

PROGRAM FOR BIOSAFETY SYSTEMS (PBS)

**Cassava (*Manihot esculenta* Crantz):
Reproductive biology and practices for
confinement of experimental field trials**

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Introduction

In June 2004, the Program for Biosafety Systems (PBS) received a letter from the Centro Internacional de Agricultura Tropical (CIAT), written on behalf of an international group of scientists involved in cassava biotechnology. This group had recently gathered at CIAT's headquarters in Cali, Colombia, for a meeting of the Cassava Biotechnology Network to discuss, among other things, recent efforts to conduct field trials of genetically-modified (GM) cassava plants in Africa and Latin America.

The letter asked for PBS's help in assembling information relevant to the design of safe field trials of GM cassava and the preparation of applications for field trials to present to regulatory authorities. Specifically, the letter requested information on: (1) the reproductive biology of cassava, (2) alternative approaches to reproductive isolation, (3) good practices to ensure confinement, and (4) information on the safety of selectable and scorable markers. Item (4) was addressed in a report completed in January 2005 entitled "Biosafety Considerations for Selectable and Scorable Markers Used in Cassava Biotechnology" (Petersen et al., 2005). PBS has prepared the present document to address items (1), (2), and (3).

CIAT's letter expressed the hope that PBS would respond to its request by producing a document that 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." We hope that the present document is able to serve that purpose.

1. Cassava Biology

1.1 Centers of Origin and Diversity

Allem (1994) originally proposed that the 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 AFLPs to estimate genetic relationships. A detailed molecular analysis based on the single-copy nuclear gene encoding glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*; 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 vegetation in Bolivia. The premise of a southern Amazonian domestication has been further supported by subsequent studies (Olsen and Schaal, 2001; Léotard and McKey, 2004).

The 98 known wild species of the New World genus *Manihot* are distributed across warm regions of the New World, 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* species and their approximate geographical distributions are shown in ANNEX 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 rain forests, they tend to be sporadic in their distributions, and never become dominant members of the local vegetation (Rogers and Appan, 1973). The habit of *Manihot* species ranges from low herbaceous vines to trees exceeding 12 meters (Rogers and Appan, 1973).

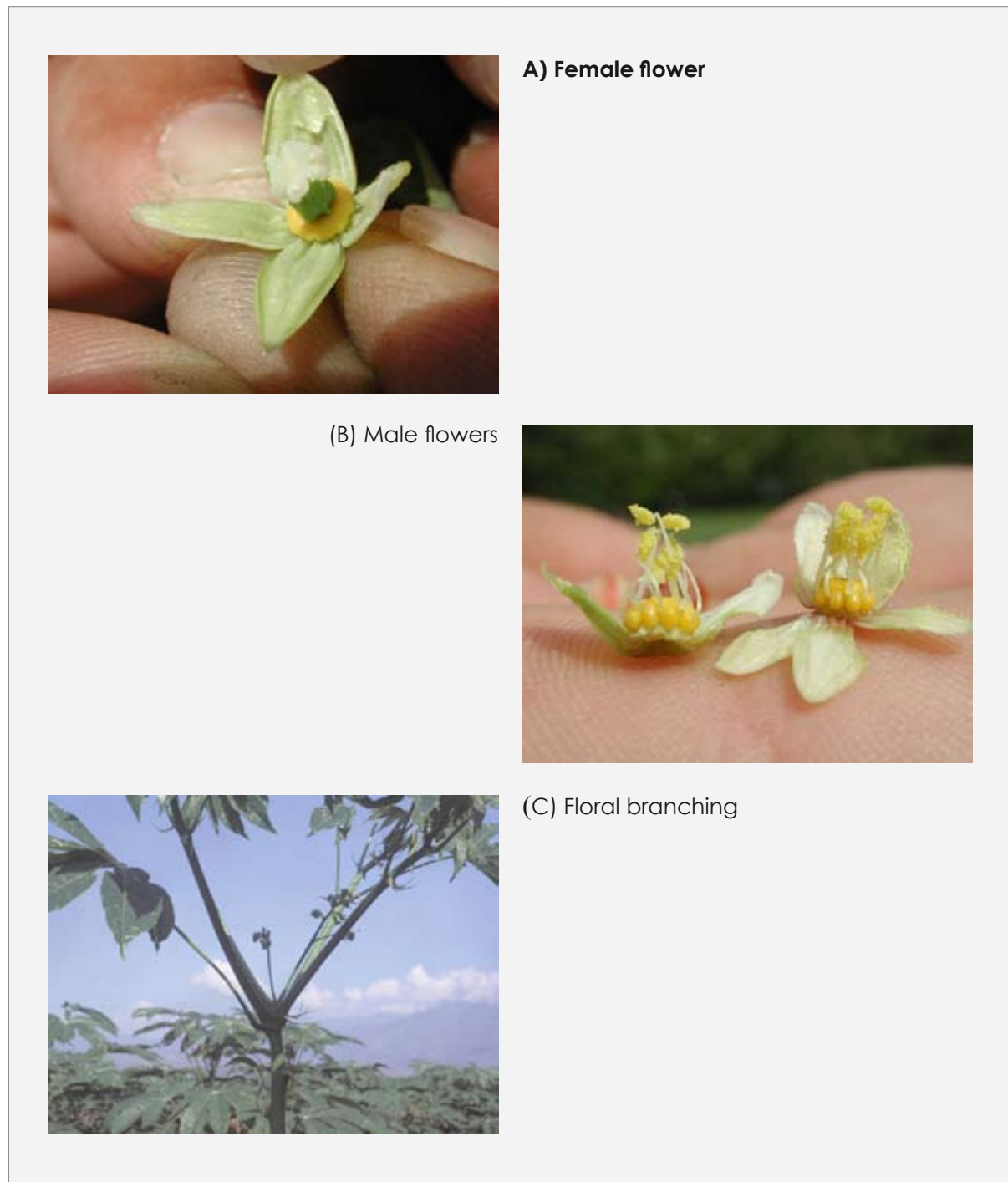
1.2 Reproductive Biology

1.2.1 Flowering characteristics

The cassava plant bears separate male and female flowers on the same plant, making it monoecious. The time interval from planting to flowering depends on the specific genotype and environmental conditions, and may vary from 1 to more than 24 months (Byrne, 1984). Male and female flowers are borne in a single 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 **Figure 1** (see next page). Flowers usually begin to open around mid-day, and remain open for about one day (Ceballos et al., 2002). On a given branch, female flowers open first and the male flowers follow 1 or 2 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 two months, both self- and sib-fertilization may occur, with the proportion of each dependent on the genotype, the environment, and the presence of pollinating insects (Kawano, 1980; Jennings and Iglesias, 2002).

Flowering may be strongly influenced by environmental factors. A particular clone may produce no flowers 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 communication, 2005). For breeding purposes, clones are classified into different growing zones (environments, ecotypes), so that breeders may take account of 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 day length – associated with temperatures of around 24°C (Keating, 1982; Alves, 2002).

Figure 1 – Flowering of cassava



Flowering is also dependent on plant habit. A flower bud typically forms when the plant branches, so that more highly branched genotypes are more prolific than those with a sparsely-branched habit. Farmers generally prefer the non-branching cassava type because it facilitates cultivation practices. Therefore, many modern cultivars fail to flower under normal growing conditions. Flower bud formation is preceded by apical branching, which is a prominent visual indication of incipient flowering, and may be used to identify plants in the pre-flowering stage.

A general scheme of the flowering process, as observed by experienced breeders at CIAT, would be:

- 1) Branching may begin as early as 2 months after planting, although 6 months is more typical.
- 2) The flowering bud (very young inflorescence) is usually observed at the branching point within 1 week of branching.
- 3) Female flowers are ready for pollination 15 days 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 days later.
- 5) Fruits become mature and are ready to open (dehisce) within 2.5 to 3 months of fertilization.

1.2.2 Pollen

The pollen grains of cassava are quite large in size and sticky, and wind pollination appears to be of little consequence. Several species of wasp (mainly *Polistes* spp.) and honeybees (*Apis mellifera*) are the main pollinators in Colombia and Africa, respectively (Kawano, 1980). Cassava pollen shows size dimorphism, the larger grains being 130 to 150 microns in diameter, whereas the smaller grains range from 90 to 110 microns (Plazas, 1991). In some varieties, the larger grains are more abundant and have better germination percentages (60%) under in vitro conditions (2 hours at 40°C) than the smaller ones. In other cultivars, the smaller grains are more common. Smaller grains typically germinate less efficiently than large ones, and may have less than 20% viability (*ibid.*).

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

1.2.3 Seed characteristics

Fertilized seed is viable two months after pollination, and fruit becomes mature about one month after that, or about three months after pollination (Ceballos et al., 2002). The fruit is a trilobular 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 an unknown percentage of the seed to their nests in the soil. 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 quality of the female parent (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 trilobular ovary. Ceballos et al. (2004) indicate that one to two viable seeds are obtained from each hand-pollination. Newly harvested seeds are dormant, requiring 3 to 6 months of storage 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 for up to 1 year, although germination percentages may decline substantially after 6 months (Rajendran et al., 2000). Under storage conditions (4°C and 70-80% relative humidity) seeds have been known to survive for up to 7 years 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 hours and 38°C for 16 hours for at least 21 days is the most appropriate for determination of cassava seed viability under laboratory conditions.

The combination of deep burial by ants, long dormancy period and heat-activated germination suggests that the ancestor of cassava evolved under conditions of sporadic natural fire, and was 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, was able to 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 very vigorous regrowth from rootstocks in areas of burned forest (K. Olsen, personal communication, 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).

Botanical seed is not typically used for commercial propagation. Genetically, any particular cassava genotype is extremely heterogeneous, 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 typically accomplished by vegetative cuttings in order to preserve the known characteristics of favored lines, 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). Heterozygous volunteers 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 stakes and require special care to become established and prosper.

1.2.4 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 with viable buds. Stakes must be transported carefully to avoid damage, and may be treated with agrochemicals to prevent pest or disease establishment in the new plants. Stakes must be matured to the point that they do not dry out too quickly when planted, but must not be over-mature (Leihner, 2002).

1.3 Crosses

1.3.1 Intra-specific crosses: cultivated cassavas

Both self and cross-pollination may occur naturally in cassava. While there appear to be no genetic barriers 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 cassava stands as a means of preserving genetic purity is of interest in both conventional breeding and in the development of genetically modified (GM)

cassava. Limited information is available on natural gene flow and distances required for reproductive isolation in cassava.

Results from the practical experience of cassava breeders may be used to help define isolation requirements. Kawano et al. (1978), while working at CIAT, determined that a 30 m isolation distance was sufficient to avoid cross-pollination, and a distance of 30 m between pure stands of different genotypes 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. Chavarriga and N. Morante, personal observation, 2005). Additionally, Kawano (1980) suggested that 500 m is an appropriate distance for 'perfect isolation' of two populations in genetic studies, based on personal experience in breeding programs. Experimental results employing modern molecular techniques are needed in order to more precisely quantify gene flow between stands of cultivated cassava, and the factors that may influence it, especially as novel genetic traits are introduced for testing and potential release.

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.

1.3.2 Inter-specific crosses: wild relatives

All *Manihot* species, including cultivated cassava, that have been studied so far have a chromosome number of $n = 36$ and show regular bivalent pairing at meiosis (Jennings and Iglesias, 2002). Thus, genetic barriers to hybridization between different species of *Manihot* would seem to be somewhat limited. However, such hybridization appears to be relatively uncommon. Substantial work has been undertaken attempting to artificially introgress genes from wild species into cultivated cassava for breeding purposes (Nassar, 1989; Nassar, 2003; Nassar et al., 1986; Hahn et al., 1990); however, such efforts have met with mixed success. For example, Nassar (2003) 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).

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 populations surviving on the margin of an abandoned cassava plantation in French Guiana (Leotard and McKey, 2004; Duputie, 2004). 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 severity 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 we cannot assume that such hybridization is common in nature.

In Africa, naturalized *Manihot glaziovii* (Ceara rubber tree) is the only reported relative of cassava. *M. glaziovii* is believed to be closely related to *M. esculenta* (Rogers and Appen, 1973; Second et al., 1997), and hybrids between cassava and *M. glaziovii* are reported to be highly fertile (Nassar, 1982). Natural hybrids with *M. glaziovii* from African collections have been identified and confirmed by morphological and electrophoretic markers (Wanyera et al., 1994) and DNA-based RFLP marker technique (Beeching et al., 1993). Certain African cultivars can also be identified as descendents of *M. glaziovii* hybrids by the same technique (*ibid.*). 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.

M. glaziovii is reported to be widely distributed in other parts of the tropics as well. Rogers and Appan (1973) have reported collections of it 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.

1.4 Agro-Ecology

1.4.1 Weediness and invasiveness

The viability of cultivated cassava in unmanaged ecosystems is limited by the habitat available. Pujol et al. (2002) found that *M. esculenta* seedlings were viable in field situations, particularly after a fire, but not under fallow conditions. Olsen and Schaal (1999) observe that "...cassava does not survive well in abandoned fields or as an escape from cultivation....cassava 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* spp. are known to naturalize over time, e.g., *M. glaziovii* in Africa. However, cassava is not considered to be a weed in agricultural settings, and is not invasive. Evaluated against Baker's twelve characteristics of weeds, only one, "discontinuous germination and long-lived seeds" (1965), appears to apply unreservedly to cassava. It should also be noted that cassava and other *Manihot* spp. can be effectively controlled by both mechanical and chemical means, if required.

1.4.2 Other environmental concerns

The confinement measures described in Section II are intended to isolate experimental plants and genes within a confined field trial site, thus minimizing any impact on the environment outside the trial site. Any effects of the GM plants on non-target organisms or on biodiversity would be limited to the trial site, for the duration of the trial and perhaps the post-harvest interval. Reproductive isolation measures ensure that novel traits borne by the experimental plants will not be transferred to nearby populations of *Manihot*, either cultivated or wild. These concerns may be relevant for general or commercial release at a future time, and are considered by the Regulatory Authority during the risk assessment process leading up to potential approval and commercial release.

1.5 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. Knowledge of outcrossing in specific circumstances can be used to underpin confinement procedures for experimental trials as well as risk management decisions for potential general release of GM cassava. Flowering times, genetic compatibility factors, insect population dynamics and proximity of target populations may all serve to limit gene flow.

Two main areas where experimental work would be of benefit are:

Data on pollen mediated gene flow between stands of cultivated cassava is needed for both conventional breeding and GM development programs. There is apparently no experimental data on gene flow related to guard rows of either cassava or other vegetation, and these results may also be useful for experimental design and confinement.

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 to design confined trials and to guide future risk management decisions. The risk of gene flow under natural conditions may be limited to a specific sub-set of wild relatives or to specific conditions, due to the natural constraints discussed above.

The measures for reproductive isolation described in Section II are designed to permit the safe implementation of experimental field trials by using methods other than isolation distances to restrict pollen mediated gene flow. Thus, experimental efforts may be on-going, while additional gene flow research is undertaken.

2. Confinement Practices for Field Trials with Cassava

When used here, the term 'trial manager' refers to those individuals designated by the Authorized Party supporting the trial to make management decision concerning the trial, and to record experimental data. Trial managers are qualified individuals acting on behalf of the Authorized Party. All trial managers will have appropriate qualifications and training, and this shall be documented in an established training file, as described below. Trial managers are distinct from other workers who are not authorized to record data and are thus not required to maintain training files.

2.1 Reproductive isolation

Measures for isolation from pollen-mediated gene flow are indicated by the insect-vectored pollination found in cassava. Similar to cotton, cassava pollen is heavy and sticky, and wind pollination is of limited concern.

At present there are 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. Thus, the procedures given here are intended to eliminate gene flow from the trial site, so that experimental work may be safely undertaken in the absence of rigorous gene flow data. Appropriate isolation distances may be proposed at a later date, when justified by the availability of such data.

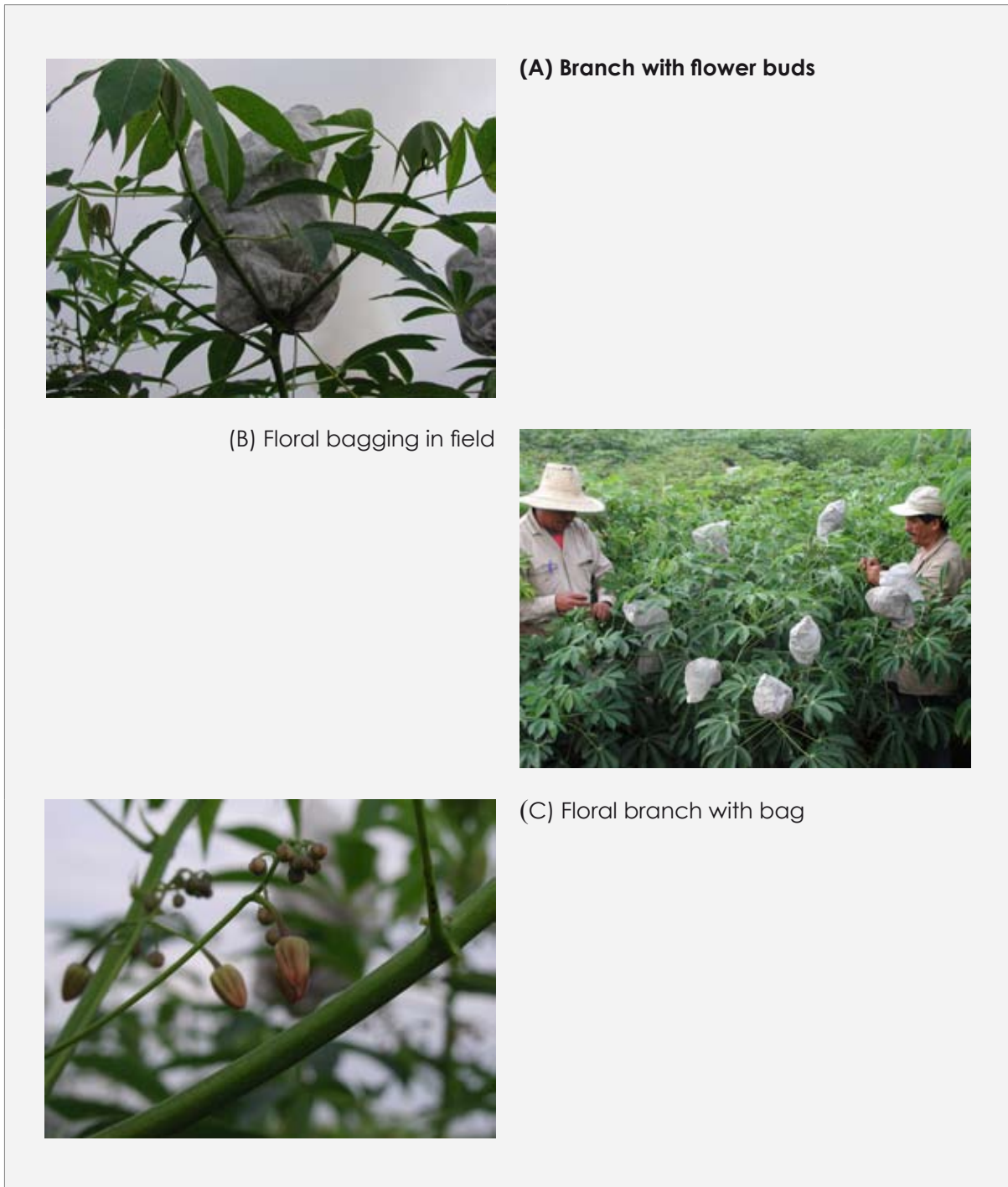
The following sections each provide a set of procedures to ensure reproductive isolation of cassava. A researcher would choose to follow the procedures described in one of the three options below, depending on the objectives of the trial:

(a) Manual removal of flower buds prior to flowering. Formation of flower buds is preceded by apical branching, facilitating positive identification of pre-flowering plants. The plot will be monitored and flower buds removed before they become fertile each week for the entire flowering period, and this process must be documented at each occurrence. Flower buds thus removed will be disposed of at the trial site by burial or incineration. Where flower buds are to be removed, the experimental plants should also be at least 30 m from any other 'prohibited plants', as described below. This isolation distance, defined by experienced cassava breeders, serves as a secondary mechanism for controlling gene flow, should manual flower bud removal be imperfect for any reason. A barrier of elephant grass or similar dense vegetation may be used in addition to the isolation distance, but is not required.

(b) Destruction of the plants prior to 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 plot area will be isolated by distance or by fencing sufficient to prevent the test plants from entering food and feed channels. The growth, development and destruction of the test plants prior to flowering will be documented by the trial manager.

(c) Floral bagging to contain pollen and seed. If flowers or mature seed are required for breeding or evaluation purposes, flower buds may be bagged to prevent pollinators from visiting the flowers. See Kawano (1980) for details on the equipment and methodology required for this process, aspects of which are shown in **Figure 2** (see next page). Male flowers shall be bagged prior to pollen shed. Female flowers must be bagged after fertilization, in order to collect any seed that may be formed as a result of manual pollinations. The trial manager shall carefully document the process, and verify the containment and collection of all seed. As with option (a), an isolation distance of 30 m is required, and a barrier of vegetation may be used, but is not required.

Figure 2 - Floral bagging for reproductive isolation.



Prohibited plants vary by geography, and include cultivated cassava and any species of *Manihot* with which cassava may be capable of hybridizing, according to geographic area. In the case of Latin America, where cassava and all wild *Manihot* species are native, prohibited species would include all *Manihot* species occurring in the geographical region where the field trial is to be conducted. Wild *Manihot* species and their geographical distributions are listed in ANNEX 1.

Table 1 - Plants prohibited from the defined reproductive isolation distance surrounding cassava confined field trials, by world area

World Area	Prohibited plants – <u>cultivated cassava</u> and :
South and Central America, Mexico	Any <i>Manihot</i> spp. (see ANNEX 1)
Africa	<i>Manihot glaziovii</i>
Asia	<i>Manihot glaziovii</i>

The isolation area surrounding the trial site will be monitored at least once each month for the presence of any prohibited plants, and any such plants found will be destroyed prior to flowering. If any prohibited plant within the isolation distance is allowed to flower and set seed, the isolation distance is subject to post-harvest restriction and monitoring. Monitoring dates, presence of prohibited plants, and the method of their destruction will be documented by the trial manager.

2.2 Material confinement

Measures taken for material confinement of cassava are influenced by the vegetative propagation system typically used for the crop. Stakes or plantlets present fewer challenges in confined handling and transport than do seeds, since the vegetative materials lose viability quickly, have no dormancy, are not likely to be lost due to their size, and may easily be prevented from establishing and surviving in the environment.

All options for reproductive isolation given above either prevent the formation of true seed, or provide for its containment and collection so that it does not remain on the trial site, helping to simplify material confinement requirements.

The following measures for material confinement are recommended for confined trials to be established using plantlets, stakes or seeds; any special requirements for the latter are so indicated.

(a) Transport and storage. Plantlets or stakes in transit will be enclosed in at least two sturdy containers, each of which shall be sufficient by itself to prevent material loss in case of accident. Examples of approved containers for in-vitro plantlets would be: glass or plastic tubes, Styrofoam boxes or trays, cardboard or fibreboard boxes. Containers appropriate for transport of stakes would include: fibre bags, sturdy cardboard, fibreboard or Styrofoam boxes. Seeds will be enclosed in three sturdy containers, the inner or 'primary container' which is in contact with the seed shall not allow seeds to be trapped or hidden within. Examples of primary containers for seed would include: sturdy plastic bags, plastic or metal cans or jars. The outer containers for seeds may be similar to primary containers mentioned, or may be sturdy boxes of fibreboard, cardboard or wood. Each container used will be labeled to indicate that the contents are 'GM plants/seeds – not for food or feed', or equivalent verbiage. A readily-understood 'Do Not Eat' symbol (e.g., a set of eating utensils within a red circle with slash) may also be included.

Storage areas for plants or seed, or contained growth facilities for plants, will be secured from unauthorized access, and will be posted with the information 'GM plants – not for food or feed' or equivalent verbiage. Experimental plants (which may include non-GM plants for use in a particular confined trial) will be kept separate from other plants, and experimental seeds will be stored separately from other seed.

(b) Disposal of containers. Any shipping container in contact with plant material or seeds will be destroyed either by incineration or burial. Alternatively, containers may be verified

and documented to be free of propagative plant material, and subsequently reused. These requirements would normally apply to only the primary (inner) container, unless a breach has occurred in that container, in which case the requirement would apply to any containers in contact with plant material. Primary containers may also be marked as 'for use with GM plant material or seeds only' or equivalent verbiage, and their use so restricted. Any containers not in contact with plant material may be reused.

(c) Security of the trial site. Security of the trial site is of utmost concern for a confined field trial. The plot area will be fenced to prevent access by man or other large mammals that may compromise material confinement or damage the plots. Access to the trial site will be limited to only authorized personnel by use of a lockable gate. Fencing of the trial site is not required where the site is located entirely within a limited-access facility or area such as an experimental farm, which provides sufficient security against ingress. A 24-hour guard may be used in conjunction with fencing to provide an extra measure of security, depending on the situation.

Each plot will be labeled or staked in order to establish its identity. The entire trial area, including guard rows where used, shall be staked in at least four corners to establish the location for post harvest monitoring. Where the trial site is fenced, fence corners or posts may be used to establish location.

(d) Equipment. Equipment used for planting, cultivation, spraying, sampling, harvesting or controlling volunteers should be cleaned of plant material capable of propagation (seeds, viable stem sections) within the trial site, before removal from the site, thus assuring the experimental material remains on the trial site. Personnel involved in these operations are also required to clean and inspect their shoes and clothing to verify that no material is removed from the trial site during these operations. Where true seed is used for planting, or where the plants are allowed to flower and produce seed, special care must be taken to ensure that no seed remains on equipment or personnel leaving the trial site. This is accomplished by adequate cleaning, followed by inspection and verification, documented by the trial manager.

(e) Personnel. Personnel involved in the direct management of the trial and the trial site will have training and qualifications sufficient for their responsibilities. Each person involved in direct management of the trial will establish and maintain a training file listing their education, work experience, qualifications, and specific training appropriate to their responsibilities. Training files will be available for inspection by the Regulatory Authority upon request.

(f) Monitoring of the trial site. The trial site will be monitored for reproductive isolation by the trial manager, according to the requirements listed above. The experimental plants will also be monitored for growth, development and any unusual characteristics at least weekly. Post harvest monitoring will be carried out according to the requirements listed below. The trial manager will document on appropriate forms all monitoring activities.

(g) Documentation. All documentation related to the trial, including the authorization letter, terms and conditions, and experimental records will be kept in an orderly fashion in a location secure from unauthorized access, and accessible to trial personnel. All records including experimental notes will be legible, recorded in indelible ink, and will include the identity of the trial. All records shall include the identity of the person making the record and the date recorded. All original records of activities on the trial site will be maintained with the experimental records, and be available for inspection by the Regulatory Authority.

(h) Sampling and harvesting. Sampling and harvesting procedures are of particular concern for confinement, especially when the plants in the plot area have been allowed to set seed. Requirements for cleaning of equipment and personnel must be applied rigorously, to prevent movement of viable seed from the trial site. This would include provision for cleaning and inspection of shoes and clothing for seed before personnel are allowed to leave the trial

site. If any harvested material from the trial site will be retained, this material will be labeled, transported and stored according to requirements outlined above.

(i) Disposal of material. Plants and plant material to be devitalized after harvest shall be burned, buried, treated with heat (e.g., autoclaved) or with appropriate herbicides, as described in 'Control of regrowth', below. The same methods will be used to destroy volunteer or prohibited plants when necessary. Seeds (in containers) to be destroyed may be incinerated, autoclaved, buried, or destroyed with chemicals or by grinding or crushing.

(j) Contingency plans. Where unauthorized or accidental release of regulated plant material has occurred or is suspected, the trial manager or other personnel will take the following steps:

- i. *Stabilize the situation.* Prevent any further loss of material or deterioration of the situation. The site of the spill must not be left unattended until the situation is stabilized.
- ii. *Prevent consumption.* Confine the spill and prevent material from being eaten by humans or livestock.
- iii. *Recover the material.* Recover all material possible. In the case of vegetative material or plantlets, steps i. through iii. are usually sufficient to prevent any serious consequences. Material recovered is subject to the same requirements for transport and labeling as outlined above.
- iv. *Notify the Authorized Party and the Regulatory Authority* within one hour of any unauthorized or accidental release.
- v. *Mark or record the location.* If seed is spilled or unintentionally released, or if other plant material cannot be completely recovered, the release site must be marked in case further monitoring is required to prevent persistence in the environment.
- vi. *Dispose of material.* If necessary, dispose of any regulated material following requirements given above.
- vii. *Consult with the Regulatory Authority.* The Regulatory Authority will determine appropriate follow-up measures for any unauthorized or accidental release.

2.3 Control of Regrowth

Reproductive isolation methods described above for confined trials of cassava either prevent the formation of seeds, or require the bagging and collection of all seeds without allowing them to fall on the ground. In these cases, seed dormancy is not a factor, and confinement measures may be focused solely on vegetative regrowth. Cassava plants will not regenerate from storage roots, and their role in persistence is not of concern. Any vegetative regrowth arising from experimental plants in the confined trial site must be identified and disposed of within the trial site before flowering, in a fashion that prevents them from being consumed by humans or livestock.

Control of regrowth from vegetative parts may be accomplished in several ways: by application of systemic or contact herbicides, by burning, or by burying. Herbicides appropriate to the control of cassava and other *Manihot* spp are: glyphosate, 2,4-D, paraquat, and phosphinothricin. If deep burial is used, vegetative material must be covered with soil to a depth of at least 30 cm. Mature vegetation may be chopped prior to burial or burning, to facilitate devitalization. In all cases of devitalization or disposal, the material to be devitalized or the disposal area will be monitored for regrowth and additional treatments of the same or different method applied, until full devitalization is achieved. The processes used for devitalization, disposal and monitoring for regrowth will be documented by the trial manager.

No *Manihot* spp. for use as food or feed may be grown on the trial site during the post-harvest interval, which should be at least 3 months. Any other crop that is not easily confused with *Manihot* spp. and that will not interfere with identification and control of volunteers may be

grown, e.g., maize or other grain. Other experimental cassava trials, including those with GM cassava, are acceptable on the trial site provided that the site is subject to confinement measures as described, and that no material from the plot is used as food or animal feed. The post-harvest interval and monitoring requirements will then commence at the end of the experimental use of the site, whether this use has been for conventional or GM cassava.

The plot area will be monitored by the trial manager at least monthly for the required post-harvest interval, to verify the post-harvest restriction against *Manihot* spp. The occurrence of any regrowth from the confined trial will be recorded, as well as the measures taken for their control and eradication. The site may be returned to general use when these requirements are completed.

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ANNEX 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).

Table prepared by Dr Kenneth M Olsen, Washington University, St Louis, MO USA.

Species	Approximate geographical range
<i>M. angustiloba</i> (Torrey) Muell.-Arg. emend Rogers & Appan	southwest USA, Mexico
<i>M. davisae</i> Croizat	southwest USA, Mexico
<i>M. walkerae</i> Croizat	southwest USA, Mexico
<i>M. aesculifolia</i> (HBK) Pohl	Mexico
<i>M. auriculata</i> McVaugh	Mexico
<i>M. caudata</i> Greenman	Mexico
<i>M. chlorosticta</i> Standley & Goldman	Mexico
<i>M. crassisepala</i> Pax & K. Hoffmann	Mexico
<i>M. foetida</i> (HBK) Pohl	Mexico
<i>M. michaelis</i> McVaugh	Mexico
<i>M. oaxacana</i> Rogers & Appan	Mexico
<i>M. pringlei</i> Watson	Mexico
<i>M. rhomboidea</i> Muell.-Arg.	Mexico
<i>M. rubricaulis</i> I.M. Johnston	Mexico
<i>M. subspicata</i> Rogers & Appan	Mexico
<i>M. tomatophylla</i> Standley	Mexico
<i>M. websterae</i> Rogers & Appan	Mexico
<i>Manihotoides pauciflora</i> (T.S. Brandegees) Rogers & Appan	Mexico
<i>M. carthaginensis</i> (Jacquin) Muell.-Arg.	Colombia, Venezuela, West Indies
<i>M. tristis</i> Muell.-Arg.	Venezuela, northern Brazil
<i>M. surinamensis</i> Rogers & Appan	Venezuela, Guayana, Suriname

Annex 1 - Wild *Manihot* species and their geographical distributions

Species	Approximate geographical range
<i>M. filamentosa</i> Pittier	Venezuela
<i>M. maguireiana</i> Rogers & Appan	Venezuela
<i>M. brachyloba</i> Muell.-Arg.	Central America, West Indies, northern & central South America
<i>M. marajoara</i> Chermonte de Miranda apud Huber	northern Brazil
<i>M. caerulescens</i> Pohl	northern, northeastern & central Brazil
<i>M. glaziovii</i> Muell.-Arg.	northeastern Brazil, introd: trop. America, Africa, India, Pacific Islands
<i>M. brachyandra</i> Pax & K. Hoffmann	northeastern Brazil
<i>M. catinae</i> Ule	northeastern Brazil
<i>M. dichotoma</i> Ule	northeastern Brazil
<i>M. epruinosa</i> Pax & K. Hoffmann	northeastern Brazil
<i>M. heptaphylla</i> Ule	northeastern Brazil
<i>M. maracasensis</i> Ule	northeastern Brazil
<i>M. pseudoglaziovii</i> Pax & K. Hoffmann	northeastern Brazil
<i>M. quinquefolia</i> Pohl	northeastern Brazil
<i>M. reniformis</i> Pohl	northeastern Brazil
<i>M. zehntneri</i> Ule	northeastern Brazil
<i>M. acuminatissima</i> Muell.-Arg.	eastern Brazil
<i>M. handroana</i> N.D. Cruz	eastern Brazil
<i>M. janiphoides</i> Muell.-Arg.	eastern Brazil
<i>M. pilosa</i> Pohl	eastern Brazil
<i>M. pohlii</i> Wawra	eastern Brazil
<i>M. sagittato-partita</i> Pohl	eastern Brazil
<i>M. warmingii</i> Muell.-Arg.	eastern Brazil
<i>M. tripartita</i> (Sprengel) Muell.-Arg.	central & eastern Brazil
<i>M. quinquepartita</i> Huber ex Rogers & Appan	northern & central Brazil
<i>M. alutacea</i> Rogers & Appan	central Brazil
<i>M. attenuata</i> Muell.-Arg.	central Brazil
<i>M. cecropiaefolia</i> Pohl	central Brazil

Species	Approximate geographical range
<i>M. crotalariaeformis</i> Pohl	central Brazil
<i>M. divergens</i> Pohl	central Brazil
<i>M. falcata</i> Rogers & Appan	central Brazil
<i>M. flemingiana</i> Rogers & Appan	central Brazil
<i>M. fruticulosa</i> (Pax) Rogers & Appan	central Brazil
<i>M. irwinii</i> Rogers & Appan	central Brazil
<i>M. jacobinensis</i> Muell.-Arg.	central Brazil
<i>M. longepetiolata</i> Pohl	central Brazil
<i>M. mossamedensis</i> Taubert	central Brazil
<i>M. nana</i> Muell.-Arg.	central Brazil
<i>M. oligantha</i> Pax	central Brazil
<i>M. orbicularis</i> Pohl	central Brazil
<i>M. paviaefolia</i> Pohl	central Brazil
<i>M. peltata</i> Pohl	central Brazil
<i>M. pruinosa</i> Pohl	central Brazil
<i>M. purpureo-costata</i> Pohl	central Brazil
<i>M. pusilla</i> Pohl	central Brazil
<i>M. quinqueloba</i> Pohl	central Brazil
<i>M. reptans</i> Pax	central Brazil
<i>M. salicifolia</i> Pohl	central Brazil
<i>M. sparsifolia</i> Pohl	central Brazil
<i>M. stipularis</i> Pax	central Brazil
<i>M. tomentosa</i> Pohl	central Brazil
<i>M. triphylla</i> Pohl	central Brazil
<i>M. weddelliana</i> Baillon	central Brazil
<i>M. violacea</i> Pohl	central Brazil
<i>M. xavatinensis</i> Rogers & Appan	central Brazil
<i>M. esculenta</i> subsp. <i>flabellifolia</i> (Pohl) Ciferri	western & central Brazil

Annex 1 - Wild *Manihot* species and their geographical distributions

Species	Approximate geographical range
<i>M. stricta</i> Baillon	Peru, western & central Brazil
<i>M. leptophylla</i> Pax	Ecuador, Peru, western & central Brazil
<i>M. grahami</i> Hooker	southeastern Brazil, northern Argentina, Paraguay, Uruguay
<i>M. inflata</i> Muell.-Arg.	southern Brazil
<i>M. corymbiflora</i> Pax	southeastern Brazil
<i>M. leptopoda</i> (Muell.-Arg.) Rogers & Appan	southeastern Brazil
<i>M. jolyana</i> N.D. Cruz	southeastern Brazil
<i>M. condensata</i> Rogers & Appan	Bolivia
<i>M. guaranitica</i> Chodat & Hassler	Bolivia
<i>M. anomala</i> Pohl	central Brazil, Paraguay
<i>M. gracilis</i> Pohl	central Brazil, Paraguay
<i>M. pentaphylla</i> Pohl	central Brazil, Paraguay
<i>M. hassleriana</i> Chodat	Paraguay
<i>M. mirabilis</i> Pax	Paraguay
<i>M. variifolia</i> Pax	Paraguay
<i>M. populifolia</i> Pax	Paraguay
<i>M. procumbens</i> Muell.-Arg.	southern Brazil, Paraguay
<i>M. affinis</i> Pax	southern Brazil
<i>M. tenella</i> Muell.-Arg.	southern Brazil
<i>M. hunzikeriana</i> Martinez-Corvetto	southern Brazil, Argentina
<i>M. anisophylla</i> (Grisebach) Muell.-Arg.	Argentina