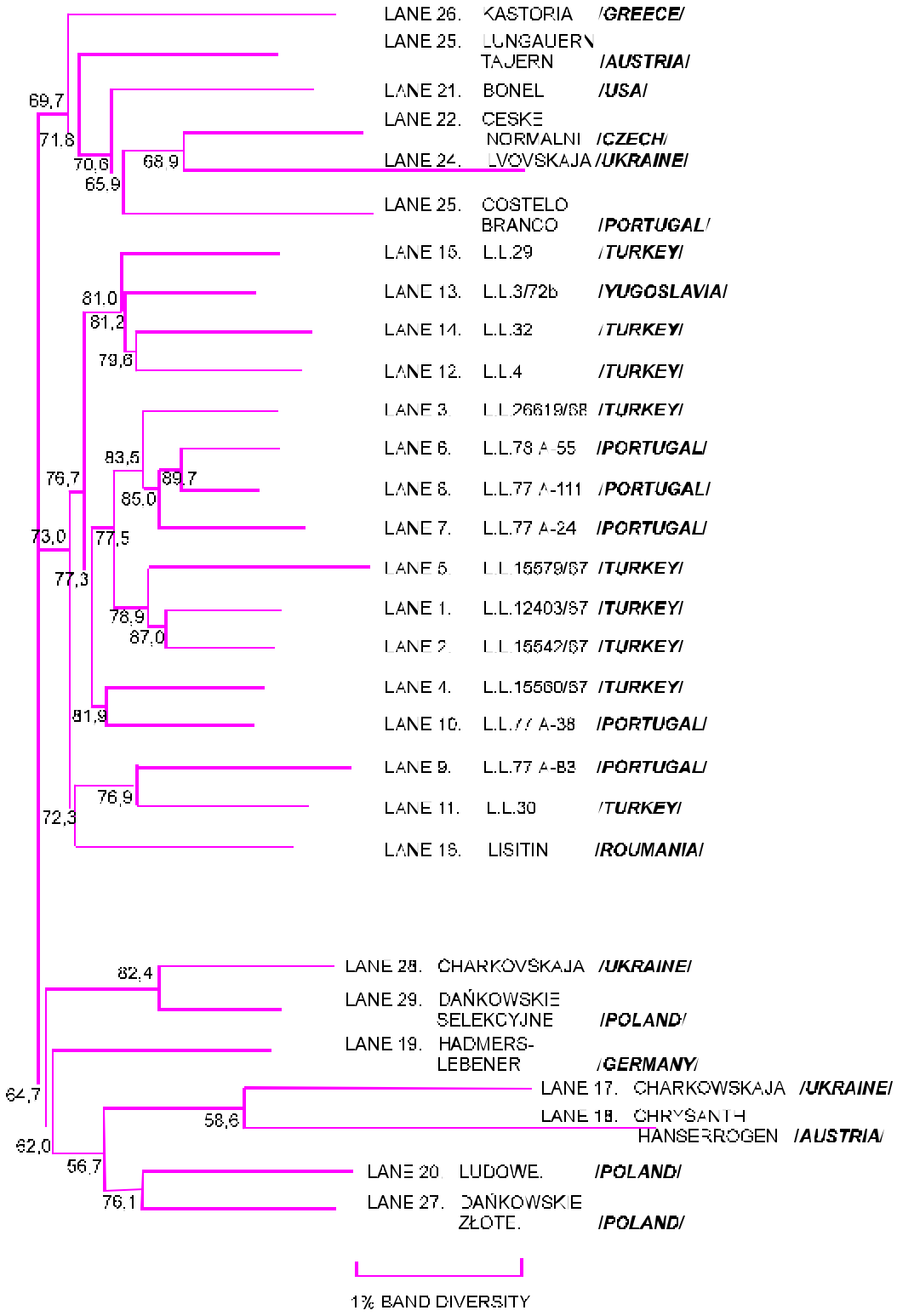

Fig. 1. Dendrogram of the modern cultivars and local landraces based on UPGMA analysis of genetic-similarity estimates (Nei's coefficient) from any available RAPD markers. On the right, names and geographic origin are indicated.

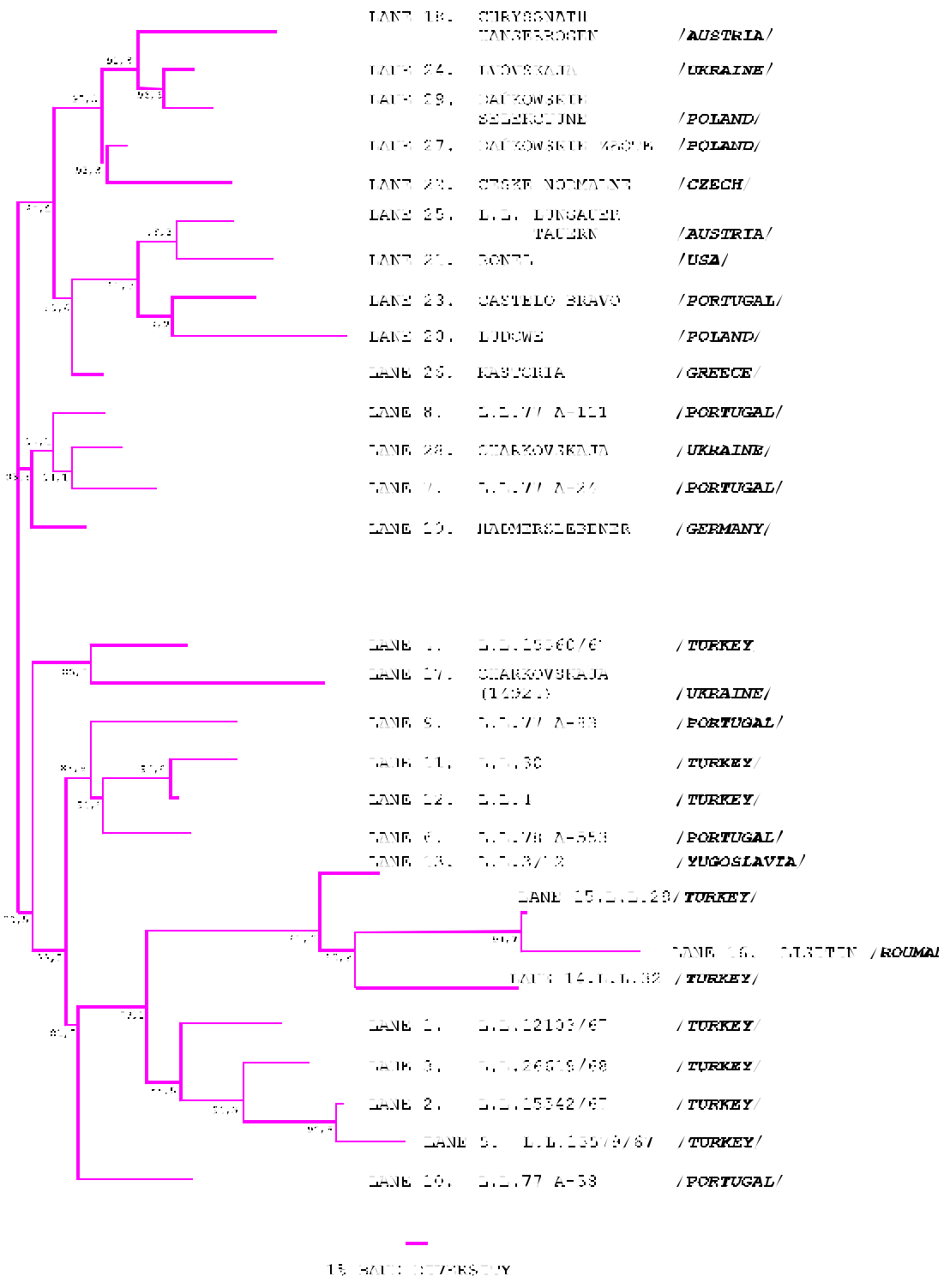

Fig. 2. Dendrogram of the modern cultivars and local landraces based on UPGMA analysis of genetic-similarity estimates (Nei's coefficient) from seed storage protein markers. On the right names and geographic origin are indicated.

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Maintenance and evaluation of the Secale *collection of the Botanical Garden of the Polish Academy of Sciences*

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Rye is a very important crop in Poland, cultivated presently on about 2 million hectares. The Botanical Garden of the Polish Academy of Sciences in Warsaw started studying rye germplasm conservation and evaluation in 1972 (Molski *et al.* 1981). The programme was initiated by professor Bogusław Molski - the first director of the garden. The seed samples were obtained through seed exchange with other genebanks or were collected during expeditions. They originated from 34 different countries (Table 1). Presently the rye germplasm collection has reached a total of 1523 accessions (Table 2). An important part of the *Secale* collection is a set of wild *Secale* species and subspecies, which number 62 accessions represented by 13 different wild species/subspecies (Table 3). They are classified according to Hammer (1990).

Table 1. Origin of the *Secale* collection of the Botanical Garden of the Polish Academy of Sciences

Country	No. of accessions[†]	Country	No. of accessions[†]
Afghanistan	9	Kenya	0 (1)
Argentina	17	Netherlands	23
Austria	53 (6)	Norway	5
Belgium	5	Pakistan	2
Brazil	26	Poland	91
Bulgaria	8	Portugal	10 (123)
Canada	28	Russia	118
China	4	Romania	10
Czech Republic + Slovakia	27	South Africa	6
Finland	24	Spain	14
France	8	Sweden	19
Germany	122 (6)	Turkey	45 (232)
Hungary	23	United Kingdom	3
India	1	USA	53
Iran	6	Uruguay	1
Italy	1	Yugoslavia (former)	1 (134)
Japan	1		

[†] First number = cultivars; second number = local landraces (in parentheses).

Table 2. *Secale* collection of the Botanical Garden of the Polish Academy of Sciences (status in 1996)

Type of accession	Number
Cultivars	857
Local landraces:	604
Wild *Secale* species:	62
Total	1523

Table 3. Wild species of *Secale* in the collection of the Botanical Garden of the Polish Academy of Sciences (classified according to Hammer 1990)

Taxon	No. of accessions
Secale cereale L.	
- subsp. *afghanicum* (Vav.) Hammer	2
- subsp. *ancestrale* Zhuk.	8
- subsp. *dighoricum* Vav.	3
- subsp. *segetale* Zhuk.	6
- subsp. *rigidum* V. et V. Antrop.	1
Secale sylvestre Host.	9
Secale vavilovii Grossh.	7
Secale strictum (Presl.) Presl.	15
- subsp. *africanum* (Stapf) Hammer	1
- subsp. *anatolicum* (Boiss.) Hammer	4
- subsp. *ciliatoglume* (Boiss.) Hammer	1
- subsp. *kuprijanovii* (Grossh.) Hammer	5

Conservation of the collected rye germplasm is realized through storage of seed samples in the seedbank and through regeneration (multiplication) of samples with lowered viability or too few seeds. Regenerated seed samples are sown in 1.5-m^2 plots at a density of 10.0 x 2.5 cm, resulting in 350 seeds per plot. At flowering time plants are isolated with linen cages to avoid cross-pollination by alien pollen (Molski *et al.* 1982b). Seed samples are harvested manually, threshed on a laboratory threshing machine, cleaned manually and dried before being placed into medium- or long-term storage. For medium-term storage seeds are kept over silica gel in glass desiccators. Samples for evaluation experiments or exchange are stored in this way. The basic collection is conserved for long-term storage in sealed vacuum foil packages in freezers at –18°C. The seed moisture content is 6-7%. Viability of samples is periodically checked using the ISTA germination test.

For regeneration and/or multiplication procedures an evaluation of the conserved germplasm is carried out. Selected phenological and morphological characters, important from the agronomic point of view, are observed. Evaluation of each accession is repeated during at least three successive years. Collected data are stored as database files. The observations include phenological studies, morpho-logical characters and agronomic features. All descriptors used for evaluation of rye collection are listed in Table 4. Special efforts were focused on protein content evaluation and testing for snow mould resistance (Molski *et al.* 1983). For example, a very interesting collection of high-protein rye cultivars was selected (Molski *et al.* 1982a). Studies on snow mould resistance showed that some wild *Secale* species and local landraces from Austria and Turkey are more tolerant to that disease than the majority of modern rye cultivars (Puchalski and Buczyńska 1985).

The maintenance and investigation of the *Secale* collection of the Botanical Garden in Warsaw-Powsin are part of the crop germplasm conservation programme coordinated by the Plant Breeding and Acclimatization Institute in Radzików and supported by a special fund of the Polish Ministry of Agriculture. Studies are conducted in close cooperation with other germplasm conservation centres around the world. The most fruitful cooperation in this regard was achieved thanks to USDA-ARS projects. From 1979 to 1996 six research projects

were conducted on rye germplasm. They were devoted firstly to protein evaluation, snow mould resistance testing and isozyme use for biosystematics and genetic studies on rye cultivars. Later, since 1988, two new projects granted from PL.-480 funds started. They were focused on the problems of genetic changes in rye seeds due to long-term storage and to rye germplasm regeneration for the long-term storage. Both projects were conducted in very close cooperation with the USDA-ARS National Seed Storage Laboratory in Fort Collins, Colorado. As a result of this cooperation the whole USDA rye germplasm was multiplied and seeds were delivered to the NSSL for long-term storage and to the USDA National Small Grains Collection in Aberdeen, Idaho for evaluation and short-term storage. Evaluation data were transmitted to the GRIN database. Among rye seed samples delivered to the NSSL and to the NSGC, unique local landraces of rye collected by Dr R.J. Metzger together with Turkish scientists during two expeditions to Turkey in 1979, and in 1984 and 1986 to Pakistan, were multiplied and evaluated. They were especially studied for the resistance to pink snow mould.

Table 4. Descriptors used for evaluation of the *Secale* collection of the Botanical Garden of the Polish Academy of Sciences

Character
Field emergence of plants – FE (%)
Grain-filling period – GFP (scale 1-9)
Wax maturity – WM (date)
Length of vegetation period from sowing to full maturity – LVP (days)
Winter hardiness – WINT (scale 1-9)
Plant height – HP (cm)
Length of ear – EL (cm)
Length of underflag leaf – LUL (cm)
Number of grains per spike – GS
Productive tillering – TILL (as number of stems with fertile spikes per plant)
Thousand-kernel weight – TKW (g)
Protein content in kernels – PROT (%)
DBC analysis
Resistance to snow mould

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Rye germplasm resources in the USDA-ARS National Small Grains Collection

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Summary

The USDA-ARS National Small Grains Collection maintains a collection of 1897 *Secale* accessions. Landraces constitute the majority of these accessions. Regenerations are based on seed viability and inventory quantities. Most accessions were regenerated recently in a joint project between the USDA-ARS National Seed Storage Laboratory and the Botanical Garden of the Polish Academy of Sciences. The rye collection is being evaluated for important quality, agronomic, disease and insect descriptors, including Russian wheat aphid.

The USDA-ARS National Small Grains Collection Rye Collection

The USDA-ARS National Small Grains Collection (NSGC) is the active collection for wheat, barley, oat, rice, rye and triticale within the National Plant Germplasm System (NPGS) (Janick 1989; Shands *et al.* 1989; ARS Information Service 1996). The NSGC is the largest of the active collections in the NPGS (ARS Information Service 1996) with more than 116 000 accessions. The National Seed Storage Laboratory (NSSL) provides the security back-up for the NSGC and the other active collections in the NPGS. The NSGC freely distributes seed to scientists worldwide. Passport and observation data are maintained on the Germplasm Resources Information Network (GRIN), which is available online at http://www.ars-grin.gov. Seed requests may be placed online or with the NSGC curator by mail or telephone. The data is also available as a software package called pcGRIN for installation on personal computers.

The NSGC rye collection consists of 1897 accessions in nine species and subspecies of *Secale* (Table 1) with origins in at least 62 countries (Table 2). Landraces constitute more than 1100 accessions with significant collections from Turkey (628 accessions) and the former Yugoslavia (271 accessions). The rye accessions were acquired through exchange with other genebanks and institutes, organized collecting expeditions and rye breeding programmes. The oldest rye accession was obtained in 1948.

Regenerations are scheduled based on seed viability and inventory quantities. Viability tests are scheduled every 10 years. Presently, viability data are available on 1713 accessions. About 97% of the tested accessions have greater than 60% viability and 73% have greater than 85% viability. A total of 1628 accessions have seed supplied from the joint regeneration project of NSSL and the Botanical Garden of the Polish Academy of Sciences [Rye Seed Regeneration for Long-Term Storage, PL-ARS-140(A)].

Table 1. Accessions in the NSGC Rye Collection by taxonomy

Species	Count
Secale cereale	17
Secale cereale subsp. *ancestrale*	6
Secale cereale subsp. *cereale*	1786
Secale cereale subsp. *segetale*	8
Secale cereale subsp. *tetraploidum*	1
Secale sp.	31
Secale strictum	33
Secale strictum subsp. *anatolicum*	6
Secale strictum subsp. *kuprijanovii*	3
Secale sylvestre	1
Secale vavilovii	4
Secale x derzhavinii	1
Total	1897

Table 2. *Secale* accessions in the NSGC by country of origin

Country	No. access	Country	No. access
Afghanistan	33	Iraq	1
Algeria	1	Ireland	1
Argentina	18	Israel	2
Armenia	5	Italy	2
Australia	5	Japan	6
Austria	34	Kenya	1
Azerbaijan	3	Latvia	1
Belarus	2	Mexico	30
Belgium	2	Morocco	6
Bosnia & Herz.	7	Poland	85
Brazil	30	Portugal	108
Bulgaria	9	Russian Fed.	36
Canada	30	Slovakia	1
Chile	27	Slovenia	1
China	3	South Africa	31
Czech Republic	4	Spain	25
Czechoslovakia	7	Sweden	11
Estonia	3	Switzerland	1
Europe	7	Turkey	628
Finland	24	Turkmenistan	1
Former USSR	28	Ukraine	14
France	5	Uncertain	4
Georgia	16	United Kingdom	7
Germany	107	United States	80
Greece	2	Unknown	8
Hungary	47	Uruguay	2
India	2	Yugoslavia	59
Iran	30	**Total**	**1897**

Russian wheat aphid resistance

The NSGC rye collection is being evaluated for a number of traits, including various quality, agronomic, disease and insect descriptors. Rye accessions were evaluated for reaction to Russian wheat aphid (*Diuraphis noxia*), an important new insect threat in the western USA. Usable levels of resistance (consisting of various levels of antibiosis, antixenosis and tolerance) were found in 45 accessions, mainly from Afghanistan and Turkey (Table 3).

Table 3. *Secale* accessions with Russian wheat aphid resistance

Accession number	Origin country	Accession number	Origin country
CIse 161	Turkey	PI 374448	Macedonia
PI 168182	Turkey	PI 374453	Yugoslavia
PI 168185	Turkey	PI 429376	Iran
PI 168187	Turkey	PI 429378	Iran
PI 168205	Turkey	PI 446029	Mexico
PI 168222	Turkey	PI 446035	Germany
PI 173587	Turkey	PI 470297	Turkey
PI 220682	Afghanistan	PI 543409	Turkey
PI 220683	Afghanistan	PI 543415	Turkey
PI 221479	Afghanistan	PI 543416	Turkey
PI 221959	Afghanistan	PI 543418	Turkey
PI 240286	Turkey	PI 543420	Turkey
PI 250745	Iran	PI 543442	Turkey
PI 250885	Iran	PI 543443	Turkey
PI 251905	Russian Fed.	PI 543522	Turkey
PI 272336	Hungary	PI 543532	Turkey
PI 289814	Iran	PI 543574	Turkey
PI 304534	Turkey	PI 543575	Turkey
PI 306497	Czechoslovakia	PI 543678	Turkey
PI 357084	Turkey	PI 543680	Turkey
PI 366495	Afghanistan	PI 543686	Turkey
PI 366496	Afghanistan	PI 543687	Turkey
PI 366498	Afghanistan		

Agronomic, seed and quality traits

Data on several agronomic, seed and quality traits were collected on accessions in the NSSL/Botanical Garden project, including awn colour, awn type, glume pubescence, grainfill period, growth habit, kernels per spike, 1000-kernel weight, leaf pubescence, lodging, plant height, grain protein, Russian wheat aphid resistance, shattering, spike density, spike type, straw breakage, straw colour and tillering. Considerable variability exists among the accessions for these traits. Grainfill period ranged from 34 to 60 days (Fig. 1). Tillering ranged from 2.0 to 14.6 (Fig. 2). Thousand-kernel weight ranged from 18.0 to 55.3 (Fig. 3). Kernels per spike ranged from 8.0 to 58.0 (Fig. 4). Grain protein ranged from 9.2 to 19.8% (Fig. 5). Landrace accessions from the former Yugoslavia appear to be a good source of higher seed protein content with many accessions at the high end of the range. The descriptor data, plus available passport data, will be utilized in the selection of a rye core subset.

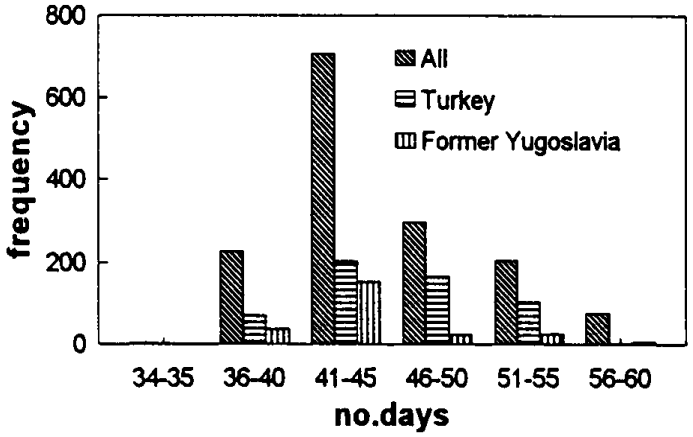

Fig. 1. Grain fill period for *Secale* accessions in the NSGC.

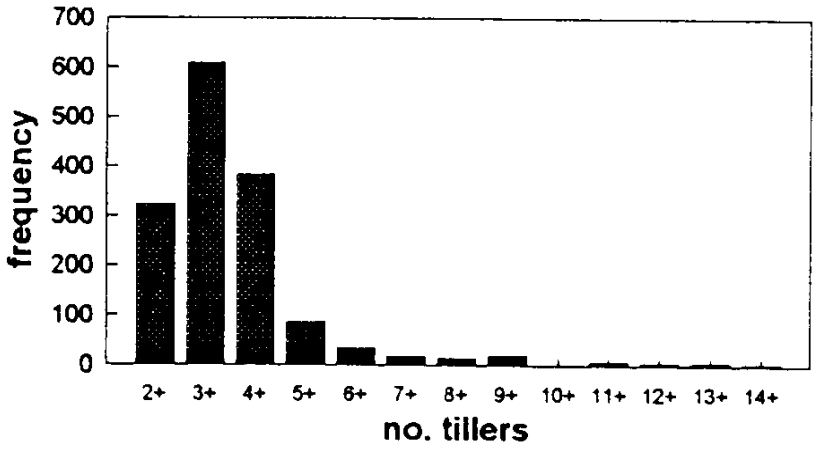

Fig. 2. Tillering for *Secale* accessions in the NSGC.

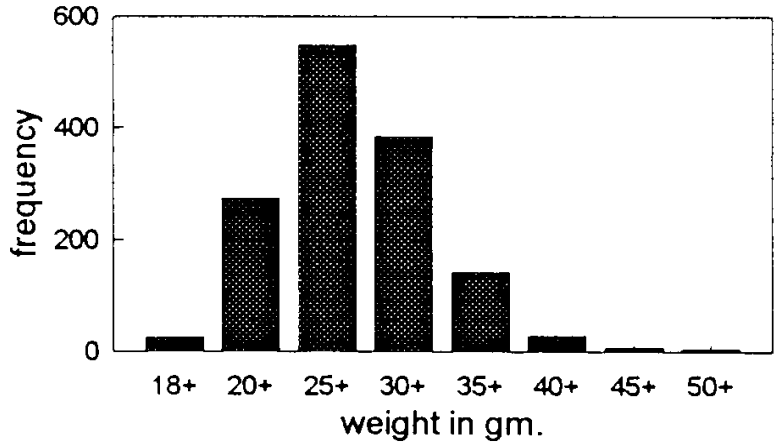

Fig. 3. Thousand-kernel weight (g) for *Secale* accessions in the NSGC.

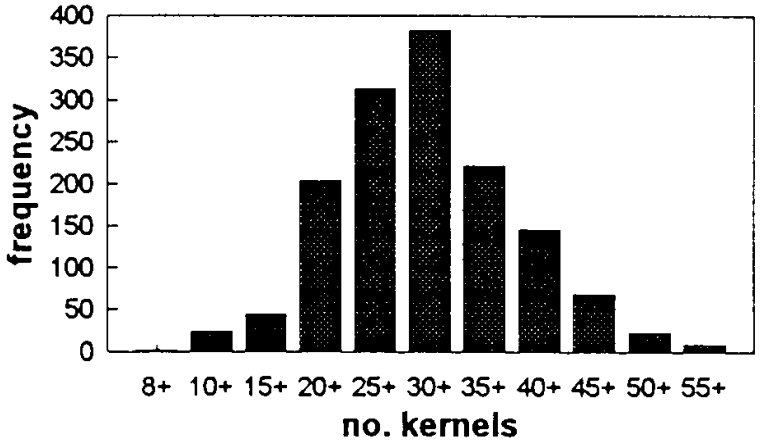

Fig. 4. Kernels per spike for *Secale* accessions in the NSGC.

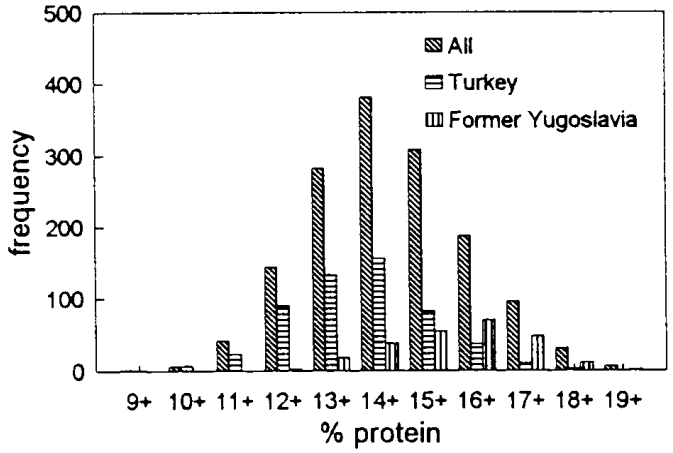

Fig. 5. Grain protein content for *Secale* accessions in the NSGC.

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Session V: Rye germplasm collections in Europe (Chair: Dr Thomas Gass)

Rye genetic resources in European genebanks

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Introduction

The first ECP/GR Secale Working Group meeting was held in Jokioinen, Finland, in August 1982. The Working Group designated the Polish Gene Bank as a crop germplasm centre for rye and recommended collation of passport data from other European rye collections.

The first edition of the rye catalogue comprised passport data of rye accessions maintained in 11 genetic resources centres. The pioneer work carried out at the Plant Breeding and Acclimatization Institute was edited under the auspices of the ECP/GR Secretariat in 1984 (Serwiński and Konopka 1984). As the first of its kind, the rye catalogue was used as a reference as well as a model for other European databases. An attempt was made to identify duplicates between collections resulting from exchange of samples. Regrettably, the undertaking was not developed into a permanent initiative; neither crop network nor permanent working group were established.

During a meeting in Prague in 1994 in the framework of the project 'Technical support to East European genebanks to improve access of privatized plant breeding to germplasm collections' the idea of the creation of crop networks was considered. The Polish Gene Bank agreed to resume the idea of the *Secale* Database continuation. The initiative was supported by the participants (Jongen and van Hintum 1994).[6]

Data collecting

Since 11 years have passed since the first data-gathering, the updated European *Secale* Database contains only recently requested data. Forty-eight institutions in 25 European countries were informed by letters, issued on 17 February 1995, about the updating initiative. We asked for passport data and information on the availability of evaluation data. Up to now, 17 institutions have provided data to PBAI Radzików (Table 1).

Data were provided mainly on diskettes; in two cases data were prepared as printouts. All files were easy to process and were accompanied by additional information on the structure and content of fields. In a few cases information was provided using the extended ASCII code. Since data from Ukraine were provided in Cyrillic, transcription was necessary. The international transcription from Cyrillic to English was used.

[6] The research was funded by IPGRI (Letter of Agreement no. 94/172 - Updating of the European *Secale* Database).

Table 1. *Secale* collections contributing to the European *Secale* Database

Institution	Acronym	Country[†]	No. of accessions
Bundesamt für Agrarbiologie	AUTBVAL	AUT {	62
Landesanstalt für Pflanzenzucht und Samenprüfung Rinn	AUTLARINN	AUT {	
Institute of Plant Introduction and Genetic Resources	BGRIIPR	BGR	337
Station Fédérale de Recherches Agronomiques de Changins	CHERAC	CHE	63
Cereal Research Institute and Breeding Institute	CZEKROME	CZE {	659
Research Institute of Crop Production	CZERUZYNE	CZE {	
Institut of Plant Genetics and Crop Plant Research-IPK	DEUGAT	DEU	878
Institut für Pflanzenbau-FAL	DEUBGRC	DEU	329
Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria	ESPINIACRF	ESP	428
Station d'Amélioration des Plantes Clermont-Ferrand	FRAINRACLF	FRA	41
CNR Instituto del Germoplasma	ITAIDG	ITA	382
Plant Breeding and Acclimatization Institute	POLIHAR	POL	1354
Botanical Garden of the Polish Academy of Sciences	POLPAN	POL	1630
Departamento de Genética Estação Agronómica Nacional	PRTEAN	PRT	33
Banca De Resurse Genetice Vegetale	ROMSUCE	ROM	45
N.I. Vavilov Institute of Plant Industry	RUSVIR	RUS	2685
Nordic Gene Bank	SWENGB		63
Aegean Agricultural Research Institute	TURARARI	TUR	512
Ukrainian Centre for Plant Genetic Resources	UKRIIRSG	UKR	171
Total			9672

[†] AUT=Austria, BGR=Bulgaria, CHE=Switzerland, CZE=Czech Republic, DEU=Germany, ESP=Spain, FRA=France, ITA=Italy, POL=Poland, PRT=Portugal, ROM=Romania, RUS=Russian Federation, TUR=Turkey, UKR=Ukraine.

In all, 9672 records containing passport data were provided to the European *Secale* Database. The passport databases contained 71 different descriptors. Three descriptors were common to all databases (accession number, name of cultivar and country of origin). Other frequent descriptors were: donor, donor number, geographical site.

Standardization of data

The database structures and data formats were different. The files received were verified with regard to the content of columns and their completeness. A smaller list of descriptors was created, containing all other descriptors. Descriptors close in meaning were joined together.

Botanical names were stored in various forms, ranging from a coded system to full names stored in three fields. The scientific name descriptor (BOTNAME) in ESDB is a combination of related descriptors in databases where infraspecific

levels are indicated by prefixes such as 'subsp.' or 'var.'. The compiler adopted scientific names of accessions according to the botanical classification currently used in the country maintaining the collection. The botanical names were verified using *Secale* monographs (Hammer *et al.* 1987; Kobylyanskyi 1989) and obvious misspellings were corrected whenever possible.

Different codes used for information such as countries, population types, donors and collectors are used in documentation systems. An attempt to standardize donor institution names was made. Different types of notation were unified according to acronyms of institutions (Serwiński *et al.* 1987) and were used with some exceptions, when the information provided was not complete enough to recognize the donor exactly.

In the databases provided, two types of country abbreviations were found: three-letter codes similar to ISO codes and two-letter abbreviations (Ukraine collections). In the ESDB, the country of origin is abbreviated as recommended by ISO 3166 codes (van Hintum *et al.* 1995).

Database structure

As a first step, a unified structure was designed for the database. From the data provided, the most common descriptors were chosen and data files from all collections were transformed using the unified structure. Less frequent descriptors, often specific for a single database, were included in 'wide' descriptors containing related data. Only management data were excluded from the structure of ESDB. An additional descriptor (GENEBANK) was added to distinguish between genebanks maintaining particular accessions. The complete database structure contains 30 descriptors.

Database content

A preliminary survey of the assembled data shows that 69% of the material has identified species names. The database contains 1208 accessions for collected materials (wild, landraces) and 1966 for breeding materials (cultivars, breeding lines). *Secale* accessions can be differentiated (5176 winter, 465 spring and 75 intermediate) on the basis of growth habit.

Identification of probable duplicates

To improve the efficiency of plant genetic resources conservation the rationalization of collections is urgently needed. Identifying and minimizing unnecessary duplication within and between collections is the first part of this effort. The problem of identifying duplicates and its principles was discussed by van Hintum and Knüpffer (1995). Passport data are useful for the identification of probable duplicates between and within collections but this can rarely be done automatically. The procedure requires detailed knowledge of breeding processes, collecting history of the particular crop, and procedures used at the genebanks for data management.

The variety name is a convenient data type for the identification of probable duplicates. A preliminary analysis of accession names showed that 20% of the records are duplicates of other accessions. The identification of probable duplicates using variety names or other designation encounters many problems. Data are stored in databases in different ways. This may be due to transcription, transliteration or translation of foreign names, abbreviation of parts of names, or

changing the order of words. Moreover, the same pieces of information may be found in different descriptors.

Parallel numbers are another source of information about accessions. Parallel numbers are the numbers given to the accessions in other collections. They are mainly indicated in the descriptors 'donor number', 'other number' (e.g. CI number commonly used for identification of accessions) or 'collection number'. These numbers offer a straightforward way of identifying probable duplicates between, and sometimes even within, collections. In the database studied, 1125 accessions have information that were obtained from other genebanks, 583 accessions have donor numbers which indicate probable duplicates (Table 2). Parallel numbers can also help in detecting spelling variants.

To assist in the identification of probable duplicates, the KWIC (key word in context) index, commonly known from bibliographic databases, was used (Knüpffer 1988, 1989). Based on accession number, donor number, collection number, other number and variety name a common descriptor describing each accession was built. By changing the order of names, number prefixes and numbers, 28 000 combinations of accession identificators, designed in this way, were obtained. This method allows detection of accessions with 'matching' similar elements of information, even if these elements are not stored in the database in the same way. All errors in passport data mentioned by van Hintum and Knüpffer (1995) were detected in the database. The efficiency of searching for duplicates increased significantly: 33% of accessions maintained in *Secale* collections throughout Europe can be preliminary identified as duplicates. The results of this analysis have been included in a simple programme where relations among accessions can be studied. However, the decision on the correction of data rests with the collection curators.

The number of duplicated accessions depends on the type of collection. Some big collections (POLPAN, POLIHAR, CZEKROME+CZEKRUZYNE) gather *Secale* materials worldwide. The number of duplicates in these collections is high. There are collections focused on indigenous materials, where the maintained materials are unique (PRTEAN, ROMSUCE, SWENGB, TURARARI). The value of a collection for conservation purposes is, to a large extent, determined by that part of the collection that is unique. Even if this part is small, the collection's value can be extremely high. Table 3 contains an estimation of the percentage of duplicates in the *Secale* collections from which data were received.

During the analysis of duplicates the problem of homonyms and synonyms which is common for commercial varieties, was not considered. The establishment of lists of homonyms and synonyms requires the collaboration of rye curators.

Table 2. The most frequent donor institutions in the European *Secale* Database

Donor[†]	No. of accessions from donor institution with donor number	No. of accessions from donor institution
DEUGAT	45	82
CZEKROME+CZEKRUZYNE	30	105
POLIHAR	86	130
SUNVIR	168	199
TURARARI	137	210
POLPAN	117	399
Total	583	1125

† Refer to Table 1 for full name of institution. SUNVIR=N.I. Vavilov Institute before dissolution of USSR.

Table 3. Indigenous materials and duplicates in *Secale* collections

Institution[†]	Country	No. of access.	% of access. origin. from the country	% of access. origin. from the country and duplicated in other collections	% duplicated access. in collection (excluding access. from the country)
AUTBVAL	AUT }	62	79	29	8
AUTLARINN	AUT }				
BGRIIPR	BGR	337	5	1	72
CHERAC	CHE	63	21	3	44
CZEKROME	CZE }	659	7	5	63
CZERUZYNE	CZE }				
DEUGAT	DEU	878	8	1	12
DEUBGRC	DEU	329	11	2	31
FRAINRACLF	FRA	41	70	0	2
ITAIDG	ITA	382	23[‡](96)	13	3
POLIHAR	POL	1354	12	3	65
POLPAN	POL	1630	7	6	55
PRTEAN	PRT	33	100	0	0
ROMSUCE	ROM	45	96	0	0
SWENGB	SWE	63	100	24	0
TURARARI	TUR	512	100	30	0
UKRIIRSG	UKR	171	46	6	26
Total [§]		6559			

[†] For full names of institutions, see Table 1.

[‡] Accessions from Mediterranean region.

[§] Data from Russia and Spain not included.

Remarks

The apparent poor quality of passport data received from genebanks makes identification of probable duplicates difficult. In some databases only a little information is stored. A more complete spectrum of information is necessary not only for studies on relations among accessions (e.g. studies on duplicates) but also for the evaluation of data contained in a single database.

Relying on perfect matches of information contained in corresponding data fields results in a low percentage of identification of probable duplicates. The KWIC index method significantly increases the efficiency of searching for probable duplicates.

The free availability of the European *Secale* Database will facilitate the rationalization of the content of contributing collections and the exchange of germplasm. The central crop database is an important tool for determining probable duplication. It is also a very useful tool for filling up gaps in collections. The European *Secale* Database will be circulated among rye specialists in Europe, and particularly to the centres which have supplied the data.

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Current status of rye germplasm conservation in Turkey

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Introduction

The introduction of high-yielding semidwarf wheat cultivars caused a decrease in the area planted with rye in Turkey, from 700 000 ha in 1970 to 190 000 ha in 1992. Nevertheless, rye is still cultivated in 60 of the 77 provinces of Turkey (Anonymous 1992).

Anatolia is considered one of the major centres of origin for rye (Scheibe 1935; Gökgöl 1969; Harlan 1975). Recoveries of rye remains dated to 6600 BC indicate the existence and cultivation of rye in Turkey during prehistoric times (Hilman 1978).

Collecting and conservation

The estimated number of rye accessions maintained in world genebanks is 18 000. This figure indicates the minor importance of rye or poor collecting activities for this crop in comparison with wheat and barley, for which 410 000 and 280 000 accessions, respectively, are in genebanks (Donald *et al.* 1987.)

The genetic diversity of cereals in Turkey attracted many scientists to survey and collect cereal species including rye. Antropovs, Berkner, Christiensen-Weniger, Gökgöl, Ervinbauer, Scheibe, Zukhovsky and Vavilov are some of the well-known scientists involved in rye survey and collecting in Turkey. These activities became more organized after the establishment of the Aegean Agricultural Institute and Gene Bank in Menemen, Izmir in 1963. Many expeditions were made to collect and conserve rye species in Turkey. These surveys revealed that rye is distributed all over Turkey, from sea level at the Aegean coast to an altitude of 2460 m on the Eastern Anatolian plateau.

Currently 585 rye accessions collected from Turkey are maintained at the National Gene Bank of Turkey in Izmir. A list of the current collection is presented in Table 1.

Table 1. The rye collection maintained at the Izmir Gene Bank of Turkey

Species	No. of accessions
Secale cereale	389
Secale montanum (anatolicum)	68
Secale ancestrale	9
Secale segetale	4
Secale dighoricum	5
Secale vavilovi	1
Secale fragile (sylvestre)	1
Secale subsp.	108
Total	585

These accessions are divided into two collections: the base collection for long-term storage, conserved under –18/–20°C, and the active collection kept under

0°C. Viability tests are performed every 10 years for long-term storage and every 5 years for medium-term storage.

Documentation

All accessions of the rye collection are documented for passport data. The accessions collected by AARI staff have complete data, but 72 seed samples provided by extension services have incomplete data.

Utilization

Rye germplasm seed distribution from the Izmir Gene Bank of Turkey is made according to general principles of genebank operation and amount of seed available for distribution. Since 1970, rye seed samples have been distributed to 19 institutions requesting germplasm from all over the world.

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Present state of rye germplasm study and conservation in the Czech Republic

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Introduction

Rye is a basic cereal crop for breadmaking in the Czech Republic. Since 1989, its area and grain production have been decreasing yearly. The study and conservation of plant genetic resources have a long tradition in Bohemia and Moravia. A number of research and breeding stations were already working on genetic resources at the beginning of this century, but only the Agricultural Research Institute (ARI) Kroměříž, Ltd. and the Research Institute of Crop Production (RICP) Prague-Ruzyně are dealing with rye genetic resources now.

At present, there are 19 institutions working on plant genetic resources of cultivated plants, but two of them are dealing with cereals (Table 1). Plant genetic resources in Czech collections of cereals are listed in Table 2.

Table 1. Cooperating institutions in the Czech Republic cereal crop genebanks, 1996

Institution	Crop
Cereal Research Institute, Krom↑i× Ltd. Havli◷kova 2787 CZ-767 41 Krom↑i× Tel.: +42(634)426111 Fax : +42(634)22725	*Triticum, Hordeum* spring, *Avena, Secale*
Research Institute of Crop Production Prague-Ruzyn↑ Drnovská 507 CZ-161 06 Praha 6-Ruzyn↑	*Triticum, Hordeum* winter, *Triticale*, *Aegilops*, and other wild species of Triticeae tribe

Table 2. Cereal genetic resources in the Czech collections (1 January 1996)

Crop		Institute	No. of accessions
Wheat	(winter)	RICP Prague	5578
Wheat	(spring)	RICP Prague	3659
Barley	(spring)		2374
Barley	(winter)	RICP Prague	1555
Rye		ARI Krom↑i×	663
Oats		ARI Krom↑i×	1863
Total			15863

As can be seen in Table 2, the largest collections are in wheat. The fewest number of collected are for rye. The current genetic resources collections and databases of rye are shown in Table 3.

Table 3. Current collections and databases of rye accessions at the ARI Krom☞↑i×, Ltd. (15 February 1996)

Accessions	Number
Populations or landraces	96
Varieties	244
Research materials	156
Wild relatives	10
Not defined	160
Total	666

Materials and methods

The methodological coordination of the study and conservation of plant genetic resources in the Czech Republic is provided (together with the Slovak Republic) by the Czech and Slovak Board of Plant Genetic Resources. The board is composed of all collection curators, genebank staff, breeders, representatives of universities and variety testing institutes. General principles are presented in the 'Methodics of Study and Conservation of Plant Genetic Resources for the Years 1992 - 1995' (Dotlačil *et al.* 1991).

Genetic resources of rye and oats are tested, maintained in active condition and stored (for short-term storage) exclusively at the ARI Kroměřiž, Ltd. Accessions of all cereals have been sent for long-term storage to the Czech Gene Bank at the RICP Prague-Ruzyně since 1994.

During the last three years all these activities had to be limited and reduced because of strong cuts in budgets of all institutes. Considering the significant decrease in the staff (generally by about 50%), it is difficult to ensure even the maintenance of some collections. All activities concerning evaluation and utilization of plant genetic resources have been drastically reduced. The direct financing of collections and the genebank should be implemented in the framework of the National Programme on Plant Genetic Resources Study and Conservation (Dotlačil *et al.* 1993).

Evaluation of rye genetic resources

The evaluation of genetic resources is specific for each crop. Therefore, collections are formed on a genus level. A 'Descriptor List of the *Secale* L. genus' has been elaborated for the evaluation of rye (Macháň *et al.* 1986). The systematic broadening of the collection, the evaluation of new accessions, the recording of collected data into a database and the multiplication of stored seed samples are the main activities at both the ARI Kroměřiž, Ltd. and other cooperating institutions.

In spite of different methods for evaluating particular crops, three steps can be recommended at least for main agricultural plants, as mentioned in the 'Methodics of Study and Conservation of Plant Genetic Resources' (Dotlačil *et al.* 1991).

- Preliminary evaluation has a quarantine function in addition to the first, simple, subjective evaluation. The extent of quarantine observations is specific for each crop according to phytosanitary regulations. After the preliminary evaluation, suitable genetic resources are included in the

collection and receive national accession numbers. In case only a small quantity of the sample is available, this step also serves for seed multiplication in artificial isolators.

- Basic evaluation of genetic resources is the main source of data for the descriptive part of the EVIGEZ information system. Comparison of genetic resources with check cultivars in plot experiments is the usual method. Evaluation is generally performed for 2 to 3 years or longer. During the evaluation, productivity and its structure, quality of the product, morphological, physiological and agronomic characters are estimated. Disease, pest and abiotic stress resistance, and occasionally other specific evaluations are usually made if necessary. The data obtained are coded in accordance with the national list of descriptors, using the 1-9 scoring scale, and recorded according to the EVIGEZ system which was developed during the 1970s and 1980s (Holubec *et al.* 1993).
- Special evaluation of genetic resources exceeds in its extent of evaluated characters the basic evaluation framework and therefore can be applied only to a part of the collection. The methodology of evaluation is, as a rule, aimed at selection of donors of particular characters, estimation of yield potential and stability, etc.

The documentation system EVIGEZ

Passport data and other accession-related data are recorded according to the EVIGEZ system (a Czech acronym for Genetic Resources Documentation), at the Department of Genetic Resources of the ARI Kroměříž, Ltd. This documentation system was developed at the RICP Prague-Ruzyně in 1976, and it was transfered into the FoxPro environment in 1989. The EVIGEZ system consists of three basic and other complementary databases. All databases are linked through a national accession number (ECN) which is used for unique identification of the accession.

The EVIGEZ system consists of:

- A passport database containing the basic data on genetic resources in the form of 33 descriptors: general information in the first part, data on breeding process in the second part, and information concerning the collection of wild species in the last part. Passport data represent the basic and most important part of the information on GR and serve for primary orientation in the collection;
- A descriptive database consisting of 110 descriptors elaborated in detail for morphological, biological and agronomic characters. Descriptive characteristics resulting from evaluation are recorded according to the National Descriptor List for *Secale* L. using the 1-9 scoring scale. Data included in the descriptive database serve primarily as a source of information for breeders and correspond with the UPOV system of assessment of important characteristics of the plant;
- Long-term storage monitoring is the third part of the information system. An assigned ECN (a national accession number) is considered a precondition for storing seed samples under controlled conditions. Another key datum, the store code, identifies seed sample location in the storage facility. The EVIGEZ documentation system is continually upgraded.

Seed storage of rye

ARI Krom↑i×, Ltd.

Both rye and oat samples are stored as active collections in common chambers, prepared for cooled conditions. At present, the air-conditioning of these chambers is based on air exchange. After the harvest and cleaning, samples are dried to 13-15% moisture. Active collections, mainly those of rye accessions, lose their viability rapidly. The samples are stored in plastic bags (each per 50 and 500 g) and placed in solid plastic boxes. The average temperature in the chamber is about 15 C. The EVIGEZ system is used for registration. The germination of grains is regularly checked. The regeneration of the seed is performed regularly depending on viability tests. Because of the progress in the research and breeding of new hybrid rye varieties using the cms (cytoplasmic male sterile) system, especially in Germany, the number of rye varieties collected for trials decreased (Macháň 1994).

Genebank at the RICP Prague-Ruzyn

The Genebank was completed in 1988 and began operations in 1989. Its total capacity for long-term storage is 100 000 seed samples. It consists of five cooled chambers, two of which (with a capacity of 55 000 samples) are cooled to +2°C. The other three chambers, operating at –15°C to –20°C have a capacity of 45 000 accessions.

Seed samples entering the Genebank are examined for their purity, germination and health. If the seeds conform with the requested parameters, they are dried to 4-8% moisture content and placed into sealed glass jars in which they are stored in moving shelves in cooled chambers.

For most species the storage method allows maintenance of satisfactory viability of seeds without regeneration for 15 to 20 years.

Seed samples from active collections are distributed free to users (mostly breeders, researchers, cooperating genebanks). They are used only in breeding and research, not for commercial purposes. Reciprocal exchange or information on the results of evaluation (use) of the sample can be requested. These data or the seeds are limited and in full correspondence with the legal variety protection.

Results

The RICP Prague-Ruzyně and ARI Kroměřiž, Ltd. are responsible for the research project 'Assessment of Genetic Resources of Cereals According to Their Diversity and Conservation'. The theoretical and practical goals of this project are as follows:

- systematic and purposeful broadening of the collections for the needs of both research and breeding
- study, characterization and assessment of cereal collections
- preparation of passport and descriptive data for the EVIGEZ database
- preparation of seeds for storage in the Genebank for research and breeding
- seed multiplication, viability tests, etc.

Collection, study and utilization of rye genetic resources have a long-term tradition in the Czech Republic. The ARI Kroměřiž, Ltd. closely cooperates with the RICP Prague-Ruzyně and is the responsible institution for all above-mentioned

activities, especially those connected with winter wheat, spring barley, oats and rye.

International cooperation in this field of activities, exchange of new methods of assessment, seeds and other results also play a very important role.

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Rye growing and germplasm conservation problems in Slovakia

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With an area of 49 036 km², Slovakia belongs to the small Central European countries. It is an industrial and agricultural country with intensive agriculture. Agricultural land covers 2 446 000 ha, of which 1 483 000 ha is arable land.

Rye is very important in breadmaking (especially for special nutritional products) and as a fodder crop. It is grown on an area of 30 897 ha, representing 2% of the arable land and 2% of the cereal production. Average yield in the last year was 3.09 t/ha and it is expected to increase in the future (Anonymous 1996). The highest average yield – 3.43 t/ha – was reached in 1991. The majority of cultivated varieties comes from Poland, Czech Republic and Germany.

Until 1970 rye was bred in four breeding stations (Radošina, Víglaš-Pstruša, Malý Šariš and Král'ová at Senec). We had varieties of domestic origin (Radošínsky Rekord, Vígl'ašská, Terrasol), but after 1962 we grew prevailingly a rye called České from Bohemia, and Kustro and Danae from Germany. Presently rye is no longer bred in Slovakia (Klinovský *et al.* 1970).

In 1951 the Research Institute of Plant Production in Piešťany was established as the only institute of this kind in Slovakia. In Piešťany genetic resources of wheat, triticale and barley were studied. Genetic resources of rye were studied in Kroměřiž (Czech Republic). After the split of former ČSFR in 1994 Slovakia began to build its own rye collection. The RIPP Piešťany coordinates a scientific and technical project 'Collection, Study and Conservation of the Cultivated Plants Gene Pool in Slovakia', within the scope of which the research task 'Rye Genetic Resources' is being addressed. The main objectives of this task are as follows:

- to collect old domestic genetic resources (released varieties, old landraces and breeding material)
- to multiply the obtained samples
- to collect a world assortment (varieties, breeding material, wild species)
- to evaluate selected characters and characteristics
- computer registration
- long-term storage
- utilization of results.

The genetic resources obtained are evaluated under field conditions at the experimental station of RIPP Piešťany within the framework of the following experiments:

- collection nursery – obtained varieties are included here with the aim of multiplication, evaluation and adaptation in given agroclimatic conditions
- evaluation nursery – selected varieties from the collection nursery are evaluated for 2 years in comparison with standard varieties.

The collection is evaluated according to the descriptors of the genus *Secale* (Macháň *et al.* 1986). During the vegetation period, the usual phenological observations are made, and errors and productivity elements are evaluated.

Until now 118 rye accessions have been collected and 12 genotypes tested (Table 1). Computer registration was made for this collection in the programme FoxPro ISGZS (Table 2). It is a new modified version of the programme EVIGEZ, developed by the Gene Bank of RICP Prague-Ruzyně in the Czech Republic.

Passport data were completed for 118 genotypes. The collection is stored in air-conditioned storage facilities in RIPP Piešťany. This year, the construction of the Gene Bank in RIPP was finished and it will be operational in the second half of 1996. The Gene Bank ensures the safe sample maintenance and long-term conservation as safety-duplication within the scope of cooperation with the Czech Gene Bank in Praha-Ruzyně.

Table 1. Number of collected varieties of *Secale*

	Winter	Spring
Varieties	93	2
Breeding material	7	–
Old varieties	12	–
Wild species	4	–
Slovakia	1	–
Czech Republic	17	–
Foreign	98	2
Total	116	2

Table 2. Computer registration of passport data

Descriptor	No. of characters	Input
Institute	2	9,K
Crop type	3	X,K
National accession number	5	9,P
Availability	1	X,K
Conservation	1	X,K
Herbarium	1	X,K
Inclusion in collection year	2	9,P
Genus, species	85	X,P
Cultivar name	30	X,P
Country of origin	3	X,K
Donor country	12	X,K
Acession number of donor country	12	X,P
Other number	12	X,P
Ploidy	1	9,K
Status of sample	1	9,K
Breeding method	1	9,K
Habitus	1	9,K
Final year of breeding	4	9,P
Year of registration	2	9,P
Year of restriction	2	9,P
Originator	10	X,K
Pedigree	120	X,P
Botanical name code	6	X,P

Input: 9 = numerical, X = alphanumerical, K = coded, P = direct.

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Conservation and evaluation of rye germplasm in Romania

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Native rye genetic resources

The Romanian flora contains about 3350 species of vascular plants. Among these, there are 182 wild species related to crop plants, 67 agricultural plant species, 73 lawn species and 42 medicinal species.

The diverse ecological conditions in Romania favoured the appearance of a great number of ecotypes and populations adapted to different ecological conditions. There are species in the wild flora that have never been used but which could constitute important native sources of vegetal products or gene sources in plant breeding, for example *Secale silvestre* and *Secale montanum* which are adapted to sandy soils.

In Romania old cultivars are still used in individual farms on relatively limited areas. Many landraces of rye are cultivated in intramontane depressions, especially in the north of the country, in Bucovina and Maramures. With the establishment of the Suceava Vegetal Genetic Resources Bank it became possible to collect, evaluate and conserve those landraces.

In situ conservation of rye germplasm, represented by landraces and traditional varieties, is achieved in individual farms and it is a dynamic conservation because the local material is permanently replaced by the improved one.

The conservation of rye genetic resources *ex situ* is done mainly by the Suceva Genebank and plant breeding centres from institutes and by agricultural research stations.

The Suceava Genebank and its collection of rye germplasm

Because of continuous loss and degradation of important plant genetic resources and diminution of biodiversity in Romania, the Plant Genetic Resources Bank was founded in Suceava. It is an institution with a national status, financed from state budget and designated to keep the national collection of phytogenetic resources.

The rye collection of Suceava Genebank contains 47 samples, including 37 landraces, 2 foreign varieties and 8 other forms. The seed samples of the active collection are kept in glass jars hermetically sealed. The conservation is realized in chambers, at 4^{o}C and humidity content of seeds between 5 and 7%. The minimum value allowed for the germination ability is 85%. A small part of the material (20%) is duplicated, kept in the donor institutes and agricultural research stations. The Genebank currently maintains only an active collection, although facilities exist to keep a base collection. Unfortunately, the refrigerating equipment does not correspond to this purpose.

The rye germplasm gathered by the Suceava Genebank is characterized and evaluated according to IPGRI descriptors in field and laboratory conditions. The Suceava Genebank has 5 ha of arable land for field research as well as specialized laboratories (biochemistry, cytology, seedology, plant protection, etc.). In the research institutes and in other research units, the rye genetic resources are studied in plant laboratories and in greenhouses. When the germination falls under 85% it is necessary to multiply the material.

In the Suceava Genebank the information system is coordinated by the Computing Office. Information concerning the rye germplasm collection is divided into three groups:

- passport data
- conservation data
- characterization / evaluation data.

In 1994 the Centre for Genetic Resources, The Netherlands, in collaboration with IPGRI, implemented a complex programme, supported financially by the Netherlands Ministry of Agriculture, Nature Management and Fisheries, to increase the quality of documentation systems and their standardization. The Suceava Genebank was included in this programme and was provided with two microcomputers (DX-386 and DX-486) and two printers. The second step of this programme is to achieve a standard in the exchange of information.

Rye germplasm collections of institutes and research stations

In 1995 Romania had a rich stock of germplasm for crop plants (over 93 000 samples), of which 71% were native. These include landraces (2.6%), wild forms (1.9%), and the rest (95.5%) predominantly breeding lines. The composition of the samples of foreign origin was similar.

The rye germplasm collections of institutes and agricultural research stations include 374 samples with 4 wild forms, 5 landraces, 183 varieties, 27 hybrids, 125 lines and 30 synthetics. Many of these samples are held by the Suceava Agricultural Research Station.

Use of rye genetic resources in Romania

The use of rye germplasm in plant breeding programmes is carried out in agricultural research stations, especially in the Suceava Agricultural Station. The ratio of rye genetic resources (native forms) used in breeding programmes is 26%.

To date, in Romania, the areas cultivated with rye are diminished, as a result of the new process of privatization of agriculture. However, the rye breeding programme is well outlined. Its main aim is to increase the quality and the production of rye crops, to achieve a constant production level and to reduce the genetic vulnerability. All rye cultivars are based on native varieties such as Gloria, Orizont, Sucevean, Ergo, etc. Other important objectives of the rye breeding programme are:

- to obtain varieties with medium or short size and an increased resistance to diseases (especially to mould) in the north of the country
- to obtain precocious varieties with a great resistance to drought for the south of the country.

In conclusion, the rye breeding activity at the national level covers the needs for production of crop material. This activity is coordinated by the Academy of Agricultural and Forest Sciences.

Characterization of the rye collection of the Warsaw Agricultural University

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The rye collection was initiated by Professor B. Kubicki in the middle of the 1970s. About 300 lines of high inbreeding level are maintained. They originate from old Polish varieties and chemical seed mutagenesis of two inbred lines.

One part of the collection comprises the variation of different characters important in breeding such as plant height, length of spike, 1000-seed weight, protein content, number of spikelets, weight of seeds from spike, spike density, earliness, combining ability, etc. The other part of the collection includes a big variation of physiological, morphological and biochemical traits valuable in genetic work. The inheritance of characters was estimated. The collection was also screened using *in vitro* techniques: (1) for regeneration ability from immature inflorescence and embryo; (2) for S-(2-aminoethylcysteine) (AEC) resistance. The lines with prolific regeneration, the lines with lack of reaction and also AEC-resistant lines were isolated.

Status of the Secale *collections in Portugal – conservation, characterization, evaluation and documentation*

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Introduction

Rye is an important crop in hinterland Portugal and in particular in the northeast region (Trás-os-Montes). This region alone contributed, on average, for the period 1991-93, 60.7% of the total national production and, for the same period, 50.3% of the national area devoted to rye production. Table 1 shows the real values used for calculation of the percentages.

Rye production and area devoted to rye are decreasing in Portugal. In 1991, the overall production was 80 358 million tonnes, while in 1993 it was 66 727 million tonnes (Table 1) (FAO 1991, 1994a). This tendency led to a growth in rye imports, from 2353 million tonnes in 1992 to 11 604 million tonnes in 1994 (FAO 1994b).

Although the national area devoted to rye decreased from 86 533 ha in 1991 to 72 516 ha in 1993, in the Trá-os-Montes region the area showed an opposite trend in the same period as it increased from 39 024 ha to 44 226 ha (Table 1).

In general, farmers grow local landraces but, in recent years, foreign varieties have been introduced. In its 1995 edition, the National Varieties' Catalogue lists two advanced cultivars of foreign origin. The introduction of these varieties can lead to genetic erosion, either by abandonment of the local populations and/or by introgression of the local varieties with the new, introduced varieties.

Although emphasis has been given to cereal germplasm – cereal accessions account for 43.8% of the total national holdings – Portugal has a wealth of rye germplasm that is urgently in need of conservation, characterization, evaluation and being made available for utilization. It is important to keep in mind that the highest lysine content in a rye world collection was observed in a population from northern Portugal, while in a screening programme for aluminium tolerance it was found that some populations from Trás-os-Montes have a behaviour similar or superior to Dańkowskie Złote, considered as tolerant (Pinto-Carnide *et al.* 1989).

Status of collections

Conservation

From a total of 14 056 cereal accessions, 769 (5.5%) are of rye. These collections are conserved in five different institutions: Genebank - National Agricultural Research Station (Estação Agronómica Nacional) at Oeiras; Portuguese Plant Germplasm Bank (Banco Português de Germoplasma Vegetal - BPGV) at Braga; University of Trás-os-Montes and Alto Douro (UTAD) at Vila Real; National Plant Breeding Station (Estação Nacional de Melhoramento de Plantas - ENMP) at Elvas; and Regional Directorate of Agriculture of Trás-os-Montes (Direcção Regional de Agricultura de Trás-os-Montes - DRATM) at Mirandela (Table 2).

Table 1. Regions, areas and production of rye in Portugal for the period 1991 to 1993

Region	Year	Area (ha)	Production (Mt)
Entre Douro e Minho	1991	7596	7419
	1992	7111	6129
	1993	6288	5976
Trás-os-Montes	1991	39024	44490
	1992	34502	44855
	1993	44226	42015
Beira Litoral	1991	3138	3159
	1992	3129	1925
	1993	3234	1949
Beira Interior	1991	33676	21656
	1992	27585	14843
	1993	16939	15802
Lisboa e Vale do Tejo	1991	271	235
	1992	166	142
	1993	276	232
Alenlejo	1991	2461	3168
	1992	2196	1537
	1993	1153	633
Algarve	1991	367	231
	1992	355	69
	1993	400	120
Total	1991	86533	80358
	1992	75043	69500
	1993	72516	66727

Table 2. Portuguese institutions involved in rye genetic resources, total number of accessions and number of accessions characterized and evaluated

	Number of accessions		
Institution	**Total**	**Characterized**	**Evaluated**
Genebank - Department of Genetics and Breeding National Agricultural Research Station - Oeiras	580	0	0
Portuguese Plant Germplasm Bank - Braga	115	0	0
Department of Genetics and Biotechnology University of Trás-os-Montes and Alto Douro - Vila Real	58	12	4
National Plant Breeding Station - Elvas	15	0	0
Regional Directorate of Agriculture of Trás-os-Montes - Mirandela	3	0	0

The accessions are maintained in good conditions, under long-term storage (around -18°C) using different types of packaging (paper, plastic and aluminium laminated foil bags).

Safety-duplication has not yet been done in a systematic and coordinated manner. In general, there is no safety-duplication of the accessions and special attention should be paid to this activity which should be included within the priorities of Genebank curators.

Characterization and evaluation

Characterization and evaluation of the accessions are an important prerequisite without which collections have a limited value. Only a few accessions (12) are characterized. This characterization was made for 15 characters according to breeders' interest and evaluated for aluminium tolerance. Four accessions were evaluated through isozymes, RFLPs and RAPDs (Table 2).

According to the characterization data three accessions are being utilized in a rye breeding programme at UTAD, Vila Real.

Documentation

Documentation, together with characterization and evaluation, plays an important role in better management, sustainable utilization and in facilitating the exchange of germplasm. The accessions conserved at the Genebank of the National Agricultural Research Station and at the Portuguese Plant Germplasm Bank have computerized passport data, while the other collections are documented manually.

Conclusions

Although the collection already contains an acceptable number of accessions, there are regions where collecting is still needed. However, new collecting missions should be planned carefully, and solidly based on the scientific analysis of the passport data of the available rye accessions, therefore avoiding duplication of work and allowing the maximization of the, always scarce, human and financial resources.

Priority should be given to the characterization and evaluation activities, therefore allowing the identification of the potentialities of the Portuguese rye germplasm, hence facilitating and promoting its sustainable utilization.

Documentation of the collections should be completed and the goal of having all passport, characterization and evaluation data computerized should be pursued.

Regeneration of allogamic material poses particular problems. However, the necessary human and financial resources should be made available so that in the future samples can be made available to the scientific community.

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Rye genetic resources at the Nordic Gene Bank (NGB)

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Introduction

The Nordic Gene Bank has collected material and information on cultivated plants in the Nordic countries since the institute was founded in 1979. Seed material has been acquired from breeders, through collecting missions, donations of single accessions and complete collections, and through repatriation of material from other countries. NGB also stores some safety-duplicates of base collections for other genebanks. Information from inventories and breeders' pedigrees, passport data for collected material and evaluations of acquired accessions and amounts and viability of the accessions are included in the databases at NGB.

Rye

Rye, and other crops, were collected among Finnish farmers during collecting missions to Finland in 1980-83. Single accessions of some local Swedish material and primitive varieties have been provided by private persons. Modern varieties have been provided by Nordic breeders, as well as breeding lines and some non-Nordic material originally kept in collections at other Nordic institutes. Inventories of Nordic varieties list 6 Danish, 14 Finnish, 3 Norwegian and 20 Swedish varieties. In addition, 5 primitive varieties of rye are listed. NGB holds seed samples of around 60% of the types described in the inventories. Nordic breeders have provided 35 breeding lines of rye to NGB's ordinary collection. A special collection of breeding material (the Müntzing collection) contains 131 accessions. NGB also stores a safety-duplicate of base collection of rye collected in Turkey. Some non-Nordic accessions of rye have become part of NGB's collection by inclusion of accessions earlier stored at the Royal Veterinarian and Agricultural University of Denmark. Part of this material was collected in Central Asia during collecting missions in the 1940s. This collection also includes a few accessions of wild *Secale* species (*S. montanum*, *S. vavilovii* and *S. fragile*).

Contributions to ECP database

NGB has provided passport data for 63 accessions to the first round of the ECP *Secale* database: 20 accessions are Nordic varieties, most of them derived directly from the original breeder; 5 are primitive varieties and 38 are Finnish landraces. More accessions may be added after results from further evaluation trials become available.

Summary of *Secale* accessions

	Accepted for storage		Responsibility
	Long-term	Short-term	pending
Nordic			
Modern varieties	20	17	–
Primitive varieties and landraces	43	27	–
Unevaluated collected material	–	–	31
Breeding lines	–	35	–
Special collections (Müntzing)	–	131	–
Non-Nordic			

Varieties	–	15	–
Landraces	–	9	–
Wild *Secale*	–	3	–

Winter rye breeding in Lithuania

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The breeding of winter rye was started in Lithuania in 1922 by Prof. D. Rudzinskas. During the first 10-year period, winter rye breeding was carried out on a very small scale. From 1932 onwards, local rye varieties or populations collected from different localities of Lithuania were studied at the Dotnuva station. Unfortunately, the collected material turned out to be of low value.

During the post-war period the basic method used in winter breeding in Dotnuva was intervarietal hybridization with individual selection. As a result the following winter rye varieties were developed and registered.

- Dotnuvos Aukstieji was developed by individual selection from the variety Rizhskaya. In 1951-54 its average yield was 2.8 t/ha. The variety was registered from 1950 to 1964.
- Lietuvos 3 – this variety was bred by crossing Viatka and Dotnuvos 8. The average grain yield was 4.2 t/ha in 1969-71. In 1957 this variety was registered in Lithuania, in 1962 in Bielorussia and Ukraine. It is a long-stalked, relatively resistant to snow mould and root diseases, winterhardy variety, with a high and stable grain yield potential. The grains are rather large.
- Baltija – this variety was developed by intervarietal hybridization of Sangaste and Dotnuva 8. According to the data of the official trials in 1969-71 it produced the average yield of 3.7 t/ha. The variety is distinguished by large grains. According to its characteristics it is similar to Lietuvos. It was registered in 1962-76.
- Kombaininiai – this variety was developed by crossing the Viatka and Petkus short-stalked varieties. According to the data of the trials in 1969-71 it produced 4.5 t/ha of grain. The straw of the variety is short. It is resistant to lodging. In 1973 it was registered in Lithuania, in 1974 in Bielorussia.
- Rukai – this variety was bred by individual selection from a complex hybrid combination, by crossing a natural tetraploid mutant EM-1 with the varieties Dotnuvele and Kombaininiai tetra. The variety is short-strawed, winterhardy, resistant to lodging, and has medium resistance to leaf diseases. Grain yield amounts to 7.6 t/ha. The grains are coarse with high protein content, 1000-seed weight is 45-52 g. The spikes are long. The variety has been on the registered list of Lithuania since 1994.
- Duoniai – this variety was bred by individual selection from a complex hybrid combination by crossing a natural diploid mutant with the varieties Saratovskaja 4, Voschod 1 and Kustro. It is high yielding, short strawed, resistant to lodging, winterhardy, medium resistant to leaf diseases. Its growth period is 2-3 days longer than that of Kustro 3 and is the same as Ciulpan 3 and Talovskaja 15. The variety has been registered in Lithuania since 1994, in Latvia since 1996.

ECP/GR *Secale* Genetic Resources Workshop

Introduction

The second *ad hoc* ECP/GR genetic resources workshop was held in Warsaw, Poland from 5 to 6 July 1996. Thirteen Delegates representing 15 of the 30 ECP/GR Member countries attended the Workshop. The Nordic Gene Bank, USDA-ARS and IPGRI were also represented (see Appendix I. List of Participants).

The Workshop was opened by Dr W. Podyma who welcomed the participants on behalf of the Polish National Coordinator, Prof. Czembor and the Polish Genebank, which is comprised of a large number of independent institutions. He mentioned that the previous *ad hoc* meeting on *Secale* (Jokioinen, Finland, 1982) had contributed to setting in motion new initiatives within ECP/GR by establishing the first ECP/GR Crop Database (Serwiński and Konopka 1994). He expressed his hope that similarly, this Workshop would not only contribute to consolidating collaboration in the area of *Secale* genetic resources, but also make a contribution toward the development of ECP/GR as a whole.

Dr T. Gass, ECP/GR Coordinator, gave an overview of the history and objectives of ECP/GR. He described the new structure of the Programme which was adopted by the Steering Committee at its meeting in Nitra (Slovakia) in 1995 and went on to emphasize that ECP/GR was made up of a multitude of inputs in kind without which no results would ever have been achieved. He expressed his hope that the Workshop would be successful in setting itself a practical workplan.

Secale collections in Europe

The discussion presented here refers to country presentations given by delegates in Session V: Rye germplasm collections in Europe (see Session V of this report).

The presentation of the Turkish rye programme highlighted the impact of widespread wheat production and consumption on the cultivation of rye. Some participants felt that the species could be considered a 'neglected crop' and that its wide genetic diversity and potential for adaptation were underutilized. It was noted that in a few countries of Europe (e.g. the Czech Republic) in which the consumption of rye bread is traditionally very high, the production currently lies below the demand. Several examples were mentioned in which *Secale* production was still, or had recently become, a real economic alternative for agriculture in mountainous or other marginal areas.

Most participants noted the serious economic constraints which their national *Secale* conservation and breeding programmes were facing.

European *Secale* Database (ESDB)

Presentation of the Database

W. Podyma presented the ESDB which was recently updated (refer to the article in Section V). In total, 9672 records containing data from 17 institutions were provided to the ESDB. The original databases contained 71 different descriptors.

The first step was to design a unified structure for the database. Sixty-nine per cent of the material has an identified species name. The database contains 1208 records of collected materials (wild, landraces) and 1966 of breeding materials (cultivars, breeding lines). The rest of the accessions are recorded as "not

determined". The information regarding the growth habit allows the identification of 5176 winter, 465 spring and 75 intermediate *Secale* accessions.

The analysis of accession names showed that 20% of the accessions are duplicates of other accessions. A more complex analysis of all passport data using the KWIC index increased the searching efficiency and allowed 33% of the accessions to be tentatively identified as duplicates.

Participants were impressed by the analysis of the ESDB which raised a number of questions regarding the unnecessary duplication of material on the one hand and, on the other hand, the lack of safety-duplication of some smaller collections composed mainly of landraces. The KWIC Index was commended as a very useful tool to further refine the searching of duplicates on the basis of accession names. It was emphasized that the analysis could be even more thorough if based on a wider range of passport descriptors. It was recognized, however, that the quality and coverage of the passport data in the ESDB is still insufficient to implement this.

Regarding the aspect of duplication, it was emphasized that a certain level of duplication cannot be avoided and that a number of institutions wish to keep large collections for research or breeding purposes. It was agreed that large collections of foreign varieties used in breeding or research **need not be declared as genetic resources in the ESDB** since they are also maintained by their country of origin, are generally available and do not fall under the national obligations to the Convention on Biological Diversity to conserve indigenous material.

Discussion on objectives and updating mechanism of the ESDB

The Group discussed the objectives of the ESDB and agreed on the following:

- facilitate the coordination and management of conservation activities
- distribute information about collections to users
- promote the interaction between curators, breeders and other users
- promote the mobilization of resources toward the conservation and use of *Secale* genetic resources
- provide support to research activities, in particular to breeding research.

It was agreed that W. Podyma will send a copy of the ESDB to all participants of the meeting by the end of July 1996. Every contributing institution will check its data, make necessary corrections and provide its update to the ESDB manager by the end of September 1996.

It was recommended that the Database Manager would request updates from genebanks every 2 years by providing detailed instructions on the format of data to be submitted for Passport data. The members of this Group will ensure that the data submitted to the ESDB from their country are complete and of high quality. The language for data submitted to the ESDB should be English.

M. Hulden informed the Group that NGB is currently evaluating material which has not yet been documented in the ESDB. After evaluation and if the material is included into NGB's rye collection, he will forward the corresponding data to IHAR (before the end of 1996).

It was noted that the UK had closed its rye breeding activities and had transferred its collections to Germany for safekeeping.

D. Gogas provided passport data of the rye collection maintained in the Greek Gene Bank. It was also noted that data from Belarus and the F.R. of Yugoslavia (Serbia and Montenegro) are missing from the ESDB. IPGRI agreed to contact the

respective National Coordinators to request that information be sent about rye collections in those countries (before September 1996).

The issue of free exchange of data was discussed and it was agreed that genebanks should provide a public service of excellent quality. It was agreed that free and intensive exchange of information is essential to the cooperation among genebanks and that ESDB can play an important role in promoting this exchange.

The Group agreed that the ESDB is an important tool for the distribution of information about the collections. IHAR was thanked for the work done to date in managing the ESDB and was asked by the Group to continue this activity.

Passport data in the ESDB

W. Podyma presented the structure of the passport data in ESDB. It was noted that the descriptors used are similar to those proposed at the recent Wheat Genetic Resources Workshop (Paris, March 1996). T. Gass presented ongoing activities in the standardization of documentation of crop genetic resources in Europe. During a Documentation Workshop to be held October 1996 in Budapest the standardization of passport descriptors for all crops will be discussed along with other issues related to the access and use of central crop databases.[7] Participants agreed that it was important that issues regarding passport data be resolved by managers of European databases for all crops simultaneously.

Characterization and evaluation data in the ESDB

The Group discussed the possibility of including characterization and evaluation data in the ESDB to make it more useful to users and in particular to breeders. While recognizing that the primary role of curators is not to evaluate collections, it was agreed that they should actively seek to increase the information associated with each accession. While re-emphasizing that the most comprehensive information for each accession is desirable, the Group agreed that a limited number of descriptors should be considered mandatory to ensure the usefulness of the ESDB.

M. Hulden presented the descriptors used by NGB and a discussion followed on the type of descriptors that would add most value to the database. A subgroup consisting of S. Roux, D. Gogas, M. Holden and M. Niedzielski was asked to prepare a list and draft the respective scales for assessment. On behalf of the subgroup M. Niedzielski later presented this list of descriptors to the whole Group. After discussion, the following list of minimum descriptors was agreed upon (the number in parentheses refers to the IBPGR Descriptors for rye and triticale, 1985):

- seed colour (descriptor 4.3.1)
- length of spike (descriptor 4.2.3)
- number of spikelets per spike (4.2.5)
- plant height (4.1.2)
- days to ear emergence (4.2.1) – in case of winter form the descriptor does not reflect the real length of plant vegetation. Start point should be determined according to practice in country.
- winterhardiness (scale 1-9)

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[7] Lipman, E., M.W.M. Jongen, Th.J.L. van Hintum, T. Gass and L. Maggioni, compilers. 1997. Central Crop Databases: Tools for Plant Genetic Resources Management. International Plant Genetic Resources Institute, Rome, Italy / CGN, Wageningen, The Netherlands.

- lodging (7.7)
- 1000-grain weight (4.3.3)
- protein content (6.3.3)
- lysine content (6.3.4)
- falling number – this descriptor is correlated with two very important characters in rye: sprouting tendency and seed quality.

The characters should be evaluated and measured according to rules accepted in the 'Descriptors for rye and triticale'. Additional information about methods of evaluation can be provided to the database.

The inclusion of disease susceptibilities in the list of minimum descriptors was discussed. The participants recognized that these characters are strongly dependent on environmental and physiological factors and should not be included in the minimum descriptor list. Nevertheless these data should be collected and included in the database whenever possible.

Quality descriptors are expensive to score and substantial support should be provided to institutions which agree to screen the collection for these traits.

The participants agreed that, in addition to the types of data mentioned above, references to any other valuable information about the accessions should also be included in the database.

The European *Secale* collection

Introduction

The need for a formal sharing of responsibilities was discussed. The Group agreed on the establishment of a decentralized European *Secale* Collection consisting of the *Secale* accessions which genebanks in Europe have agreed to maintain on behalf of all the Member Countries of ECP / GR.

Objectives and scope

The following objectives were agreed upon for a European *Secale* Collection:

- ensure the safe conservation of *Secale* genetic resources in Europe
- formalize the sharing of responsibilities
- comply with countries' obligations under the CBD to conserve indigenous genetic resources
- enhance the use of the *Secale* genetic resources collections in Europe.

It was agreed that the European *Secale* Collection will include:

- all wild and cultivated species of the Secale genus
- material of the following types:
  - cultivated varieties (cultivars) in current use and newly developed varieties
  - obsolete varieties
  - primitive cultivars or landraces
  - wild and weedy species, near relatives of cultivated varieties
  - special genetic stocks
- material for which distribution is not restricted
- material of indigenous origin (bred or collected)

- material collected or obtained from other countries (providing reference to that country in the 'original country of origin' field)
- material missing from other collections or of which safe conservation is doubtful.

The inclusion in the collection of registered varieties is useful as these provide valuable quantitative traits and represent the most frequent source of material for breeding. It is, however, recognized that in many countries, access to this material requires prior informed consent from breeders. Breeding lines should be included only if they are well documented.

Workplan for the establishment of the European Secale *Collection*

On the basis of the fields 'country of origin' and 'institute of origin', the Manager of the European Secale Database will suggest for each accession a 'Reference Collection'. After agreement with the other *Secale* collection holders, the 'Reference Collection' will formalize its commitment by depositing a list of these accessions with the Database Manager (one copy to be kept by the ECP/GR Coordinator). The designated accessions will be marked (European *Secale* Collection) in the Database.

Accessions of foreign origin will be listed in a summarized form by the Database Manager and circulated among the Group to facilitate eventual repatriation of material.

Responsibilities

The collection of reference

- Ensure that the material is maintained under long-term conditions in compliance with International Standards
- Ensure that a safety-duplicate of at least 1500-2000 viable seeds is deposited in a genebank within another ECP/GR country. It is recognized that more seeds will be necessary in case of genetically heterogeneous accessions (standard for base collections)
- Communicate to the ESDB Manager the name of the host genebank for the safety-duplicate as well as the date on which the material was safety-duplicated
- Maintain an active collection with sufficient quantities of seed (12 000 seeds are recommended) from which the declared accessions are distributed if requested
- Provide access to the declared accessions to all users from ECP/GR Member Countries (exception is made for registered varieties - see above)
- Announce to the ESDB Manager if the material can no longer be maintained and actively seek a new reference collection willing to maintain the material. If no new host genebank can be found, then maintain the material under long-term conditions for at least another 2 years.

The European *Secale* database manager

- Effect regular updates (every 2 years) of the database and make them available to the collection holders
- Effect changes to the database when informed by the collection holders
- Rapidly forward to the 'Collection of Reference' any requests for seed
- Facilitate the repatriation of material by distributing relevant information about accessions conserved in foreign countries.

The genebank hosting safety-duplicates

- Maintain a sufficient quantity of the safety-duplicated material in long-term storage conditions in compliance with international standards
- Not distribute the material
- Clearly designate the safety-duplicate as such in any of its accession lists
- Immediately notify the reference collection in case of any problem
- Not carry out viability tests
- Not regenerate the safety-duplicated material.

W. Podyma agreed to draft a list of accessions and corresponding Primary Collections and forward it to the participants by end of September 1996.

IPGRI will draft a Letter of Agreement by which institutions and countries can designate the germplasm they commit to conserve on behalf of the Member countries of ECP/GR (by end of September 1996).

Issues related to enhancement of the use of *Secale* genetic resource

The Group discussed various issues related to enhancing the use of *Secale* genetic resources. It was agreed that the accessibility and quality of the information provided within the ESDB are key factors influencing the degree of use of the collections. In addition to this, it was suggested that most promising accessions should be evaluated in a number of different locations. Participants agreed that this could be done in addition to the main conservation work and would yield valuable results for breeders and other users. It was pointed out, however, that additional funding would be required to carry out this activity.

Use of genebank material in research to facilitate the evaluation and characterization of the material should be enhanced by attracting funding from universities, national science academies and other sources of research funding. This type of activity would be intermediary between regular genebank activity (documentation and maintenance) and the use of the material by breeders. Along with the decrease of direct public funding, the necessity to find alternative ways of funding becomes obvious. Establishing diverse links between genebanks, research institutes and breeding institutes may also help to make genetic conservation better known and appreciated.

The Group agreed that the next ECP/GR *Secale* Workshop should focus on the aspect of enhancing use of *Secale* germplasm, addressing *inter alia*:

- the accessibility of information about collections
- the multilocational evaluation of promising accessions
- the conservation of *Secale* genetic stocks in genebanks.

Conclusion

The participants reviewed the report of the workshop and decided to adopt it as a workplan for the enhancement of ESDB, and as an initial agreement for the establishment of the European *Secale* Collection. The coordination of these activities within the framework of the ECP/GR Cereals Network is considered essential to further strengthen the cooperation among the participants.

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Appendix II. Related information

Invited guests

Prof. Katarzyna Duczkowska-Małysz
Undersecretary of State
Ministry of Agriculture and Food Economy

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Plant Breeding and Aclimatization Institute

Mr Marian Jerzy Nasiadko, Director
Department of Agricultural Production
Ministry of Agriculture and Food Economy

Mr Wiesław Wawiernia
Department of Agricultural Production
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Dr Roger Wentzel
Agricultural Counsellor
Office of Agricultural Affairs
US Embassy in Warsaw, Poland

Prof. Saturnin Zawadzki, Secretary
Division of Agricultural and Forestry Sciences
Polish Academy of Siences

Mrs Antonina Korzeniowska
Department of Agricultural Production
Ministry of Agriculture and Food Economy

Technical attendants

Mr Zdzislaw Bujalski

Ms Monika Ciepłowska

Ms Monika Gazda

Mr Jacek Piekarniak

Ms Wieslawa Potkańska
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Mr Jaroslaw Nowosielski
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