Reproductive Biology of Cassava (*Manihot esculenta* Crantz) and Isolation of Experimental Field Trials

Mark E. Halsey, Kenneth M. Olsen, Nigel J. Taylor, and Paul Chavarriaga-Aguirre*

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is a vitally important food source for many people in developing tropical countries. There are significant opportunities for improving the compositional qualities and pest resistance of cassava, and modern biotechnology is expected to play an important role in these improvements. The testing and development of genetically modified cassava will of course be subject to regulatory review, and experimental field trials must be performed in a fashion that prevents gene flow from the regulated plants. Methods to ensure reproductive isolation will be derived from a fundamental understanding of the biology of the crop. A current and comprehensive document on cassava reproductive biology is not yet available but is essential to guide regulators and scientists in planning and evaluating measures for reproductive isolation of confined field trials. This paper compiles a current view of the reproductive biology of cassava for use in experimental design and regulation of confined field trials. With the current state of knowledge on gene flow and seed dormancy in cassava, three methods for reproductive isolation of regulated experimental plots may currently be recommended: (i) removal of flower buds before flowering, (ii) destruction of plants before flowering, and (iii) floral bagging to contain pollen and seed. Areas for further research in cassava biology and biosafety are suggested.

M.E. Halsey and N.J. Taylor, Donald Danforth Plant Science Center, St. Louis, MO 63132; K.M. Olsen, Dep. of Biology, Washington University, St. Louis, MO 63130-4899; P. Chavarriaga-Aguirre, CIAT, AA 6713, Cali, Colombia. M.E. Halsey, current address: United Soybean Board, St. Louis, MO 63141. Received 18 May 2007. *Corresponding author (p.chavarriaga@cgiar.org).

Abbreviations: CIAT, Centro Internacional de Agricultura Tropical; GM, genetically modified; PBS, Program for Biosafety Systems; USAID, U.S. Agency for International Development.

Cassava (*Manihot esculenta* Crantz) is a staple food for many people in large parts of tropical Africa, South America, and Asia. According to the United Nations Food and Agriculture Organization, "cassava is an essential part of the diet of more than half a billion people" (FAO, 2000). The starchy root of cassava is most frequently grown as a food source by small farmers in developing countries. Because of its role as a subsistence crop in tropical agriculture, and the vegetative propagation system typically used in cassava culture, research into the agronomy, genetics, and improvement of this important crop is often neglected by scientists in industrialized countries and by commercial entities. Instead, such research is frequently performed by public-sector scientists and research institutions in developing countries, who typically lack the resources that may be brought to bear on major temperate crops of industrialized countries, such as maize (*Zea mays* L.) and soybean [(*Glycine max* L. (Merr.)].

There are many significant opportunities for improving cassava, especially in terms of nutritional qualities, reduction of cyanogenic content, pest resistance, and compositional qualities (Taylor et al., 2004). Progress in these areas stands to greatly ben-

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677 S. Segoe Rd., Madison, WI 53711 USA

efit the many subsistence farmers and their families who rely on cassava and its products (Kawano, 2003). Traditional breeding of cassava is constrained by a number of intrinsic factors, including high levels of genetic heterozygosity, variable flowering patterns, and low seed set and germination (Jennings and Iglesias, 2002). Because of these difficulties, modern biotechnology and especially genetic modification through recombinant DNA methodology may be expected to play a significant—indeed, an essential—role in future improvements of cassava.

In March 2004, a meeting of the Cassava Biotechnology Network, an international group of scientists involved in research on cassava biotechnology, took place at the Centro Internacional de Agricultura Tropical (CIAT), Colombia. The group discussed recent efforts to conduct field trials of genetically modified (GM) cassava plants in Africa and Latin America. Members identified issues of biosafety with regards to the design and execution of regulated and confined field trials as a key to facilitating future research on GM cassava. In particular, a compilation of current information on reproductive biology and reproductive isolation was requested to establish sound procedures to prevent gene flow from regulated field trials of GM cassava.

As a result of these discussions, CIAT requested assistance from the Regulatory Approval Strategies component of the Program for Biosafety Systems in developing a document to address this critical need. The Program for Biosafety Systems (PBS) is a biosafety consultancy funded by the U.S. Agency for International Development (USAID); the Regulatory Approval Strategies component of PBS is housed at the Donald Danforth Plant Science Center in St. Louis, MO. The Centro Internacional de Agricultura Tropical expressed the hope that this document would be "an essential resource for developing country institutions and their partners when assembling biosafety dossiers—ensuring soundness and a degree of consistency for those aspects of transgenic cassava testing that are not construct-specific" (L.T. Kent, personal communication, 2005). The present review of reproductive biology and suggested measures for reproductive isolation of regulated and confined field trials were undertaken by PBS in response to CIAT's request.

CENTERS OF ORIGIN AND DIVERSITY

Allem (1994) originally proposed that modern cultivated cassava, *M. esculenta* subsp. *esculenta*, originated directly from the extant wild subspecies *M. esculenta* subsp. *flabellifolia*. This close relationship has since been supported by studies of Roa (1996) and Roa et al. (1997, 2000) using amplified fragment length polymorphisms (AFLPs) to estimate genetic relationships. A detailed molecular analysis based on the single-copy nuclear gene encoding glyceraldehyde 3-phosphate dehydrogenase (Olsen and Schaal, 1999) indicated that cassava was domesticated specifically from populations of *M. esculenta* subsp. *flabellifolia* occurring along the southern rim of the Amazon basin, in the Brazilian states of Acre, Rondônia, and Mato Grosso, and likely extending south into similar conditions in Bolivia. The premise of a southern Amazonian domestication has been further supported by subsequent studies, which consistently show that genetic variation in cassava is almost entirely a subset of the genetic variation occurring in the wild *M. esculenta* populations from this geographical region (Olsen and Schaal, 2001; Léotard and McKey, 2004; Olsen, 2004).

The 98 known wild species of the New World genus *Manihot* are distributed across warm regions of the Americas, from southern Arizona to Argentina (Rogers and Appan, 1973). There are two centers of species diversity in the genus; most species occur in northern South America (~80 species), and a secondary center of diversity occurs in Mexico and Central America (17 species, plus the related taxon *Manihotoides pauciflora*). A list of *Manihot* and *Manihotoides* species and their approximate geographical distributions are shown in Table 1. Central Brazil has the highest diversity of *Manihot* species and is home to about 40 wild species. Most *Manihot* species occur in dry or seasonally dry conditions. Although a few species are found in rainforests, they tend to be sporadic in their distributions and never become dominant members of the local vegetation (Rogers and Appan, 1973). The growth habit of *Manihot* species ranges from low herbaceous vines to trees exceeding 12 m in height (Rogers and Appan, 1973).

REPRODUCTIVE BIOLOGY

Flowering Characteristics

The cassava plant is monoecious and bears separate male and female flowers on the same plant. The time interval from planting to flowering depends on the specific genotype and environmental conditions and may vary from 1 to more than 24 mo (Byrne, 1984). Male and female flowers are borne on the same branched panicle, with female flowers at the base and male flowers toward the tip. The flowers are small, with the male flower being about 0.5 cm in diameter and the female flower slightly larger. Male and female flowers and a flowering panicle are shown in Fig. 1. Flowers usually begin to open around midday, remaining open for about 1 d. In a given inflorescence, female flowers open first and the male flowers follow from one to a few weeks later, a characteristic called protogyny. By the time male flowers open, the female flowers on the same branch have been fertilized or have aborted. However, because flowering on a single plant may last for more than 2 mo, pollen from a flower may fertilize other flowers on the same plant, or flowers on surrounding plants, with the proportion of each dependent on the genotype, the environment, and the presence of pollinating insects (Kawano, 1980; Ceballos et al., 2002; Jennings and Iglesias, 2002).

Flowering may be strongly influenced by environmental factors. A particular clone may produce no flowers

Table 1. Wild *Manihot* and *Manihotoides* species and their approximate geographical distributions. Taxonomy follows Rogers and Appan (1973), except for *M. esculenta*, where the wild relative of cassava is referred to as *M. esculenta* subsp. *flabellifolia*, following Allem (1994).

Species	Approximate geographical range
M. angustiloba (Torrey) Muell.-Arg. emend Rogers & Appan	southwest USA, Mexico
M. davisae Croizat	southwest USA, Mexico
M. walkerae Croizat	southwest USA, Mexico
M. aesculifolia (HBK) Pohl	Mexico
M. auriculata McVaugh	Mexico
M. caudata Greenman	Mexico
M. chlorosticta Standley & Goldman	Mexico
M. crassisepala Pax & K. Hoffmann	Mexico
M. foetida (HBK) Pohl	Mexico
M. michaelis McVaugh	Mexico
M. oaxacana Rogers & Appan	Mexico
M. pringlei Watson	Mexico
M. rhomboidea Muell.-Arg.	Mexico
M. rubricaulis I.M. Johnston	Mexico
M. subspicata Rogers & Appan	Mexico
M. tomatophylla Standley	Mexico
M. websterae Rogers & Appan	Mexico
Manihotoides pauciflora (T.S. Brandegee) Rogers & Appan	Mexico
M. carthaginensis (Jacquin) Muell.-Arg.	Colombia, Venezuela, West Indies
M. tristis Muell.-Arg.	Venezuela, northern Brazil
M. surinamensis Rogers & Appan	Venezuela, Guayana, Suriname
M. filamentosa Pittier	Venezuela
M. maguireiana Rogers & Appan	Venezuela
M. brachyloba Muell.-Arg.	Central America, West Indies, northern & central South America
M. marajoara Chermonte de Miranda apud Huber	northern Brazil
M. caerulescens Pohl	northern, northeastern, and central Brazil
M. glaziovii Muell.-Arg.	northeastern Brazil; introduced throughout tropical America, Africa, India, Pacific Islands
M. brachyandra Pax & K. Hoffmann	northeastern Brazil
M. catingae Ule	northeastern Brazil
M. dichotoma Ule	northeastern Brazil
M. epruinosa Pax & K. Hoffmann	northeastern Brazil
M. heptaphylla Ule	northeastern Brazil
M. maracasensis Ule	northeastern Brazil
M. pseudoglaziovii Pax & K. Hoffmann	northeastern Brazil
M. quinquefolia Pohl	northeastern Brazil
M. reniformis Pohl	northeastern Brazil
M. zehntneri Ule	northeastern Brazil
M. acuminatissima Muell.-Arg.	eastern Brazil
M. handroana N.D. Cruz	eastern Brazil
M. janiphoides Muell.-Arg.	eastern Brazil
M. pilosa Pohl	eastern Brazil
M. pohlii Wawra	eastern Brazil
M. sagittato-partita Pohl	eastern Brazil
M. warmingii Muell.-Arg.	eastern Brazil
M. tripartita (Sprengel) Muell.-Arg.	central and eastern Brazil
M. quinquepartita Huber ex Rogers & Appan	northern and central Brazil
M. alutacea Rogers & Appan	central Brazil
M. attenuata Muell.-Arg.	central Brazil
M. cecropiaefolia Pohl	central Brazil
M. crotalariaeformis Pohl	central Brazil
M. divergens Pohl	central Brazil
M. falcata Rogers & Appan	central Brazil
M. flemingiana Rogers & Appan	central Brazil
M. fruticulosa (Pax) Rogers & Appan	central Brazil
M. irwinii Rogers & Appan	central Brazil
M. jacobinensis Muell.-Arg.	central Brazil
M. longepetiolata Pohl	central Brazil
M. mossamedensis Taubert	central Brazil
M. nana Muell.-Arg.	central Brazil
M. oligantha Pax	central Brazil
M. orbicularis Pohl	central Brazil
M. paviaefolia Pohl	central Brazil
M. peltata Pohl	central Brazil
M. pruinosa Pohl	central Brazil
M. purpureo-costata Pohl	central Brazil
M. pusilla Pohl	central Brazil
M. quinqueloba Pohl	central Brazil
M. reptans Pax	central Brazil
M. salicifolia Pohl	central Brazil
M. sparsifolia Pohl	central Brazil
M. stipularis Pax	central Brazil
M. tomentosa Pohl	central Brazil
M. triphylla Pohl	central Brazil
M. weddelliana Baillon	central Brazil
M. violacea Pohl	central Brazil
M. xavatinensis Rogers & Appan	central Brazil
M. esculenta subsp. *flabellifolia* (Pohl) Ciferri	western and central Brazil
M. stricta Baillon	Peru, western and central Brazil
M. leptophylla Pax	Ecuador, Peru, western and central Brazil
M. grahami Hooker	southeastern Brazil, northern Argentina, Paraguay, Uruguay
M. inflata Muell.-Arg.	southern Brazil
M. corymbiflora Pax	southeastern Brazil
M. leptopoda (Muell.-Arg.) Rogers & Appan	southeastern Brazil
M. jolyana N.D. Cruz	southeastern Brazil
M. condensata Rogers & Appan	Bolivia
M. guaranitica Chodat & Hassler	Bolivia
M. anomala Pohl	central Brazil, Paraguay
M. gracilis Pohl	central Brazil, Paraguay
M. pentaphylla Pohl	central Brazil, Paraguay
M. hassleriana Chodat	Paraguay
M. mirabilis Pax	Paraguay
M. variifolia Pax	Paraguay
M. populifolia Pax	Paraguay
M. procumbens Muell.-Arg.	southern Brazil, Paraguay
M. affinis Pax	southern Brazil
M. tenella Muell.-Arg.	southern Brazil
M. hunzikeriana Martinez-Corvetto	southern Brazil, Argentina
M. anisophylla (Grisebach) Muell.-Arg.	Argentina

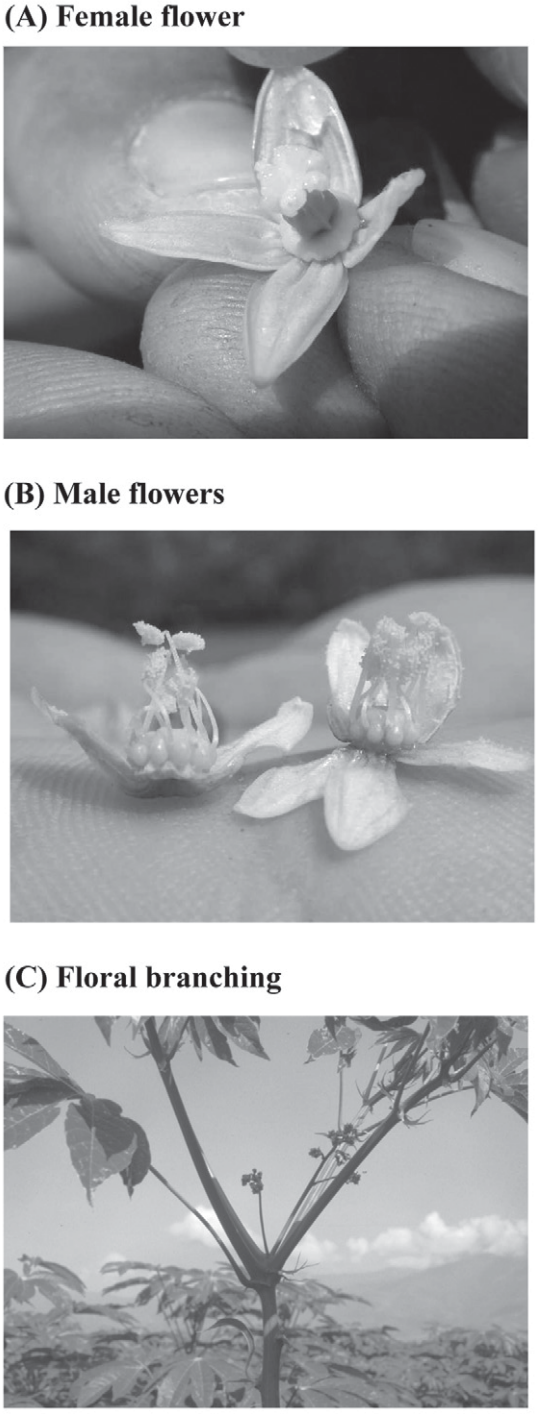

Figure 1. Flowering of cassava.

in one environment, produce only aborted flowers or fail to produce viable seed in another environment, and yet flower profusely and set seed in a third environment (N. Taylor, personal observation, 2005). For breeding purposes, clones are classified into different ecotypes so that breeders may take into account the flowering habits of the plants they wish to cross (Ceballos et al., 2002). For some clones, induction of flowering appears to depend on long photoperiods—up to 16-hour daylength—associated with temperatures of about 24°C (Keating, 1982; Alves, 2002).

Flowering is also dependent on plant habit. A flower bud typically forms when the plant branches, so that more highly branched genotypes flower more prolifically than those with a sparsely branched habit. Since flower-bud formation is preceded by apical branching, a prominent visual indication of incipient flowering is available to identify plants in the immediate preflowering stage.

The following is a general scheme of the flowering process, as observed by experienced breeders at CIAT:

1. Branching may begin as early as 2 mo after vegetative planting, although 6 mo is more typical.
2. The flowering bud (very young inflorescence) is usually observed at the branching point within 1 wk of branching.
3. Female flowers are ready for pollination 15 d after floral initiation. An indication of receptivity is the presence of a drop of nectar within the flower.
4. Male flowers on the same branch open 20 to 30 d later.
5. Fruits mature and are ready to open (dehisce) within 2.5 to 3 mo of fertilization.

Pollen

The pollen grains of cassava are relatively large in size and are sticky. Wind pollination therefore appears to be of little consequence (Rogers and Appan, 1973), with several species of wasp (mainly *Polistes* spp.) and honeybees (*Apis mellifera*) considered the main pollinators in Colombia and Africa, respectively (Kawano, 1980). Cassava pollen shows size dimorphism within the same genotype, the larger grains being 130 to 150 μm in diameter, whereas the smaller grains range from 90 to 110 μm. In some clones, the larger grains are more abundant, whereas in other clones the smaller grains are more common. The larger pollen grains have been observed to have better in vitro germination (60% germination after 2 h at 40°C) than the smaller ones, which may have less than 20% germination (Plazas, 1991).

Cassava pollen loses viability rapidly after it is shed. Leyton (1993) found 97% seed set with pollen used immediately after its collection, 56% seed set with pollen stored for 24 h at 25°C, and 0.9% seed set (one seed from 102 pollinations) after 48 h of storage. In practice, breeders take care to perform pollinations within 1 h after collection of pollen to help ensure successful fertilization; pollen viability seems to decline substantially after this time (P. Chavarriaga and N. Morante, personal observation, 2005).

Seed Characteristics

Developing seeds are viable 2 mo after pollination, and the fruit becomes mature about 1 mo after that, or about 3 mo after pollination (Ceballos et al., 2002). The fruit is a trilocular schizocarp, and seeds are ovoid-ellipsoidal, approximately 100 mm long and 4 to 6 mm thick (Alves, 2002). Dehiscence is explosive; the seed initially falls close

to the mother plant but then may be further dispersed by ants, which carry seeds to their underground nests. Through these two mechanisms of autochory followed by myrmecochory, a seed may be dispersed up to several meters from its place of origin (Elias and McKey, 2000; Elias et al., 2001).

Seed production and viability are variable, depending largely on the vigor and number of flowers borne by the parent plant (Kawano, 1980). Jennings (1963) reports that one viable seed per fruit is normally achieved in controlled pollinations, from a maximum of three possible in the trilocular ovary. Ceballos et al. (2004) indicate that one to two viable seeds are obtained from each hand pollination. Newly harvested seeds exhibit physiological dormancy and require 3 to 6 mo of storage at ambient temperature before they will germinate (Jennings and Iglesias, 2002).

Cassava seeds are adapted to ant dispersal, with large energy reserves that allow deep burial and a long dormancy period (Pujol et al., 2002). Seeds can remain viable when stored under ambient conditions for up to 1 yr, although germination percentages may decline substantially after 6 mo (Rajendran et al., 2000). Under cool-temperature storage conditions (4°C and 70–80% relative humidity) seeds have been known to survive for up to 7 yr with no loss of germination (N. Morante, personal communication, 2005). The persistence of natural seed banks has not been well documented, but they may endure for many years (Elias et al., 2000).

Seed germination is favored by dry heat and complete darkness. Ellis et al. (1982), working with two-dimensional temperature gradient plates, found that germination occurred most often when temperatures exceeded 30°C for part of the day, with a mean temperature of at least 24°C. They suggest that an alternating temperature regime of 30°C for 8 h and 38°C for 16 h for at least 21 d is the most appropriate for determining cassava seed viability under laboratory conditions.

The combination of deep burial by ants, a long dormancy period, and heat-activated germination suggests that the ancestor of cassava may have evolved under conditions of sporadic natural fire and was thus uniquely suited to domestication under slash and burn agriculture (Pujol et al., 2002). In this view, the seed bank of ancestral cassava, protected from environmental hazards by deep burial and physiological dormancy, could await the triggering effect of lightning-caused fires, allowing seedlings to avoid competition with established vegetation. In addition to seed, the rootstocks of the crop's ancestor were also likely well adapted to fire; the wild relative *M. esculenta* subsp. *flabellifolia* has been observed to show vigorous regrowth from rootstocks in areas of burned forest (K. Olsen, personal observation, 2005). Domesticated cassava was thus "pre-adapted to slash and burn agriculture, which enabled spread of this plant into habitats much wetter than those occupied by its wild ancestors. [Further,] cultivation by stem cuttings may have originated via attempts by foraging peoples to supplement the density in newly burned areas" (Pujol et al., 2002, p.377).

Botanical seed is not usually used for commercial propagation of cassava. Genetically, any particular cassava clone is highly heterozygous, and propagation from sexual seed results in wide and unpredictable diversity of phenotypes, which is of interest to breeders but presents difficulties in propagation (Ceballos et al., 2004). Propagation of cassava is therefore accomplished by vegetative stem cuttings to preserve the known characteristics of favored clones, as described below. Amerindian peoples of South America frequently encourage volunteer seedlings in their native gardens, in the hope of selecting a superior clone, which is then propagated vegetatively (Salick et al., 1997; Elias et al., 2000; Elias et al., 2001). Heterotic volunteer seedlings resulting from natural outcrosses are preferentially retained, since they are larger and much more vigorous than inbred seedlings, which can suffer from inbreeding depression (Kawano, 1980). This practice contributes to the maintenance of genetic diversity in cultivated populations (Pujol et al., 2005). Seedlings are initially smaller than plants developed from vegetative cuttings and require special care to become established and prosper.

Vegetative Propagation

Cassava is normally propagated by means of stem cuttings, which are known horticulturally as 'stakes'. Stakes are typically at least 20 cm long, and have 4 to 5 nodes each with a viable bud. Stakes must be transported carefully to avoid damage and may be treated with agrochemicals to prevent pest or disease establishment in the new plants (Leihner, 2002).

CROSSES

Intraspecific Crosses: Cultivated Cassavas

Both self- and cross-pollination may occur naturally in cassava. While there appears to be no genetic barrier to fertilization between clones of cultivated cassava, the need for synchronous flowering represents a major hurdle in cassava breeding (Ceballos et al., 2004) and is presumably no less a barrier to natural gene flow between diverse stands of cultivated cassava.

The reproductive isolation of different stands of cultivated cassava is of interest in conventional breeding programs, to maintain the characteristics of different breeding lines, as well as in the confinement of trials of GM cassava. Limited information is available on natural gene flow and associated distances required for reproductive isolation in cassava.

Results from the empirical experience of cassava breeders have been used in the past to help define isolation

requirements. While working at CIAT, Kawano et al. (1978) determined that a 30-m isolation distance was sufficient to avoid cross-pollination, and a distance of 30 m between stands of different clones became the established standard of breeders at CIAT to prevent cross-pollination (CIAT, 1974). Genetic isolation of test plots in conventional breeding programs at CIAT is also accomplished using an arrangement of 4 m of alley, 8 m of androsterile cassava, and another 4 m alley, for a total of 16 m distance (P. Chavarriaga and N. Morante, personal observation, 2005). Additionally, based on extensive personal experience in breeding programs, Kawano (1980) suggested that 500 m is an appropriate distance for "perfect isolation" of two populations in genetic studies. However, these standards were based on visual observations and were established before the advent of modern molecular techniques. New methods are now available to quantify more precisely the gene flow between stands of cultivated cassava and to elucidate the factors influencing it.

It is important to note that because of the typical vegetative propagation used for cassava, the consequences of gene flow from either experimental or commercial material are likely to be of less concern than with other crops. Even if a low level of successful outcrossing were to occur from experimental plants to the flowers of neighboring cassava, any novel traits would not be passed on in the typical vegetative propagation system used for cassava.

Interspecific Crosses: Wild Relatives

All *Manihot* species, including cultivated cassava, that have been studied so far have a chromosome number of $2n = 36$ and show regular bivalent pairing at meiosis (Jennings and Iglesias, 2002). Thus, species karyotypes do not rule out the possibility of interspecific hybridization among *Manihot* species. However, such hybridization appears to be uncommon. Substantial work has been undertaken attempting to artificially introgress genes from wild species into cultivated cassava for breeding purposes (Nassar, 1989, 2003; Nassar et al., 1986; Hahn et al., 1990), and such efforts have met with mixed success. Nassar (2003), for example, reported no fruit set and no viable seed from 145 flowers of *M. pohlii* hand pollinated with pollen of cassava. Natural (insect-mediated) crosses of cassava with *M. neusana* and *M anomala* were more successful than hand pollinations but still resulted in less than 5% hybrid seed from an arrangement in which rows of cassava (as the pollen parent) were alternated with rows of the wild species (Nassar, 1989).

The more closely related the wild species is to cultivated cassava, the more successful hybridization seems to become—16 successful crosses at CIAT between cassava and the conspecific wild progenitor *M. esculenta* subsp. *flabellifolia* resulted in "thousands of seeds," whereas only five seeds of unknown viability were obtained from two crosses with *M. aesculifolia*, according to Roa et al. (1997, p. 748).

The natural hybridization of cassava with its closely related wild relatives has been reported and confirmed using modern molecular methods (Second et al., 1997). Natural hybrids with *M. esculenta* subsp. *flabellifolia* or *M. pruinosa* have been identified as arising from feral cassava populations surviving on the margin of an abandoned cassava plantation in French Guiana (Duputié, 2004; Léotard and McKey, 2004; Duputié et al., 2007). The sexually compatible wild relatives in this case are either the immediate ancestor of cultivated cassava or a closely related species. From the results of both artificial and natural hybridizations, it seems likely that genetic or physiological factors play a significant role in restricting gene flow from cassava to related populations. The probability of gene flow, as well as the stringency of measures required to prevent it, may thus diminish rapidly with increasing evolutionary distance between the species. Most *Manihot* species do not hybridize readily with cassava (Olsen and Schaal, 2001), and thus it cannot be assumed that such hybridization is common in nature. Nonetheless, the potential for interspecific introgression between cassava and wild *Manihot* species should not be discounted.

Manihot glaziovii (Ceara rubber tree) is the only relative of cassava that is reported to be naturalized in Africa. *M. glaziovii* is thought to be closely related to *M. esculenta* (Rogers and Appan, 1973; Second et al., 1997), and hybrids between cassava and *M. glaziovii* are highly fertile (Nassar, 1982). Natural hybrids between cassava and *M. glaziovii*, identified by morphological and electrophoretic markers (Wanyera et al., 1994) and DNA-based restriction fragment length polymorphism (RFLP) markers (Beeching et al., 1993), have been collected in Africa. Certain African cultivars can also be identified as descendents of *M. glaziovii* hybrids by the same technique (Beeching et al., 1993). Naturally occurring hybrid stands have been reported (Lefevre, 1988). These reports reflect hybridization presumably occurring with the two species in close proximity over long periods of time; the probability of gene flow from a particular stand of cassava to *M. glaziovii* over specific distances and a finite time period, as would be the case with an experimental confined field trial, remains to be established.

Manihot glaziovii seems to be widely distributed in other parts of the tropics as well. Rogers and Appan (1973) have reported collections in Asia from Laos, Sri Lanka, Malaysia, Indonesia, Philippines, and India, as well as from the New World tropics and islands in the Pacific Ocean.

WEEDINESS AND INVASIVENESS

The viability of cassava in unmanaged ecosystems is limited by the habitat available. Pujol et al. (2002) found that *M. esculenta* seedlings were viable in the field, particularly after a fire, but not in competition with untended vegetation. Olsen and Schaal (1999, p. 5587) observed that

"cassava does not survive well in abandoned fields or as an escape from cultivation. . . . [C]assava is propagated almost exclusively by stem cuttings, minimizing unintentional spread of the crop by humans."

The low fecundity and physiological dormancy of seeds also limits the spread and establishment of the crop into unmanaged habitats. Feral stands of cassava are reported to exist on the margins of abandoned plantations in South America, and other *Manihot* species are known to naturalize over time, such as *M. glaziovii* in Africa. However, cassava is not considered to be a weed in agricultural settings and is not invasive. Evaluated against Baker's (1965) 12 characteristics of weeds, only one, "discontinuous germination and long-lived seeds," appears to apply unreservedly to cassava.

REPRODUCTIVE ISOLATION

Measures for isolation from pollen-mediated gene flow must take into account the insect-vectored pollination found in cassava. Similar to cotton (*Gossypium* L.), cassava pollen is heavy and sticky, and wind pollination is of limited concern. Unfortunately, there is presently only limited data on isolation distances required to prevent pollen-mediated gene flow between stands of cassava and between cassava and its various wild relatives. Until the probability and risks associated with gene flow from experimental field trials can be assessed, it is critical to establish biosafety standards that will effectively prevent off-site gene flow. The procedures recommended here are thus intended to eliminate gene flow from a trial site, allowing experimental work to be safely undertaken in the absence of rigorous gene flow data. Refined isolation distances may be proposed at a later date, when justified by experimental data.

Each of the following sections provides a set of procedures to ensure reproductive isolation of cassava. A researcher would choose to follow the procedures described in one of the three options below, depending on the objectives of the trial:

1. Manual removal of flower buds before flowering. Formation of flower buds is preceded by apical branching, which allows positive identification of preflowering plants. In this method, experimental plots are monitored and flower buds are removed before they become fertile. Generally, weekly inspection for the duration of the entire flowering period is sufficient to prevent maturation and fertilization of the flowers. Inspection should be commenced before flowering; this may be as early as 6 wk after vegetative planting, depending on the particular clone and environment. For regulated trials, it is important that inspection and flower bud removal be documented at each occurrence. Removed flower buds should be disposed of within the trial site, typically by burial. If desired, especially for trials with regulated GM plants, the experimental plants may also be isolated by a distance of 30 m or so from any other reproductively compatible plants, either cultivated or wild cassavas with which the experimental plants are capable of hybridizing. This isolation distance serves as an additional margin of safety for reproductive isolation and prevents the loss of regulated plant material from the trial site. If an isolation distance surrounding the trial site is to be enforced, the area should be monitored at least once each month for the presence of any sexually compatible plants, which must be destroyed before they reach the reproductive stage.
2. Destruction of the plants before flowering. Where the required experimental data may be obtained before the flowering stage, and the plants in the test site destroyed before flowering, there is less concern for reproductive isolation. The trial site should be isolated by distance or by fencing sufficient to prevent the test plants from entering food and feed channels. For regulated trials, the growth, development, and destruction of the test plants before flowering must be carefully documented.
3. Floral bagging to contain pollen and seed. If flowers or mature seed are required for breeding or evaluation purposes, flower buds, or the entire plant, can be bagged to prevent pollinating insects from visiting the flowers. See Kawano (1980) for details on the equipment and methodology required for this process, aspects of which are shown in Fig. 2. Male flowers should be bagged before anthesis. Female flowers must be bagged after fertilization to collect any seed that may be formed as a result of manual pollinations. As mentioned above, careful documentation to verify the containment and collection of all seed must be enforced. If the seed collected is required to be devitalized, this may be done by burning, grinding, or autoclaving. As with manual removal of flower buds before flowering, an isolation distance of 30 m and/or a barrier of vegetation may be used as an additional margin of safety but is not strictly required.

Reproductive isolation methods described above for experimental trials of cassava either prevent the formation of seeds or require their collection before they fall to the ground. This allows researchers to avoid issues of seed dormancy, which may be quite long, and focus solely on preventing vegetative regrowth. Cassava plants will not regenerate from storage roots, and the role of these organs in persistence is not of concern. To ensure that the experimental plants do not persist in the environment, vegetative regrowth arising from stems or other aboveground

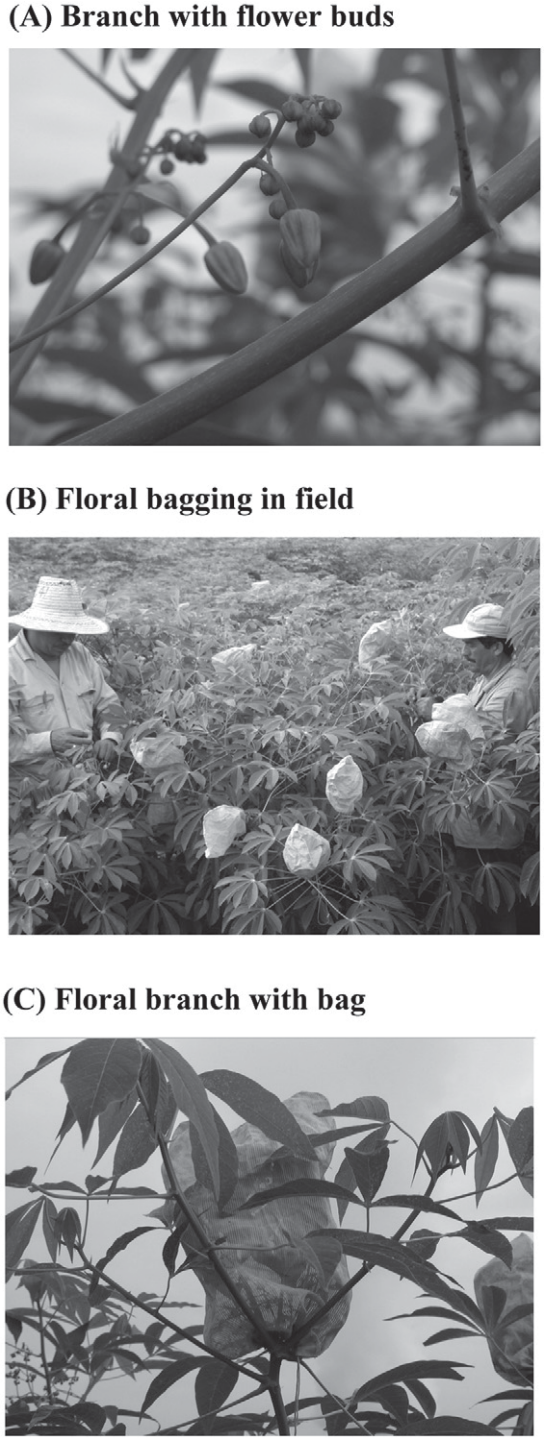

Figure 2. Floral bagging for reproductive isolation of cassava.

parts must be devitalized before flowering. For regulated trials with GM plants, consumption of the plant material by humans or livestock should be prevented.

Control of regrowth from vegetative parts may be accomplished by herbicides, by burning, by burying, or by otherwise devitalizing the vegetative parts, such as chopping or grinding with commercial equipment designed for this purpose. If deep burial is used, it is recommended that vegetative material be covered with soil to a depth of at least 30 cm to prevent reemergence. Mature vegetation may be chopped before burial or burning, which should help destroy the material, speed decomposition, and prevent regrowth. Generally, the plot area should be monitored for regrowth for several months, or sufficient time to allow regrowth under local rainfall patterns. It is recommended that any crop to be grown in the area immediately after the regulated trial be chosen so as not to interfere with the recognition and destruction of regrowth of the experimental plants.

AREAS FOR FURTHER RESEARCH

As has been noted, there is little direct experimental data on gene flow in cassava that is useful in constructing standards for reproductive isolation. Existing information is experiential and often qualitative, rather than experimental and quantitative. Focused experimentation using modern techniques is needed on outcrossing between stands of cultivated cassava, from cassava to its different wild relatives under natural conditions, and on measures that may serve to minimize or prevent such gene flow, especially isolation distances. Such information on outcrossing in specific circumstances would be valuable to establish confinement procedures for regulated trials with GM plants, as well as conventional breeding programs. Flowering times, genetic compatibility factors, insect population dynamics, surrounding guard rows of vegetation and proximity of target populations are all variables that may be expected to affect gene flow.

Experimental evaluation of gene flow from cassava to specific wild relatives in nature, as related to the physical and genetic proximity of the plants, is needed both to design safe experimental trials and to guide future risk management decisions that will be required before any general release of GM cassava. The risk of gene flow under natural conditions may be limited to a specific subset of wild relatives or to specific conditions, due to the natural constraints discussed above. Until the true nature of the biosafety issues posed by the development of new and potentially GM cassava lines is known, the measures of reproductive isolation described here should be applied to experimental trials, especially those with regulated GM plants.

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