LEONARDO AUGUSTO ZEBRAL RODRIGUES

CRYOPRESERVATION OF CASSAVA GENOTYPES

LAVRAS – MG

2012

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, área de concentração em Biologia Molecular, para a obtenção do título de Doutor.

Orientador

Dr. Luciano Vilela Paiva

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Alfredo Augusto Cunha Alves, PhD;	EMBRAPA
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RESUMO GERAL

Mandioca (*Manihot esculenta* Crantz) é um alimento básico para a alimentação humana. É a terceira mais importante fonte de carboidratos nos trópicos, ocupando a sexta posição quando considerada a produção global. Nos últimos anos vários grupos de pesquisa têm relatado métodos para cultura de tecidos vegetais de mandioca, oferecendo importantes aplicações para as coleções de germoplasma, tais como a conservação, a facilidade de intercambio, micropropagação, erradicação de doenças, entre outros. Assim, um dos objetivos deste estudo foi ampliar essas investigações, avaliando os fatores associados à regeneração *in vitro* de duas cultivares de mandioca. Segmentos uninodais de plantas de mandioca crescidas *in vitro* foram cultivados até a obtenção de gemas axilares alongadas. Meristemas foram excisados e cultivados em quatro diferentes meios de cultura. A regeneração, o enraizamento e a formação de calos foram avaliados. Como esperado, uma diferença significativa foi encontrada entre os meios de cultura testados, em grande parte devido à presença ou ausência dos diferentes reguladores de crescimento vegetal. Para ambas as cultivares o melhor meio de cultura foi suplementado com 0,2 $mg.L^{-1}$ de cinetina e 1,0 $g.L^{-1}$ de carvão ativado. Neste meio, houve uma boa regeneração e enraizamento, além de uma completa ausência de calos. Em sequência a isso, vários métodos de criopreservação foram testados para o desenvolvimento de um protocolo de criopreservação eficiente para a conservação dessas cultivares. Usando a técnica de vitrificação, com uma solução de pré-tratamento modificada contendo 0,5% de Tween-20, alcançamos uma taxa de regeneração superior a 50% após exposição ao nitrogênio líquido.

Palavras-chave: Mandioca. Micropropagação. Meristemas apicais. Criopreservação. Vitrificação.

ABSTRACT

Cassava (*Manihot esculenta* Crantz) is a staple food crop for human consumption is the third most important source of carbohydrates in the tropics, and ranks sixth place in overall production. In recent years several research groups have reported methods for plant tissue culture of cassava, offering important applications for germplasm collections, such as conservation, ease of exchange, micropropagation, eradication of diseases, among others. One objective of this study was to further these investigations by evaluating factors associated with *in vitro* regeneration of two cultivars of cassava. Uninodal segments of *in vitro* grown cassava plants were cultivated until axillary buds elongated, shoot tips were excised from the shoots and cultured in four different culture media. Regeneration, rooting and callus formation were evaluated. As expected, a significant difference was found between the culture media tested due largely to differences in the presence or absence of plant growth regulators. For both cultivars the best culture medium was supplemented with a 0.2 $mg.L^{-1}$ kinetin and 1.0 $g.L^{-1}$ of activated charcoal. In this medium, we good regeneration and rooting and the complete absence of callus. Subsequent to this, several cryopreservation methods were tested to development of an efficient cryopreservation protocol for the conservation of these cultivars. Using a vitrification technique with a modified pre-treatment solution containing 0.5% Tween-20 a regeneration rate after liquid nitrogen exposure of more than 50% was achieved.

Keywords: Cassava. Micropropagation. Shoot tips. Cryopreservation. Vitrification.

SUMÁRIO

1 INTRODUÇÃO GERAL

Plant genetic resources are a natural reservoir of genes with potential use for the sustainable production of essentials to humanity, such as food, fiber and medicines, as well as being a means of preserving the genetic heritage of endangered species. Most cultures of success are derived from and dependent on its wild ancestors. However, this biodiversity is being destroyed at an alarming rate due to growth disorganized and uncontrolled exploitation of ecosystems and natural resources.

Plant genetic resources conservation carried in the natural habitats of species populations (*in situ*) or outside them (*ex situ*) has been proposed as a more rational way of preventing unexpected events, such as the occurrence of new pests and diseases, and the loss of resistance of certain crops resulting from the process of genetic erosion.

In situ conservation offers several advantages such as, the plant population under this condition is open to free genetic exchange, selectively advantageous mutations may accumulate and spread among the populations, healthy competition among members of the population helps it to better adapt and possess greater genetic diversity. However, *in situ* conservation is always under the threat of natural forces and calamities. Besides, for some species *in situ* conservation may not be suitable due to their small population size, wide dispersal, genetic drift due to inbreeding, competition from invasive species, and high vulnerability to abiotic and biotic stresses. In this case, is better the conservation of genetic resources, away from their natural habitat. The *ex situ* plant genetic resources conservation can be carried out in an efficient and economical way by seed conservation, in seedbanks. However, to overcome these limitations, studies in biotechnology have intensified, and being the cryopreservation is the best available technique with the potential to ensure

long-term conservation of germplasm of plant species. Cryopreservation is defined as the conservation of biological material in liquid nitrogen at -196° C, or its vapor phase at -150° C. Through this procedure the entire cellular metabolism is drastically reduced and biological deterioration is virtually paralyzed and, therefore, considered the most promising method for long-term preservation of cells, tissues and plant organs. These explants can be regenerated into new plants at any time, without the risk of genetic variation.

Thus, tissue culture techniques together with cryopreservation are of great interest for the medium and long-term conservation of plant germplasm, particularly of tropical species. However, study and evaluation of different protocols it is necessary. As in any others crop, *in vitro* culture techniques and cryoprotocols in cassava are genotype and hence cultivar specific Here in this work, we conducted a study with different culture media to evaluate the performance of *in vitro* shoot tips of two cassava cultivars. Furthermore, different protocols for cryopreservation were tested and, after some adaptation, one of these protocols showed to be efficient for cryopreservation of shoot tips of two cassava cultivars studied here.

2 REFERENCIAL TEÓRICO

2.1 Cassava

Cassava (*Maninhot esculenta* Crantz) is a woody shrub originating in Latin America, probably in the Amazon Basin (Leotard *et al.,* 2009; Nassar and Ortiz, 2007; Allem, 2002; Hillocks, 2002; Olsen and Schaal, 1999). Cassava belongs to the Division Magnoliophyta, Class Magnoliopsida, Order Euphorbiales, Family Euphorbiaceae and subfamily Crotonoideae (McKey *et al.,* 2010; Tafur, 2002; Viana *et al.,* 2002; El-Sharkawy, 2004; Puonti-Kaerlas, 1998; Fauguet Fargetta, 1990). Among the 150 species, *M. esculenta* Crantz is the only commercially important species in the genus *Manihot* (Ricardo *et al.,* 2007; Alves, 2002; Cebalos and Cruz, 2002a), with the physiological characteristic of the ability to store starch in its roots (Otsubo *et al.,* 2002).

It was domesticated less than 10,000 years ago when early European sailors recognized the advantages of the crop and carried it to Africa and Asia. It is cultivated in all tropical and subtropical regions of the world, between latitudes 30° N and 30° S (Christopher, 2008; Nassar, 2004). The rainfall for good crop development ranges from 600 to 1,200 mm, with an average temperature of 25° C (Sediyama *et al.,* 2007) and a photoperiod of 12 hours (Bolhuis, 1966; Alves, 2006). Cassava is a very rustic crop that grows well under marginal conditions where few other crops could survive. A large proportion of cassava varieties are drought tolerant, can produce a crop in degraded soils, and are resistant to most diseases and pests. The crop is naturally tolerant to acidic soils, and offers the convenient flexibility that it can be harvested when the farmers need it. This adaptive capacity for growth, the need

for low inputs and low levels of cultivation technology and its good yield potential, make cassava an ideal staple food for many countries.

Cassava, along with rice and corn, constitute an important source of energy for more than 800 million people in the developing world (El-Sharkawy and de Tafur, 2010; Nassar and Ortiz, 2010; Nassar *et al.*, 2008). It is the most important root and tuber crop in the tropics and ranks as the sixth most important food crop in terms of global annual production (FAOSTAT, 2010). Current global cassava production is around 250 million tons, of which Africa accounts for half (51%), while Asia and Latin America produce 34% and 15%, respectively (FAO/FAOSTAT, 2011) (Figure 1).

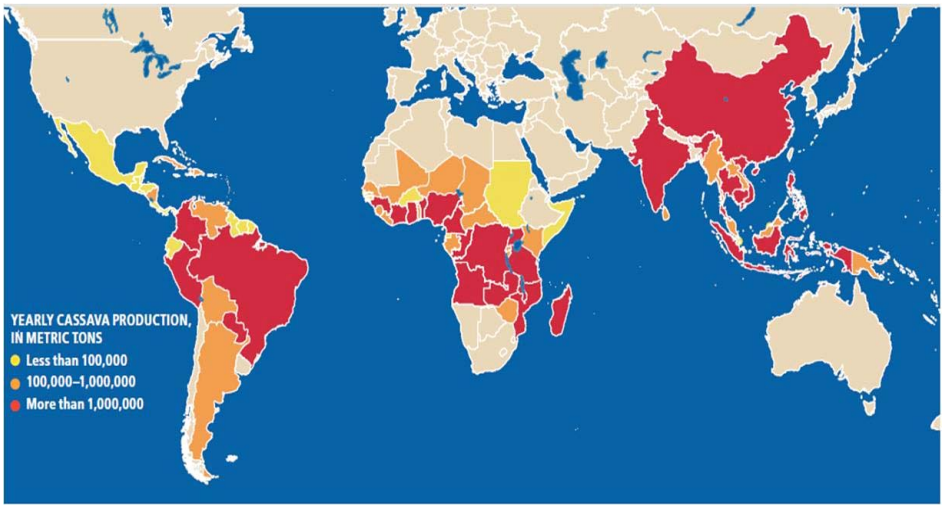

Figure 1 – Worldwide cassava production areas. Cassava is a favorite of subsistence farmers throughout the tropics and especially in Africa.

Until recently, cassava and its products were not well known outside the tropical and subtropical regions where it grows (Figure 2).

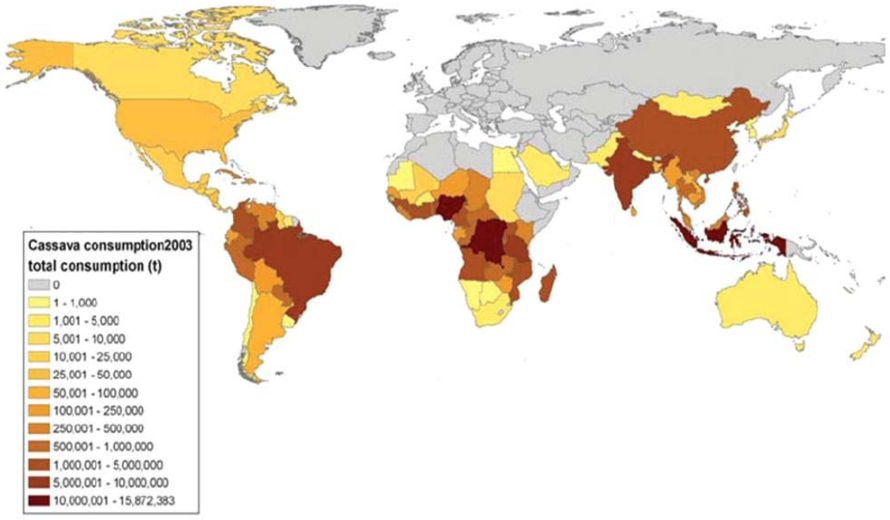

Figure 2 – Total consumption of cassava in 2003. Cassava is a major part of the diet for over 800 million people in approximately 80 countries. It is widely grown for consumption in the tropics and subtropics, in a range of habitats including semi-arid savannas and mountains regions.

Cassava can be propagated either by vegetative propagation or by sexual seed. However, the former is the most common practice used by farmers for multiplication and planting purposes. Propagation from true seed occurs occasionally in farmers fields and, as such, is a starting point for the generation of useful genetic diversity (Alves, 2002). Unfortunately, vegetative propagation causes low productivity due to physiological aging caused by the constant multiplication and infestation by diseases that are transmitted through successive generations (Silva *et al.*, 2002).

Cassava is monoecious, with female flowers opening 10-14 days before the male ones on the same branch. Self-pollination can occur because male and female flowers on different branches or on different plants of the same genotype can open simultaneously (Jennings and Iglesias, 2002). Flowering depends on the genotype and the environmental conditions. Branching occurs when an inflorescence is formed. Because erect, non-branching types, are frequently

preferred by farmers, the crossing of elite clones in certain regions may become more difficult because of the scarcity of flowers. Synchronization of flowering remains a difficult issue in cassava breeding. Some clones flower relatively early at 4 or 5 months after planting whereas others flower only 8 to 10 months after planting. Because of this, and the time required for the seed to mature, it takes generally at least a year to obtain seeds of planned crosses. On average, between one and two seeds per pollination are obtained. Seeds often have a dormancy period for a few months after maturity, and they require relatively high temperatures (30-35º C) for optimum germination.

2.2 Genetics Resources

Genetic variability and genetic diversity is of great importance for plant geneticists, breeders and taxonomists (Prince *et al.,* 1995). In populations, the genetic composition and genetic diversity are derived from wild progenitors and is influenced by evolutionary processes such as mutation, recombination, genetic drift, migration, natural selection and adaptation to different environments. In general, the knowledge of genetic diversity and the relationship among sets of germplasm as well as their potential breeding values would be beneficial to all phases of crop improvement (Hartl and Clark, 1997).

Plant genetic resources are a natural reservoir of genes with potential use for the sustainable production of essentials to humanity, such as food, fiber and medicines, as well as being a means of preserving the genetic heritage of endangered species (Berjak *et al.,* 1989). Today many commercial cultivars are products of breeding employing crosses with wild species. This wild species are a gene bank to be widely exploited in breeding. However, this biodiversity is being destroyed at an alarming rate due to unorganized growth resulting in the uncontrolled exploitation of ecosystems and natural resources. *In situ*

conservation and *ex situ* conservation of germplasm of local breeds, varieties and wild relatives of domestic species is proposed as a rational way to preserve genes which can be used in breeding programs to develop new varieties that can prevent losses due to unexpected events such as new pests and diseases, or the loss of resistance in cultivars due to biotype evolution of the pests or genetic erosion in the crop (Faleiro, 2004 and Bajaj, 1995).

Conservation of plant genetic resources is necessary to maintain food security and agrobiodiversity. Worldwide many plant species are threatened and therefore conservation is extremely important because of their potential disappearance. Over exploration of forest species, destruction of natural ecosystems caused by human activities, such as climate change and industrialization due to urbanization, have contributed further increasing the extinction danger.

Plant genetic resources conservation can be carried out either in the natural habitats of species populations (*in situ*) or outside them (*ex situ*) (Iriondo, 2001). The conservation of genetic resources implies the maintenance of collections *in situ* at their place of occurrence, or *ex situ* (Valois, 1998). In this case, individuals can be maintained, seeds, embryos and other plant structures under different conditions, depending on the material: in the field or in greenhouses, at low temperatures in culture medium with low salt concentration (conservation *in vitro*) or cryopreserved (Gonzalez-Benito *et al.,* 2004; Bajaj, 1995 and CGIAR, 1993).

A goal of conservation of genetic resources in a natural habitat, *in situ* conservation (Ravindran *et al.,* 1999; Tikader and Thangavelu, 2002), is to protect the genetic resources from natural and man-made destructive forces. Since the native land of a particular plant species is rich in genetic diversity, *in situ* conservation is a good way to conserve the entire genetic diversity of wild and weedy relatives of crop species (Vaughan and Chang, 1992). *In situ*

conservation offers several advantages such as, the plant population under *in situ* conservation is open to free genetic exchange allowing evolutionary process to progress by the accumulation of selectively advantageous mutations which can spread among the populations. It also provides an environment with healthy competition among members of the population to better adapt and capture genetic diversity (Engles, 2001). However, *in situ* conservation is always under the threat of natural forces and calamities. For some species *in situ* conservation may not be suitable due to their small population size, wide dispersal, genetic drift due to inbreeding, competition from invasive species, and high vulnerability to abiotic and biotic stresses.

Ex situ conservation includes the conservation of genetic resources away from their natural habitat in botanical gardens, research institutes, experiment stations, nurseries or home gardens and seed gene banks is called (Tikader *et al.,* 2009). *Ex situ* plant genetic resources conservation can be carried out in an efficient and economical way by seed conservation, in seedbanks. The most common and easiest method of *ex situ* germplasm conservation is storing seeds at low relative humidity and low temperature. However, seed storage is not feasible for some plants. Seeds of some species are recalcitrant or intermediate and they cannot survive desiccation below a relatively high critical water content value (desiccation-sensitive), and because of this cannot be stored frozen and therefore lose viability much faster (Hong *et al.,* 1996; King and Roberts, 1980; Berjak et al., 1990). The seedbank also is not feasible for plants vegetatively propagated, including *Dioscorea* spp. (yam), *Solanum* spp (potato), *Musa* spp. (banana), *Manihot* spp. (cassava), *Colocasia esculentum* (taro), and *Ipomoea batatas* (sweet potato). The genetic conservation of these species must be carried out in field collections, *in vitro* or in cryopreservation. Field plantings are often used due to a low requirement for technology yet are labour intensive and are exposed to losses due to disease or pest attacks or natural disasters.

The genetic variability available within *Manihot* has not been fully explored or screened. Therefore, this genetic wealth has not been fully exploited, and should offer interesting possibilities for the future. In part, the limited evaluation of cassava genetic variability is because most cassava germplasm collections are preserved in fields where the maintenance is difficult, cumbersome and expensive.

Currently there are reports estimating that nearly 2/3 of the cassava genetic diversity exists only in *in situ* collections, with only 1/3 being securely conserved in *ex situ* collections. In the case of cassava, the Manihot Genetic Resources Network representing 15 National Agricultural Research Systems (NARS), the International Institute for Tropical Agriculture (IITA), CIAT and the International Plant Genetic Resources Institute (IPGRI) , now called Biodiversity, recognized that cassava germplasm conservation should be broadened with increased *ex situ* conservation of cassava genotypes, to more thoroughly conserve the genetic diversity of the primary genepool and valuable genes and gene complexes in other Manihot spp. (IPGRI, 1994).

According to Vieira (2000), active collections of *Manihot* germplasm are held in several institutions each with the responsibility to maintain genetic diversity, collect genetic resources, facilitate exchange with other genebanks, multiply collections for distribution and continuously characterize the collections. The collections are in contrast to the base collections which are back-ups of the active collection which are for long-term conservation and generally not used for study, exchange or characterization unless the active collection is lost or compromised (Pistorius, 1997; Sackville and Chorton, 1997).

The largest *ex situ* cassava collections are held by CIAT (Colombia) with about 6500 accessions, followed by EMBRAPA (Brazil) with about 4500 accessions. Other important collections are those in CTCRI (India), INIA (Peru),

NRCRI (Nigeria), IITA (Nigeria), IAN (Paraguay), SRCV (Benin) and PGRC (Ghana).

Plant breeding has one of the highest rates of return among the investments in agricultural research. It has been reported that the remarkable increase in the productivity of many crops during the twentieth century was due to genetic gains achieved through crop breeding. Cassava has also benefited from technological inputs though breeding (Kawano, 2003). New varieties in Africa, Asia, and Latin America and the Caribbean satisfy the needs of farmers, processors, and consumers, bringing millions of dollars in additional income to small farmers. Besides, these new technologies in the area of tissue culture, germplasm conservation, genetic transformation and molecular biology have also made positive contributions for this important crop (Calderón-Urrea, 1988; DeVries and Toenniessen, 2001; Fregene *et al.,* 1997, 2000; Puonti-Kaerlas *et al.,* 1997).

2.3 *Ex situ* conservation

In vitro plant regeneration can be achieved via *in vitro* culturing of shot-tips, axillary buds, and somatic embryogenesis and organogenesis from callus obtained from different explants. Conservation of genetic resources can utilize all *in vitro* regenerations systems. In the simplest terms, *in vitro* technology is based on the property of plants to regenerate whole plants from single cells (totipotency). Unfortunately, regeneration from single cells or less differentiated cells is not desirable under most conservation systems as this type of regeneration is also associated with a higher risk of somaclonal variation. Therefore, for genetic resources conservation, systems that will regenerate true, such as from a pre-formed meristem are more desirable.

Since regenerants originating from cloning should be identical to the mother plant, a small piece of plant material can be considered as germplasm and it can be used as storage material (Shii *et al.,* 1994). However, the successful propagation depends on several steps, including initiation of cultures free of contamination, establishment of shoot-producing or embryogenic cultures, the rooting of shoots or the outgrowth of embryos into plants, and the acclimatization of those plants to the *ex vitro* environment. A number of endangered species have been propagated *in vitro* and several research teams have used *in vitro* cultures not only as a method for clonal propagation and safe exchange of plant material but also for medium-term *ex situ* germplasm conservation.

In vitro storage based on tissue culture techniques has been used as an alternative strategy for conservation of genetic resources of plants. This procedure is used at the two biggest cassava germplasm banks. At CIAT (CIAT web site) in Colombia, over 6,500 cassava accessions are kept in *in vitro* storage and at the Empresa Brasileira de Pesquisa Agropecuaria (The Brazilian Agricultural Research Corporation, EMBRAPA, Brazil), more than 4000 cassava accessions are maintained in this condition (Fukuda *et al.,* 2008). Other than actively growing cultures requiring extensive resources to maintain, two other types of *in vitro* conservation methods are employed for conserving plant genetic resources, slow growth method and cryopreservation. In slow growth or short-term storage conditions, the cultures are kept under growth-limiting conditions, thereby extending the intervals between subcultures (Razdan and Cocking, 2000). The growth rate of the cultures can be limited by various methods, including reduced temperature or light intensity or manipulation of the medium by adding osmotic agents or growth retardants or reducing the nutrient salts (Bajaj, 1989; Withers, 1984). Short-term storage is especially important for vegetatively propagated plants, recalcitrant seed species and plants with

unavailable or nonviable seeds (Ashmore, 1997). Species kept under these *in vitro* conditions include, *Allium* spp., *Cocos nucifera*, *Theobroma cocoa*, *Vitis, Prunus*, *Citrus* spp., *Saccharum*, *Solanum* spp., *Musa* spp., *Colocasia esculentum*, *Manihot* spp., and *Ipomaea batatas* (Henshaw, 1975; Zapartan and Deliu, 1994; Withers, 1995; Ashmore, 1997; Withers and Engelmann, 1997; Engelmann and Engels, 2002; Gonzalez-Benito *et al.,* 2004; Paunesca and Holobiuc, 2005; Sarasan *et al.,* 2006). Slow growth techniques are available for a wide range of plant species, but only used routinely for genetic resource conservation of a limited number of species. This is because of challenges in the management of collections even if the intervals between transfers are extended and also because of concerns of genetic instability caused by somaclonal variation (Shikhamany, 2006).

For long-term preservation of genetic materials, the slow growth method is less useful as it requires repeated regeneration and may suffer from genetic changes and pathogen contamination (Ashmore, 1997). Thus for long-term preservation cryopreservation is often the system of choice for vegetatively propagated crops. In cryopreservation, the plant materials are stored at −196 ºC in liquid nitrogen (LN). At this temperature, cell division and metabolic activities remain suspended and the material can be stored without any genetic changes for long periods. In fact, cryopreservation is the only cost-effective method for long-term conservation of vegetative propagated plants like cassava.

Different types of plant cells, tissues and organs can be used for cryopreservation of plant genetic resources. Nonetheless, the most common organs used for cryopreservation of vegetatively propagated plants are shoot tips or dormant axillary buds (Towill and Forsline, 1999). Shoot tips 1–3 mm long are widely used for cryopreservation due to their high genetic stability and ability to recover a plant (Ashmore, 1997). However, the protocols for for *in*

vitro propagation are not fully developed for many species and can be very genotype-specific.

2.4 Cryopreservation techniques

There are many different cryopreservation methods which include advantages and disadvantage that should be considered according to species, cultivar, origin of tissue to be cryopreserved and, also the facilities available (Reed, 2001). Basically, we can distinguish the methods based on freeze-induced dehydration of cells (classical methods) and, more recently, those based on tissue vitrification including, vitrification, encapsulation-dehydration, encapsulation-vitrification, and droplet vitrification (Engelmann, 2000).

Plant cryopreservation procedures were first developed following the success of animal cell cryopreservation. They were based on chemical cryoprotection and slow cooling, followed by rapid immersion in liquid nitrogen. Termed slow controlled freezing or two-step freezing this method combines the application of penetrating cryoprotective substances such as DMSO and controlled freeze dehydration, often preceded by cold or sugar hardening or osmotic dehydration. Using decreasing temperature at a relatively slow rate, usually 0.5 ºC/min until -4º C and 0.1º C/min until -40º C, followed by rapid immersion of samples in liquid nitrogen. Ice crystals are formed in the extracellular solution and water is removed from the intracellular one, leading to cellular dehydration and therefore avoiding intracellular ice formation (Engelmann, 1997a).

These cryopreservation procedures have been highly successful with callus and cell cultures, consisting of rather uniform and small cellular units (Schrijnemakers and van Iren, 1995; Lynch, 2000). However, this technique is

usually not very effective to cryo-preserve larger structures which comprise different cell types, such as shoot apices. Nevertheless, there are reports of successful examples of two-step shoot-tip cryopreservation, with vegetatively propagated species such as cassava or potato (Benson *et al.,* 1989; Fukai *et al.,* 1991; Engelmann, 1997a).

Different protocols have been developed for the cryopreservation of plant tissues. Vitrification technique developed by Sakai *et al*. (1990), can be defined as the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling (Fahy *et al.,* 1984). The freezing procedure referred to as vitrification comprises a pretreatment (loading solution) at room temperature, followed by exposure to a vitrification solution at 25 or 0º C, rapid cooling and warming, and final removal of the vitrification solution by washing samples with an unloading solution consisting of liquid culture medium supplemented with higher sucrose (Withers and Engelmann 1997). Different Plant Vitrification Solutions have been successfully developed by various research groups (Langis and Steponkus, 1990; Sakai *et al.,* 1990). The most frequently used and efficient plant vitrification solution so far is PVS2 (MS-based culture medium with 0.4 M sucrose supplemented with 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% (w/v) DMSO) (Sakai *et al.,* 1990).

Cryopreservation techniques based on vitrification are usually simple to carry out and applicable to complex structures such as embryos and shoot apices (Withers and Engelmann, 1997). The primary disadvantage is that some vitrification solutions, such as PVS2, can be toxic to plant tissues.

There are other two cryo-methods derived from synthetic seeds technology include encapsulation-dehydration and encapsulation-vitrification (Lambardi *et al.,* 2006). The method involves the inclusion of explants in a Na-alginate solution (1-5%, 3%) and their subsequent polymerization (in a high $CaCl_2$ medium (50-100 mM $CaCl_2$ solution) resulting in a hardened alginate

bead containing the tissue. In encapsulation-dehydration the procedure is continued by subsequent culture of the bead in a series of high concentrated sucrose solution (0.3-1.5 M), followed by physical dehydration (silica gel) or air drying (laminar flow cabinet) to a moisture content of 18-25% and direct immersion in liquid nitrogen. This method is simple and has been successful for apices of numerous species from tropical origin including cassava and sugarcane and of temperate origin like pear, apple, grape and eucalyptus (Dereuddre, 1992; Engelmann, 1997a; Sakai, 2004). The culture of explants on sucrose enriched medium (0.3-0.7%) prior to encapsulation, usually improves survival after desiccation and freezing (Takagi et al., 1997). However, some species do not tolerate the high sucrose concentrations employed limiting the method efficiency. For encapsulation-vitrification, the encapsulated plant tissue is exposed to a vitrification solution, without any further physical desiccation.

In many temperate species, incubation of germplasm at low temperature (generally 1° C to 10° C) to cold acclimate the tissue, for periods ranging from days to weeks, increases survival after freezing (Reed, 1990; Wu *et al.,* 2000). During cold acclimation cellular changes such as numerous smaller vacuoles, more abundant mitochondria and rough endoplasmic reticulum and accumulation of certain proteins occur (Shikhamany, 2006).

The droplet vitrification technique can be considered a modification of encapsulation-vitrification. It consists on treating shoot apices in drops of PVS2 placed on aluminium foil strips, which are rapidly immersed in liquid nitrogen (Mix-Wagner *et al.,* 2003). The term droplet refers to droplets of cryoprotectant on an aluminium foil, into with explants are placed for cooling and rewarming. The original idea of using aluminium foils came from Kartha *et al.* (1982), who cryopreserved cassava shoot tips on foils in plastic Petri dishes using a two-step cooling method. The foil strips make it easier to handle a large amount of tissues at once quickly into and out of liquid nitrogen. Also, aluminium is a very good

heat conductor important for quick cooling and rewarming of samples (Schäfer-Menuhr, 1996).

This method has been applied successfully to more than 150 varieties and, is now one of the methods that receive considerable attention in large scale cryopreservation of germplasm collections including banana, sweet potato, taro, potato and garlic (Panis *et al.,* 2009; Halmagyi *et al.,* 2004).

For the recovery of apices after cryostorage, rapid thawing is usually required to avoid recrystallisation (Towill and Jarret, 1994). Vials containing the apices are usually immersed in a water bath at 35-45 ºC.

Some studies have shown the importance of the appropriate post-thawing culture conditions to enhance organized growth. For example, potato cv. Desirée shoot tips showed higher recovery when incubation during the first week after thawing was performed under low light intensity (Benson *et al.,* 1989). In many cases, selection of a suitable growth medium for apex recovery may be necessary. Adjustment of growth regulator concentration (Withers and Endelmann, 1997) or even medium salt formulation (Pennycooke and Towill, 2001; Decruse and Seeni, 2002) could be required for the normal development of frozen shoot apices. Some media supplements such as iron chelating agents or surfactants have been shown to improve recovery (Benson *et al.,* 1995; Pennycooke and Towill, 2001).

2.5 Cassava Cryopreservation

Since cassava is a vegetative propagated crop, shoot tips excised from *in vitro* plants are widely used for cryopreservation. Shoot tips are preferred because are pre-formed meristematic organs, providing more genetic stability (Charoensub *et al*., 2004).

Various authors have successfully developed different cryoprotocols for cassava shoot tips (Kartha *et al.,* 1982; Bajaj, 1983; Engelmann *et al.,* 1994; Charoensub *et al.,* 1999, 2003 and 2004; Danso and Ford-Lloyd, 2011 and 2003). The first protocol for cassava shoot tip cryopreservation was developed by Kartha *et al*. (1982), and used a cryoprotective treatment with MS-basal medium solution containing 15% DMSO and 3% sucrose, followed by slow controlled freezing. After shoot tips were exposure into LN_2 they obtained some direct regrowth, however, also reported a high incidence of callus. Bajaj (1983), reported success in cryopreservation of cassava shoot tips using a pre-treatment with MS-basal salts supplemented with 5% DMSO, 5% sucrose and 5% glycerol. After shoot tips were exposured into LN_2, they obtained similar results to those reported by Kartha. Years later Engelmann *et al.* (1994), tested the encapsulation-dehydration technique with cassava shoot tips using a 24 hours pretreatment in 0.75 M sucrose or 72 hours in 0.5 M sucrose, followed by desiccation to approximately 30% moisture content (fresh weight basis) and freezing by direct immersion in LN_2. He obtained good results with 60% shoot tip survival, however, also reported the occurrence of callus on the regrowth medium employed. Charoensub *et al*. (1999 and 2003) also demonstrated that cassava shoot tips could be cryopreserved through vitrification using one pre-treatment with MS-basal medium containing 0.3 M sucrose for 16 hours, followed the cryoprotective treatment with a MS-basal medium containing 2 M glycerol and 0.4 M sucrose for 20 minutes at room temperature, dehydration

with Plant Vitrification Solution (PVS2) for 45 minutes and freezing by direct immersion of samples in LN_2. Using this protocol, six of the ten cultivars studied achieved more than 70% shoot tip survival. They concluded that this protocol is promising as a practical method for cryopreservation of cassava germplasm but further study was necessary to apply this technique to a wide range of cassava diversity. Charoensub *et al*. (2004) then reported the use of encapsulation-vitrification for cryopreservation of cassava shoot tips using a preculture on MS-basal medium containing 0.3 M sucrose for 16 hours, encapsulation in 3% calcium alginate, osmoprotection at room temperature for 90 minutes with MS-basal medium containing 2 M glycerol and 0.6 M sucrose, and then dehydrated with PVS2 for 4 hours at 0º C before direct immersion in LN_2. Four cultivars were tested with results as high as 80% shoot tip survival for three cultivars. More recently, Danso and Lloyd (2011), reported cryopreservation for cassava shoot tips and embryogenic calli by slow freezing and vitrification. Their study showed a faster cooling rate for embryogenic callus when compared with shoot tips upon immersion in LN_2. Using these protocols the shoot tip explants showed only post-thaw callus formation. In contrast, all embryogenic calli explants survived after post-thaw by slow freezing and using vitrification he did not saw viability.

Although many studies achieved good results none of these protocols was tested for large scale routine cryopreservation of cassava germplasm. Some one of these protocols showed excellent post-thaw shoot formation, however, when it was tested in other cassava cultivars the results were not as good (2-21%) (Escobar *et al.,* 2000).

The aim in this work was to make a comprehensive review of *in vitro* propagation of cassava with emphasis on conservation of plant germplasm. The results of this study will facilitate the development of micropropagation systems of cassava (Paper 1), allowing the introduction of new cultivars by using *in vitro*

culture media and conditions most appropriate for the cultivar studied. In addition, all techniques described above were tested and only one of them showed suitable results for cryopreservation of cassava shoot tips (Paper 2). This knowledge may facilitate the expansion as well as protection of the germplasm collection, and also, can contribute to the maintenance of *in vitro* culture systems allowing the introduction of new cultivars in the germplasm banks of this important crop.

3. GENERAL CONSIDERATIONS

Protocols for micropropagation, regeneration and acclimatization were developed that will assist in the propagation of cassava clones of interest, providing quality genetic material homogeneous and free of pathogens, which can be used for cryopreservation.

A cryopreservation protocol based on vitrification was successfully implemented which should aid in the conservation of germplasm of this important crop. However, more research is needed to evaluate the applicability of this technique with genetic resources collections.

In addition, several variations to the cryopreservation protocols were tested. We found that cold aclimatation (-1º C in the dark for 16 hours followed by 20º C with light for 8 hours) was lethal to cassava. When we tested the encapsulation-dehydration procedure we found that the cultivar CM 507-37 was more tolerant of higher sucrose concentrations but both cultivars were very sensitive to high sucrose. When we tested slow cooling shoot tips survived nucleation (-9º C) but did not survive slow cooling to -35º C. We are also testing droplet vitrification, however, these are still in progress and do not allow us to make conclusions.

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Paper 1

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Studies on culture media and explants source for micropropagation and *in vitro* conservation of cassava (*Maninhot esculenta* Crantz)

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Abstract

Cassava is one of the most important root crop in the tropics, ranks third, after rice and corn, as a staple food root-crop for more than 800 million people in the developing world. Since it is a vegetative propagated crop, the use of *in vitro* propagation is very important to preserve the germplasm. Several protocols for *in vitro* cassava micropropagation have been used, in which the main results were large differences in the performance of different accessions to a specific medium. The goal of the present paper is to evaluate the performance of contrasting cassava genotypes under different culture media in order to achieve the best results for establishing a comprehensive protocol for cassava micropropagation. Shoot apical meristems were used for *in vitro* regeneration in two cassava cultivars (CM 507-37 and COL 1468). Shoot tips were taken from uninodal segments with 0.5-0.7 cm length with 14-21 days-old and cultivated in four different media. The culture media used in this study was 1) MS (Murashige & Skoog, 1962), supplemented

with 1.0 mg.L^{-1} IAA; 2) MS-basal supplemented with 0.2 mg.L^{-1} Kinetin, 1.0 g.L^{-1}actived charcoal; 3% sucrose and agar at pH 5.7; 3) MS-basal supplemented with 0.04 mg.L^{-1} BAP, 0.05m g.$L^{-1}$$GA_3$; 0.02m g.$L^{-1}$NAA; 2% sucrose and agar at pH 5.7; 4) MS-basal supplemented with 0.02 mg.L^{-1} BAP, 0.1m g.$L^{-1}$$GA_3$; 0.01m g.$L^{-1}$NAA; 3% sucrose and agar at pH 5.6. The best results on regeneration and rooting without callus formation were obtained in the treatment containing 0.2 mg.L^{-1} Kinetin, 1.0 g.L^{-1}actived charcoal.

Keywords. Cassava. Micropropagation. Germplasm collection. Shoot tips.

Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial woody shrub (1-4 m in height) with domesticated plants derived from one or more species of the genus *Maninhot* (Ricardo *et al.,* 2007; Alves, 2002). The cultivated species may be derived from the wild progenitor *M. flabellifolia* (Fregene *et al.,* 1994; Roa *et al.,* 1997), first domesticated 5,000–7,000 years BC in marginal areas to the Amazon region (Leotard *et al.,* 2009; Nassar and Ortiz, 2007; Allem, 2002; Hillocks, 2002; Olsen and Schall, 1999). During the 16th through 19th centuries European explorers recognized the value of cassava as a food and cash crop and spread its cultivation around the world. Cassava is now widely grown and consumed in the tropics and subtropics, in a range of habitats including

semi-arid savannas and mountain regions (Christopher, 2008). Cassava is a major part of the diet for over 800 million people in approximately 80 developing countries (Nassar *et al.,* 2008; Fregene and Puonti-Kaerlas, 2002; Bonierbale *et al.,* 1997).

Many thousands of cultivars, adapted to different environmental conditions, have been developed and differ in their ability to produce under biotic and abiotic stresses (Bamidele *et al.,* 2008; Ceballos *et al.,* 2004). Cassava can be propagated by seed, however due to genetic segregation of the seedlings is a major drawback in sexual propagation (de Vries *et al.,* 2010; Rajendran *et al.,* 2005). Therefore, the primary means of propagation of cassava is vegetative-propagation and hence, the use of biotechnology-based methods such as *in vitro* micropropagation is very powerful offering several important applications including facilitated germplasm conservation and exchange (George, 2008), breeding through genetic transformation (Taylor *et al.,* 2004, 2001; Puonti-Kaerlas *et al.,* 1997), micropropagation (Oliveira *et al.,* 2000), disease eradication (Helliot *et al.,* 2002) and others.

Since the first reports of cassava tissue culture (Eskes *et al.,* 1974; Kartha *et al.,* 1974), several protocols have been used for different

purposes (Oliveira *et al.,* 2000; Taylor *et al.,* 2001; Konan *et al.,* 1994; Mathews *et al.,* 1993; Raemakers *et al.,* 1993; Roca *et al.,* 1992; Stamp and Henshaw, 1982, 1987). In microprogagation, there are large differences in the performance of different cultivars to specific medium culture medium. As in any others crop, *in vitro* culture techniques in cassava are genotype and hence cultivar specific (Acedo and Labana, 2008; Puonti-Kaerlas, 1998). The establishment of a culture medium efficient for the maintenance of *in vitro* cultures remains a great challenge (Medina *et al.,* 2007; Onuoch and Onwubiku, 2007). Therefore, alternative, robust *in vitro* systems could increase the number of cassava cultivars maintained in germplasm banks and make these germplasm collections more widely accessible to producers and breeding programs.

Genotype, explant type and size, the nutrient composition of the culture medium and the culture conditions are major sources of variation in *in vitro* culture (Fan *et al.,* 2011; Gaj, 2004; Khanna and Raina, 1998; Thorpe, 1994). In this paper we present, the results of a comparative study of the efficiency of plant regeneration from excised shoot tips from two contrasting cassava cultivars (CM 507-37 and COL 1468) on four

different culture media. The study involved the most important plant regulators for cassava (kinetin, BA, auxin and giberelin) (Gonzalez, 1998) and, in addition to the individual effects of genotypes and culture media, interactive effects have also been analyzed. The selected cultivars are well known in the literature with one cultivar being drought tolerant and other drought sensitive (El-Sharkawy, 2006, 2003; Okobenin *et al.*, 2003). The results of this study will facilitate the development of micropropagation of cassava by providing insight into meristem culture for disease therapy allowing the introduction of new cultivars *in vitro* and hence will facilitate the expansion of the germplasm collection of this important crop.

Material and Methods

Plant material and growth conditions. Previously established cassava *in vitro* plants of variety CM 507-37 and COL 1468 were obtained from Centro Internacional de Agricultura Tropical (CIAT, Cali/Colombia). *In vitro* plants were maintained on a MS (Murashige and Skoog, 1962)-based medium developed by CIAT (CIAT $12A_3$), supplemented with 0.2 $mg.L^{-1}$ kinetin, 3% sucrose, 1.0 $g.L^{-1}$ charcoal

(Sigma C6289,) 7.0 $g.L^{-1}$ agar (Sigma A7002), pH 5.7-5.8 (Velasquez and Mafla, 1999). Shoot cultures were transferred every 2 months and grown at 25º C constant temperature under a 12 h photoperiod with an irradiance of 50 $\mu mol\ s^{-1}\ m^{-2}$.

Explant preparation and shoot tip isolation. Uninodal segments (0.5-0.7 cm in length) consisting of one lateral bud were cut from 2-3 month old stock shoot cultures and densely cultured (40 segments/dish) in Petri dishes on the CIAT $12A_3$. Since the most uniform results from preliminary experiments on axillary bud break were from the second to fifth nodal positions from the apex (data not shown), all shoot tips used in these experiments were isolated from these nodal positions. After 14-21 days axillary buds from all uninodal segments from both cultivars developed leaves and a well formed growing shoot. Two different sized shoot tips (0.8-1.0 and 1.8-2.0 mm) consisting of the meristematic dome with one or two leaf primordial were aseptically excised from the actively growing shoot with a sterile 22g needle in a laminar flow chamber using stereomicroscope (10-40X). The excised shoot tips were immediately inoculated to avoid dehydration onto four different culture media to test

the influence of media on the growth and development of the excised shoot tips. The four media tested were:

1. CIAT 4E (Roca *et al.,* 1991), containing MS salts, 0.04 $mg.L^{-1}$ 6-benzylaminopurine (BAP), 0.05 $mg.L^{-1}$ Giberellic acid (GA_3), 0.02 $mg.L^{-1}$ naphthaleneacetic acid (NAA), 1.0 $mg.L^{-1}$ thiamine HCl, 100 $mg.L^{-1}$ inositol, 2% sucrose and agar, at pH 5.7
2. CIAT $12A_3$ (Velasquez and Mafla, 1999), containing MS salts, 0.2 $mg.L^{-1}$ kinetin, 1.0 $mg.L^{-1}$ thiamine HCl, 100 $mg.L^{-1}$ inositol, 1.0 $g.L^{-1}$ activated charcoal, 3% sucrose and agar, at pH 5.7;
3. MS + IAA (Murashige and Skoog, 1962), containing MS salts, 1.0 $mg.L^{-1}$ indole-3-acetic acid (IAA), 1.0 $mg.L^{-1}$ thiamine HCl, 100 $mg.L^{-1}$ inositol, 3% sucrose and agar, at pH 5.6;
4. Charoensub *et al*. (2003), containing MS salts, 0.02 $mg.L^{-1}$ BAP, 0.1 $mg.L^{-1}$ GA_3, 0.01 $mg.L^{-1}$ NAA, 1.0 $mg.L^{-1}$ thiamine HCl, 100 $mg.L^{-1}$ inositol, 3% sucrose and agar, at pH 5.6.

Experimental design and data analysis. The experimental design was a completely randomized 2 x 4 x 2 factorial (two cultivars, four culture media and two shoot tip sizes), with each replicate consisting of 5

shoot tips from each variable placed onto each of two Petri dishes (55 x 15 mm) containing 10 mL media. The experiment was replicated three times. Weekly observations on shoot survival based on the presence of a green shoot, shoot growth and root and callus frequency were counted (Viana *et al.,* 2002). The data were analyzed by an analysis of variance (ANOVA) using the SISVAR Statistical Program (Ferreira, 2008) and the average of the treatments were compared by Tukey 5%.

Results and Discussion

Uninodal explants from tender stem cuttings of two cassava cultivars were densely cultured on CIAT $12A_3$ media. For cultivar CM 507-37, it took 14 days for the axillary buds of all uninodal explants to form shoots with leaves. In contrast, cultivar COL 1468 cultivar needed the 21 days for all axillary buds to form shoots from uninodal explants from which shoot tips could be excised. From both cultivars, growth of excised shoot tips were tested on the four different MS-basal media listed in Table I.

Table I. The four different MS basal media with their supplements.

Media name	IAA	NAA	BA	GA	Kinetin	Sucrose	Charcoal	pH
	mg/L					g/L		
CIAT-4E	0	0.02	0.04	0.05	0	20	0	5.7
CIAT-12A$_3$	0	0	0	0	0.2	30	1	5.7
MS + IAA	1	0	0	0	0	30	0	5.6
Charoensub	0	0.01	0.02	0.1	0	30	0	5.6

The use of these four plant growth regulators have been used for *in vitro* growth of cassava (Kartha *et al.,* 1974). Roca (1984), reported successful multiplication of cassava using MS-basal medium supplemented with 0.04 mg.L^{-1} BAP, 0.05 mg.L^{-1} GA$_3$ and 0.02 mg.L^{-1} NAA. Pan (2001), reported that ithe auxin (NAA) and cytokinin (BAP) were important for cassava micropropagation. Finally, Guohuo (1998), reported that the use of kinetin induced organogenesis in cassava, but often also caused callus formation. Rather than an extensive factorial experiment looking at different levels and combinations of these PGRs, the present study tested media and plant growth regulator combinations reported to be successful in the literature.

Excised shoot tips started responded within 5-10 days after isolation. The responses included swelling of the shoot tip, callus

formation, shoot growth and root formation. After 30 days visual differences between treatments could readily be observed (Figure 1 and 2).

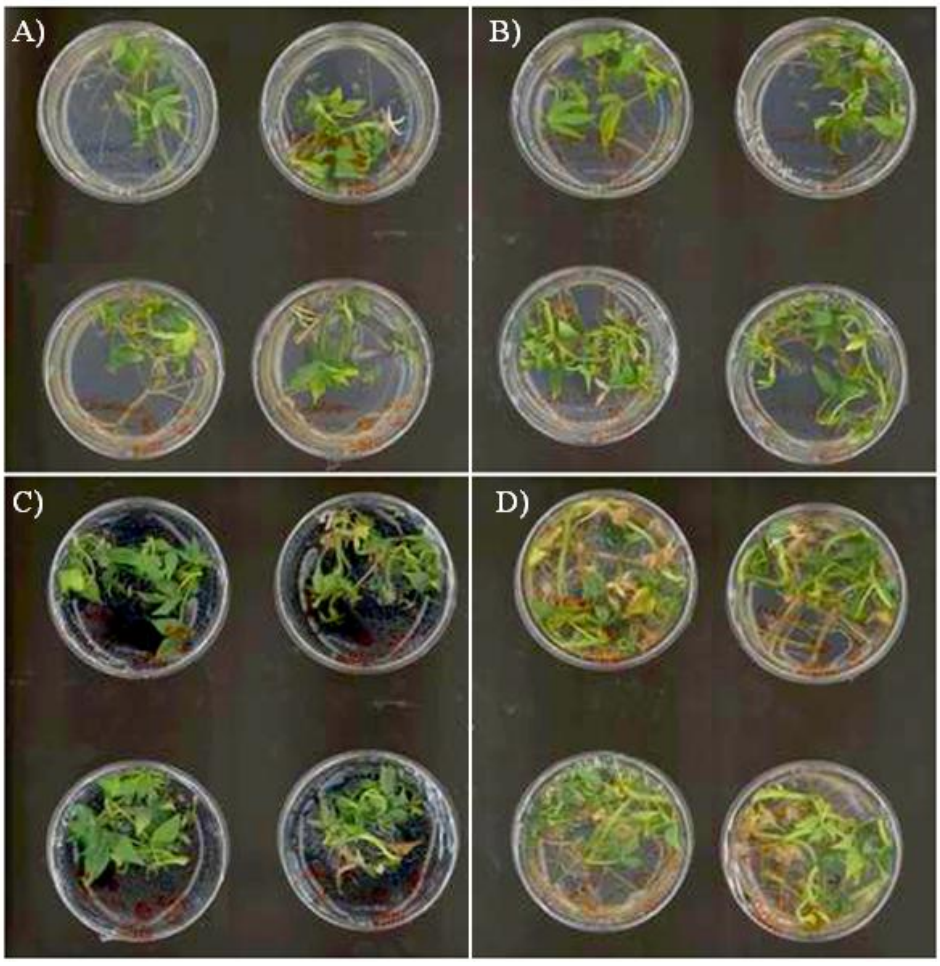

Figure 1. Comparison of the response of two different sizes of excised shoot tips (1.0 – upper row and 2.0 mm – lower row) from cassava cultivar CM 507-37 after 30 days on four different culture media, Charoensub (A), CIAT 4E (B), CIAT $12A_3$ (C) and MS + IAA (D).

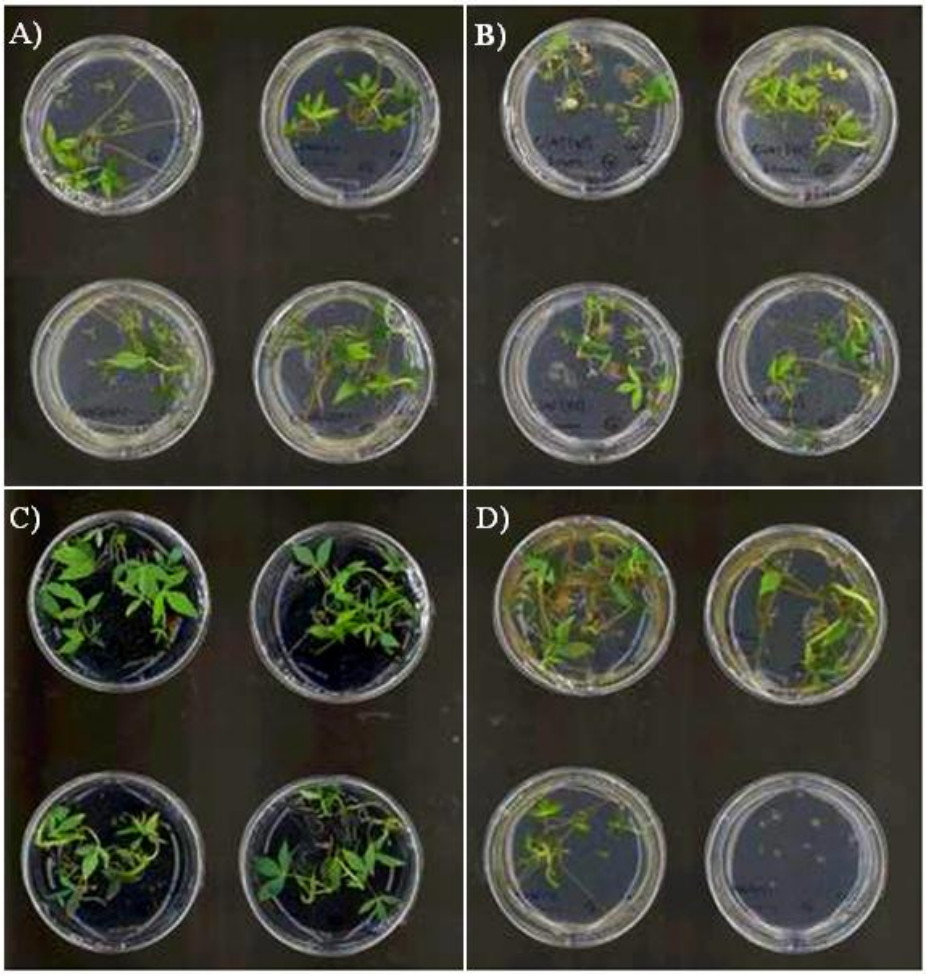

Figure 2. Comparison of the response of two different sizes of excised shoot tips (1.0 – upper row and 2.0 mm – lower row) from cassava cultivar COL 1468 after 30 days on four different culture media, Charoensub (A), CIAT 4E (B), CIAT $12A_3$ (C) and MS + IAA (D).

The shoot tip regeneration was highest on CIAT $12A_3$ for both cultivars (CM 507-37 95% and COL 1468 82%). No significant differences in growth were found in cultivar CM 507-37 between CIAT

$12A_3$ (95%), CIAT 4E (93%) and Charoensub (87%) media. In contrast, for cultivar COL 1468 the CIAT $12A_3$ media was significantly better than the others (Charoensub 67%, CIAT 4E 65% and MS + IAA 62%) (Figure 3).

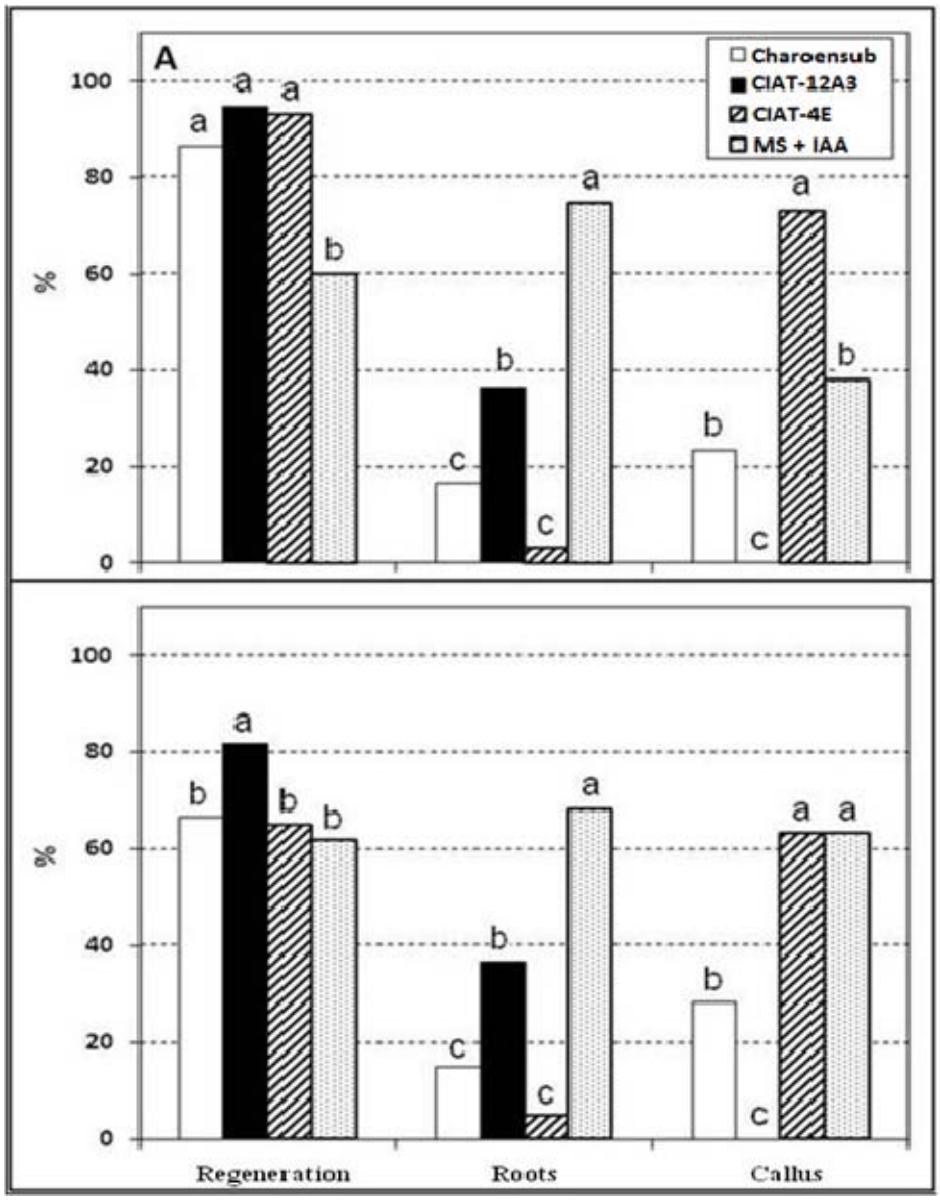

Figure 3. Effect of different culture media on the shoot, root and callus formation from excised shoot tips after 30 days for cassava cultivars CM 507-37 (A) and COL 1468 (B). Bars with the same letter indicate no significant difference based on Tukey's Test ($P<0.05$).

For both cultivars CIAT $12A_3$ medium not only gave the higher percent of shoot tip regeneration but this medium also allow the best shoot tip development and uniformity between the shoot tips. The basic formulation of CIAT $12A_3$ is MS-basal salts supplemented with 0.2 $mg.L^{-1}$ kinetin and 1.0 $g.L^{-1}$ activated charcoal. Cytokinins facilitate not only cell differentiation but also cell division leading to morphogenesis and organogenesis in cassava (Pan, 2001; Guohua, 1998). In addtion, the use of activated charcoal likely contributed to better shoot initiation and root development in addition to having a protective effect by absorbing detrimental substances such as phenolic compounds (Van Winkle and Pullman, 2003). Bertozo and Machado (2010), reported significantly higher rates of regeneration from shoot tips of *Ricinus communis* when using a culture medium supplemented with active charcoal.

The efficiency of the rooting from shoot tips of these two cultivars was also determined for the four different culture media. The highest root formation, for both cultivars (CM 507-37 75% and COL 1468 68%) was seen on the MS + IAA medium which contained only auxin (IAA 1.0 $mg.L^{-1}$). Auxins stimulate cell elongation and division, and rhizogenesis is usually achieved by treatment with auxin alone (de Klerk *et al.,* 2008).

Oliveira *et al*. (2000), reported that the callus formation in cassava micropropagation systems from apical or lateral buds followed by organogenesis is undesirable due to the potential occurrence of somaclonal variants and inhibition of seedling growth. In this sense, for the purpose of this study the best medium would be the one that resulted in least callus formation. Using this criteria, CIAT $12A_3$ was again the superior choice as there was no callus formation observed on this medium from either cultivars. In contrast, callus was induced on all other media with the highest percent of shoot tips forming callus was from CM 507-37 on CIAT 4E (73%), followed by MS + IAA (38%) and Charoensub (7%). For COL 1468, similar results were obtained with the culture media CIAT 4E and MS + IAA (63% for both) and followed by Charoensub medium (17%.)

Razdan (2005), reported that the use of gibberellin promoted the elongation of adventitious shoots but also induced callus formation when used in combination with auxins and cytokinins in low concentration. Unnikrishnan and Sheela (2000) reported that supplementing the culture medium with BAP induced the formation of callus in cassava and this

behavior may be accentuated when BAP is used in combination with NAA.

In this work we saw that the interaction of NAA, BAP and GA_3 in the CIAT 4E culture medium in relatively small concentrations were sufficient to induce a higher rate of callus formation in both cultivars. Moreover, the supplementation of the MS-basal with 1.0 $mg.L^{-1}$ IAA also resulted in a high rate of callus formation.

In general, for both cultivars, the effect of initial size of the shoot tip on the tip shoot regeneration, rooting and callus formation followed the same trend in all culture media used (Table II).

Table II. The influence of initial shoot tip size on the frequency (%) of shoot (>2 leaves), root and callus formation after 30 days of shoot tip isolation for cassava cultivars CM 507-37 (CM) and COL 1468 (COL). Letters following the data in each column denote significant difference based on Tukey's Test ($P<0.05$).

		>2leaves		Roots		Callus	
		CM	COL	CM	COL	CM	COL
Size	1mm	78.33 b	63.33 b	25.83 b	25.83b	26.67 a	26.67 b
	2mm	89.17 a	74.17 a	40.00 a	36.67 a	32.50 a	45.00 a

Also, the larger shoot tip responded significantly better in both cultivars giving a higher percentage of regeneration, and rooting and a

lower percentage of callus (note there was no significant difference in callus formation between the two sizes of shoot tips for CM 507-37).

Conclusions

An *in vitro* response is the result of a complex interaction of factors involving the physiological state of the plant, the genotype and the cultivation conditions. According to the findings of this study we concluded that the culture medium most suitable for the multiplication of cassava genotypes CM 507-37 and COL 1468 is CIAT12A_3. Using this medium we obtained the best shoot induction, with excellent growth, development and uniformity of plantlets. Additionally, this medium provided a good rooting frequency and complete absence of callus for both cultivars.

The concentrations of the regulators (BAP, NAA and GA_3) used in the preparation of CIAT 4E and Charoensub culture media are quite low, yet based on these results, these varieties of cassava are very sensitive to plant growth regulators and the combination of these regulators had an synergistic effect inducing rooting and callus formation.

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Paper 2

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THE LONG-TERM CRYOPRESERVATION OF CASSAVA USING VITRIFICATION

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Abstract

Preliminary experiments with non-cryostored shoot tips showed that the presence of waxes in the shoot tips does not permit a great shoot tip exposure to cryoprotectants solutions. The addition of Tween-20 0.5% in pre-treatment solution showed increase in this exposure. Besides, pre-treated shoot tips were dehydrated at room temperature with a PVS2 solution for different lengths of time prior to liquid nitrogen. The results showed good survival of shoot tips after LN exposure. Therefore, the best result was outlined as follows: pre-growth of explants for 21 days;

excision of 1 mm long shoot tips; loading for 20 min in modified LS at room temperature; dehydration in PVS2 at room temperature for 20 min; immersion in liquid nitrogen (LN); rewarming at 45º C for 1 min and subsequent treatment with 1.2 M sucrose for 20 min followed for cultive on solid culture medium based on MS-basal supplemented with 0.02 $mg.L^{-1}$BAP, 0.1 $mg.L^{-1}$ G_{A3} and 0.01 $mg.L^{-1}$ NAA.

Keywords: Cassava, cryopreservation, shoot tip, vitrification, germplasm collection.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) belongs to the botanical family Euphorbiaceae (56, 82, 92, 66), and is the only commercially important species in the genus *Manihot* (72, 3). Cassava is native to the Amazon region in South America (54, 62, 2, 45) and is cultivated throughout the lowland tropics (16). Current global cassava production is approximately 250 million tons, of which Africa accounts for half (51%), while Asia and Latin America produce 34% and 15%, respectively (33). It is the most important root and tuber crop in the tropics, ranks third, after rice and corn, as a staple food root-crop for more than 800 million people in the developing world (25, 61, 60) and is the sixth most important food crop in terms of global annual production (33). In addition to food, cassava is used as a raw material in the production of bio-fuels (20, 7), glue (12) and glucose syrup (53, 8).

The development of sterile *in vitro* plant tissue culture technology offers several important applications including facilitated germplasm conservation and exchange (39), development of genetic transformation systems (85, 83, 69), micropropagation (65), disease irradication (43) and others. Initial attempts to regenerate cassava plants through *in vitro* techniques were made by Eskes *et al.,* 1974 (29) and Kartha *et al.,* 1974 (51). Since these first reports, protocols for *in vitro* plant regeneration of cassava through callus culture, axillary buds, shoot tip culture and embryogenic cell suspensions have been reported (65, 83, 52, 55, 70, 75,

80, 81). As in any others crop, *in vitro* culture techniques in cassava are genotype and hence cultivar specific (1, 68). This genotype-specific response in culture is a challenge for genetic resources programs and collections where handling of many genotypes of wide genetic diversity needs to be done. Therefore, alternative, more robust *in vitro* systems would increase the number of cassava cultivars accessible for more rapid regeneration and distribution from these collections.

This is particularly important for vegetatively-propagated crops such as cassava where large *ex situ* collections are maintained as field plantings (26, 27). The largest *ex situ* cassava collection, housed at the Centro Internacional de Agricultura Tropical (CIAT) in Colombia is represented by at least six thousand accessions from more the 90 countries (37, 11, 36, 48, 63). It is estimated that nearly 2/3 of the cassava genetic diversity exists only in *in situ* collections (natural habitats) with only 1/3 being securely conserved in *ex situ* collections (outside the natural habitats) (44).

Conservation of plant genetic resources ensures maintenance of agrobiodiversity. The most common method for preserving plant genetic resources is through seed or as whole plants in the field for vegetatively-propagated plants. There are, however, several challenges with these fields genebanks (42) such as the requirement of large land areas and the continuous risk of losses due to biotic and abiotic stresses. In addition, field collections are labor intensive to maintain (46). Due to these issues, other methods are being developed such as *in vitro* culture to complement traditional approaches to conservation of plant germplasm.

Cryopreservation techniques developed over the last 35 years are now the best option for the long-term storage of plant genetic resources and require only a minimum of space and maintenance (9, 22, 23, 73). Cryopreservation is the storage of biological material at ultra low temperatures (below -150º C) using liquid nitrogen (LN) as the cryogenic medium (93). Through this procedure the entire cellular metabolism is drastically reduced and biological deterioration is virtually paralyzed and, therefore, considered the most promising method for long-term preservation of cells, tissues and plant organs. These explants can be regenerated into new plants at any time, without the risk of genetic variation (9, 21).

Different types of plant cell, tissue and organs can be cryopreserved, including embryogenic cell suspensions, callus, pollen, somatic and zygotic embryos and shoot tips or meristems (73, 74, 87). However, for some vegetatively propagated crops such as cassava (*Manihot spp*.), potato (*Solanum spp.*), banana (*Musa spp.*), sweet potato (*Ipomoea batatas*), and others, the most widely used organs for cryopreservation are shoot tips excised from *in vitro* plants. In these cases shoot tips are preferred because they contain pre-formed meristematic organs, providing genetic stability (34). There have been several reports of successful cryopreservation of cassava shoot tips (50, 6, 24, 13, 14, 15, 17, 18). The report for cassava shoot tip cryopreservation used a MS-basal medium solution containing 15% DMSO and 3% sucrose, followed by slow controlled-rate freezing (50). After shoot tips were exposure to LN some growth was obtained however there was also a high incidence of callus. Bajaj (1983) (6), reported cryopreservation of cassava shoot tips using a MS-basal solution containing 5% DMSO, 5% sucrose and 5% glycerol pre-treatment. After exposure to LN, similar results were reported as by Kartha *et al*. (1982) (50). Years later Engelmann (1994) (24), tested the encapsulation-dehydration technique with cassava shoot tips utilizing a 24 hours pre-treatment in a cryoprotective treatment consisting of 0.75 M sucrose or 72 hours pre-treatment with 0.5 M sucrose, followed by desiccation to approximately 30% moisture content (fresh weight basis) and freezing by direct immersion of samples in LN. He obtained about 60% shoot tip survival, however he also reported the occurrence of callus on the regrowth medium. Charoensub *et al*. (1999, 2003) (13, 15), reported that cassava shoot tips could be cryopreserved through vitrification using a pre-treatment for 16 hours of a MS-basal medium containing 0.3 M sucrose, followed by a cryoprotective treatment with a MS-basal medium containing 2 M glycerol and 0.4 M sucrose for 20 minutes at room temperature. Shoot tips could then be dehydrated with Plant Vitrification Solution (PVS2) for 45 minutes followed by freezing by direct immersion of samples in LN. Using this protocol, six of the ten cultivars studied achieved more than 70% shoot tip survival after LN, and he concluded that this protocol appears promising as a practical method for cryopreservation of cassava germplasm but further study was necessary to apply this technique to a wide range of cassava genotypes. Charoensub *et al*. (2004) (14), also reported the use of encapsulation-vitrification for cryopreservation of cassava shoot tips. This report

utilized a preculture on MS-basal medium containing 0.3 M sucrose for 16 hours, encapsulation in 3% calcium alginate, osmoprotection at room temperature for 90 minutes on a MS-basal medium containing 2 M glycerol and 0.6 M sucrose, and then dehydration with PVS2 for 4 hours at 0º C before direct immersion of samples in LN. With this method, shoot tip survival in three of the four cultivars studied was 80%. More recently, Danso and Lloyd (2011) (17), reported cryopreservation for cassava shoot tips and embryogenic calli by slow freezing. Their study showed a faster cooling rate for embryogenic callus as compared with shoot tips when immersed in LN. Using these protocols the shoot tip explants showed only post-thaw callus formation. In contrast, all embryogenic calli explants survived after post-thaw by slow freezing.

Clearly, there are many reports reports on the cryopreservation of cassava however none of these protocols has been tested for large scale routine cryopreservation of cassava germplasm. Some of these protocols showed excellent post-thaw shoot formation, however, when tested with other cassava cultivars, it had low survival (2-21%) (28).

The aim of this study was to evaluate a cryomethod based on the cryopreservation protocol described by Charoensub *et al*. (1999, 2003) (13, 15). It is known that the culture medium can have a major influence on the performance of shoot tips and hence we tested post-cryo survival on two different culture media. In addition, during preliminary vitrification studies it was noted that shoot tips floated with limited contact to the pre-treatment (0.3 M sucrose) and the vitrification solutions (PVS2). Several treatments were tested including shaking and here we report on the use of the surfactant tween in the medium to aid in contact of the shoot tips with the PVS2.

MATERIALS AND METHODS

Plant material and growth conditions

Previously established cassava *in vitro* plants of variety CM 507-37 and COL 1468 were obtained from Centro Internacional de Agricultura Tropical (CIAT, Cali/Colombia) and used in this study. *In*

vitro stock plants were grown in Magenta GA-7 boxes (Magenta Corp., Chicago, IL.) containing CIAT $12A_3$ medium (Velasquez and Mafla 1999) consisting of MS-salts (59) supplemented with 0.2 $mg.L^{-1}$ kinetin, 3% sucrose, 1.0 $g.L^{-1}$ charcoal (Sigma C6289) 7.0 $g.L^{-1}$ agar (Sigma A7002), pH 5.7-5.8. Cultures were subcultured every 2 months and grown at 25º C constant temperature under a 12 h photoperiod (50 $\mu mol.s^{-1}.m^{-2}$).

Explant preparation and shoot tip isolation

Uninodal segments (0.5-0.7 cm in length) consisting of one axillary bud were taken from 2-3 month old stock cultures and densely cultured (40 segments/dish) in Petri dishes (90 x 55 mm) on CIAT $12A_3$ medium under the culture conditions described above. Since the most uniform results from preliminary experiments on axillary bud break were from the second to fifth nodal positions from the shoot tip (data not shown), all shoot tips used in these experiments were isolated from these nodal positions. After 14-21 days uninodal segments developed leaves and a well formed growing shoot. Shoot tips (1.0 mm) consisting of the meristematic dome with one or two leaf primordial were aseptically isolated with a sterile 22g needle in a laminar flow chamber using a stereomicroscope (10-40X). .

Preculture

Excised shoot tips were immediately inoculated onto preculture medium avoid dehydration nd were precultured for 16-24 hrs on small Petri dishes (55 x 15 mm) containing solidified MS-basal medium supplemented with 0.3 M sucrose under indirect light in the culture conditions specified above.

Vitrification

The precultured shot tips were removed from the preculture medium and placed 1.8 mL cryotubes (10 shoot tips per cryovial)

containing 0.5 mL Loading Solution (LS) consisting of MS supplemented with 2 M glycerol and 0.4 M sucrose (78) for 20 minutes at 25º C. After this time the LS was removed using a sterile syringe and needle, and then, 0.5 mL of Plant Vitrification Solution, PVS2 (79) which contain 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide (DMSO) in MS liquid medium containing 0.4 M sucrose with pH 5.8, was added and gently mixed. Different exposure PVS2 times (10, 20, 30, 45 and 60 minutes) were tested with fresh PVS2 added half way through the exposure time. Cryotubes from each of the different exposure times were plunged in LN. Controls were treated identical to the LN submerged shoot tips only were not exposed to LN. To test the effect of Tween 20 on the survival of the crypreserved shoot tips, 0.5% Tween 20 (v/v) was added to the LS solution and vitrification was done as described above using 20, 30 and 40 minutes of PVS2 exposure.

Thaw and Re-growth

Cryotubes were left in LN for a minimum of 24 hrs prior to thawing in a 45º C water-bath for 1 minute. Following thawing, the PVS2 was drained from the cryotubes and 0.5 mL of MS-basal medium containing 1.2 M sucrose was added. After 20 minutes, the shoot tips were transferred onto Petri dishes containing sterilized filter paper discs to remove the excess high-sucrose medium. Shoot tips were then cultured on CIAT $12A_3$ or Charoensub media and placed in a growth room under the same conditions listed above. Assessments were made every 15 days by measuring the number of green explants, the number of explants with more than 2 leaves and explants with roots or callus.

Experimental design and data analysis

The experimental design for the first experiment was a completely randomized 2 x 6 factorial (two cultivars, six exposure times), with each replicate consisting of 30 shoot tips from each variable placed in three 55 x 15 mm Petri dishes (10 shoot tips per Petri dish) containing 10 mL media. The second experiment used a 1 x 2 x 4 x 2 factorial s (one cultivar, two culture media, four exposure times, and two tween (+ and -)

treatments, with each replicate consisting of 15 shoot tips from each variable placed in three 55 x 15 mm Petri dishes (10 shoot tips per Petri dish) containing 10 mL media.

Each experiment was replicated three times. Every 15 days observations were made on shoot survival based on green shoot, shoot growth and root and callus frequency were counted. Data were analyzed by an analysis of variance (ANOVA) using the SISVAR Statistical Program (35) and the average of the treatments were compared by Tukey 5%.

RESULTS AND DISCUSSION

To increase the osmotolerance, excised cassava shoot tips were pretreated with 0.3 M sucrose for 16-24 hours. Pre-treatment had no detrimental effects on shoot tip growth as excised shoot tip only controls (no pre-treatment) grew as well as the shoot tips that went through the pretreatment process (data not shown). Pre-treatment pre-conditions tissues for cryopreservation by vitrification by providing a balance between adequate dehydration by PVS2 and the damage by osmotic stress and chemical phytotoxicity (30). This damage is controlled by optimizing the duration of exposure of explants to PVS2 solution, cryoprotecting with loading solution prior to PVS2 exposure and by conditioning treatments. Therefore, the optimal time in PVS2 varies with plant species, cultivars, size of materials and others variables (58, 77).

To determine the optimal time of PVS2 exposure 25º C, pre-treated shoot tips were dehydrated with a PVS2 solution for different lengths of time prior to liquid nitrogen exposure (Figure 1 A and B).

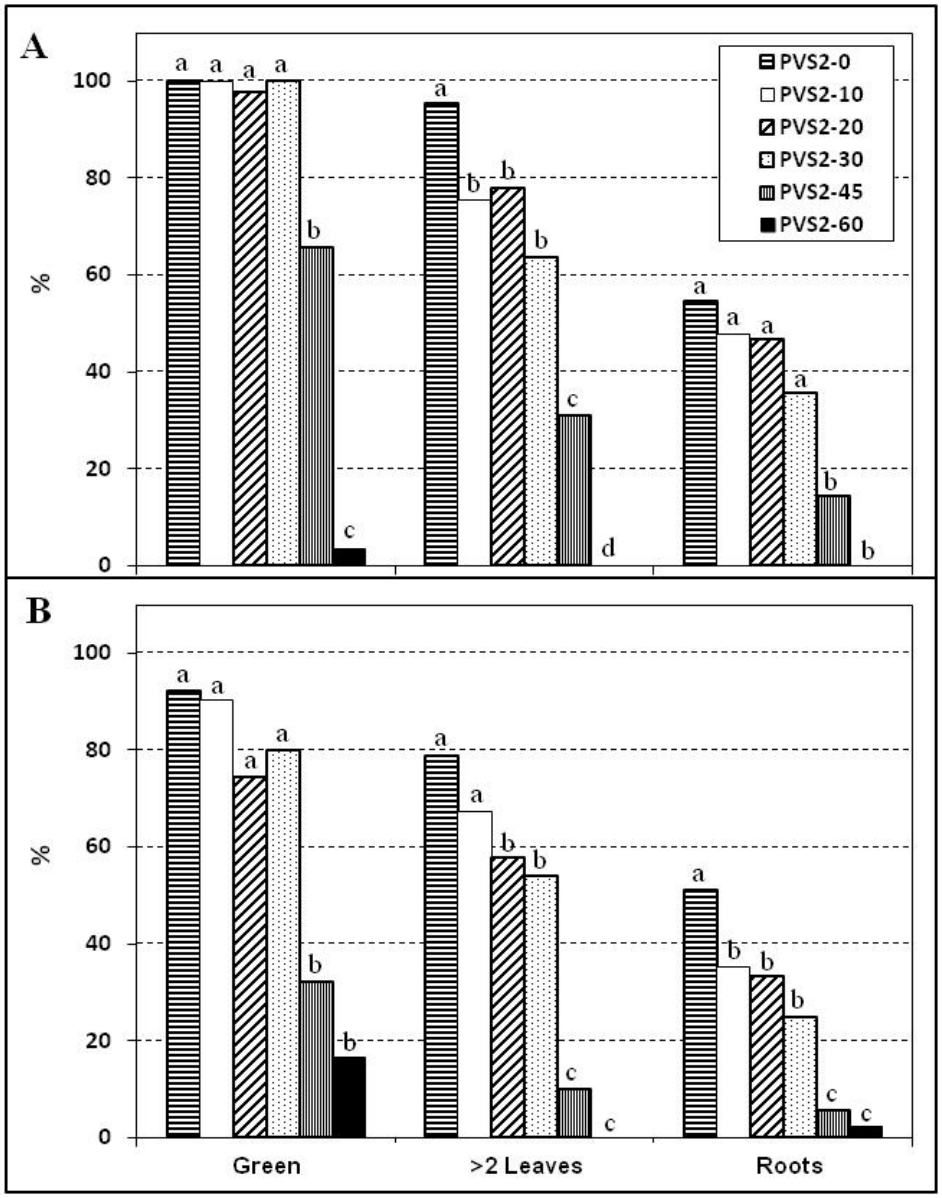

Figure 1 - Percent of shoot tips remaining green, growing and with roots from cassava cultivars CM 507-37 (A) and COL 1468 (B) after exposure to PVS2 for different time periods. Data were recorded 30 days after treatment. Values are means of at least three replicates. Different letters indicate significant differences at P>0.05 by Tukey's Test.

Results from our preliminary PVS2 time course experiments (Figure 1) indicate that PVS2 treatment of more than 30 min is lethal to the two cassava cultivars tested as exposure times greater than 30 min caused a significant drop in green shoot tips. Further, PVS2 exposure times as short as 10-20 minutes showed significant decreases in shoot tip growth during this same 30-day period. Due to the sensitivity of the shoot tips to PVS2, it came as no surprise that no shoot tips survived cryopreservation following PVS2 exposure under these conditions (data not shown).

Interestingly there are reports for cassava shoot formation from vitrified shoot tips with PVS2 exposure with maximum survival following 45 minutes exposure. However, with the cultivars used in this study, we could not repeat the reports of regrowth of cassava shoot tips following PVS2 exposure. Although some shoot tips remained green for over 80 days following LN-exposure, no growth or morphological changes were visible and it was clear that under the conditions tested the detrimental effects of PVS2 prevented exposure long enough to protect the shoot tips from LN-exposure.

By pooling the data per cultivar, it is clear that cultivar CM 507-37 was significantly more tolerant of the PVS2 (Table 1).

Table I - Rates of cassava shoot tips responses (% of shoot tips) for two cultivars (CM507-37 and COL 1468) after treatment with PVS2 expressed as an average of means obtained from three replications regardless of exposure time on PSV2.

	CM 507-37	COL 1468
Green (%)	77.76 a	64.29 b
Leaves (%)	57.28 a	44.69 b
Roots (%)	33.16 a	25.40 b

To test the effect of the culture medium on the regenerative capacity of the shoot tips after PVS and LN exposure shoot tips were regenerated on two different culture media (Table 2).

Table II - Effects of two different recovery media and different PVS exposure times on the % of shoot tips of cassava (cv. CM 507-37) remaining green, having growth with more the two leaves or root and callus formation without and with LN exposure.

	Exposure	Green (%)		>2 Leaves (%)		Roots (%)		Callus (%)	
Treatment	time (min)	Charoensub	CIAT $12A_3$	Charoensub	CIAT $12A_3$	Charoensub	CIAT $12A_3$	Charoensub	CIAT $12A_3$
PVS2 Control	0	100	100	88.9	97.8	4.4	26.7	6.7	0
	20	97.8	100	86.7	97.8	17.8	24.5	26.7	0
	30	100	100	77.8	93.4	28.9	37.8	48.9	0
	40	97.8	76.3	37.8	37.7	13.3	40	51.1	0
PVS2 + LN_2	20	28.9	0	4.4	0	4.4	0	28.9	0
	30	23.3	0	2.2	0	5.5	0	47.8	0
	40	44.5	8.9	11.1	4.4	8.9	0	75.6	0

CIAT $12A_3$ culture medium is routinely used for cassava micropropagation and its basic formulation is MS basal supplemented with 0.2 $mg.L^{-1}$ kinetin and 1.0 $g.L^{-1}$activated charcoal (91). This medium showed good results prior to exposure to LN, however after LN exposure it showed no significant regrowth. In contrast, the culture medium of Charoensub *et al*. (2003), is a variation of the CIAT 4E culture medium which was also developed for micropropagation. CIAT 4E is also an MS-based medium but is supplemented with 0.01 $mg.L^{-1}$ NAA, 0.02 $mg.L^{-1}$ BAP and 0.1 $mg.L^{-1}$ GA_3. On Charoensub medium the control no LN exposured shoot tips great equally well as on CIAT $12A_3$, however, after exposure to LN shoot tip growth was greater on Charoensub medium indicating an interaction of growth medium and perhaps growth regulators is beneficial for cassava after freezing in LN and suggesting that cryopreservation performance can be improved.

Clearly the medium and/or plant growth regulator content a significant effect on survival and regeneration of cryopreserved shoot tips. Genotype specific plant growth regulators requirements have been reported for different cassava cultivars (5, 31, 41, 64, 67). Other authors

also found that modifications in the recovery medium could significantly improve the regeneration capacity of the cryopreserved explants (4). In particular, variations in the growth regulator content of recovery medium influenced the percentage of regeneration (10, 19, 57, 88).

Several hypotheses had been tested to obtain better regrowth after exposure of shoot tips to a vitrification process. It is known that cassava has a leaf epidermis with wax deposits and other lipids in addition to a cutin wall cell (79, 88). These attributes could contribute to the fact that cassava shoot tips float the LS and PVS2 solutions preventing good contact with the solutions. This could result poor acquisition of an appropriate osmotic potential and, therefore not offer any protective effects to the shoot tips during LN exposure. Shaking or stirring of the shoot tips sis not significantly increases the wetting of the shoot tips in these solutions (data not shown). The addition of 0.5% (v / v) Tween-20 to the LS solution prevented the shoot tips from floating on the surface of the medium and hence likely increased exposure of the shoot tips to these solutions (Figure 2).

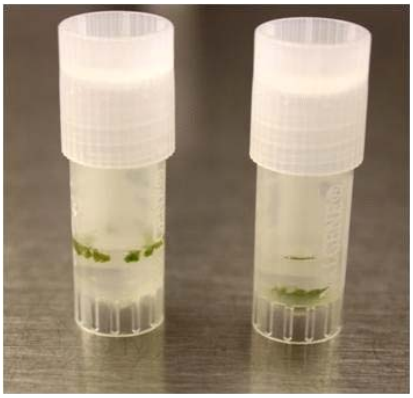

Figure 2 - The effect of pre-treatment with LS solution without Tween-20 (left vial) and with Tween-20 (right vial). Shoot tips in LS solution (left vial) and shoot tips in LS solution + Tween 20 (right vial).

There are reports on a positive interaction of Tween with waxy plant tissues. Its use in low concentrations and for short periods of time has been associated with slight increases in the rate of photosynthesis and

transpiration, as well as increasing exposure to other substances in an aqueous environment (47). However, inhibitory effects of high concentrations of Tween were also reported. High concentrations of Tween-20 can cause phytotoxic damages and decreased photosynthesis and transpiration (87).

To test the effect of Tween 20 in the LS solution, PVS2 vitrification was made with cultivar CM 507-37 where half of the shoot tips was treated for 20 minutes with the LS solution containing Tween-20 0.5% (v/v) and, the other half, was treated for the same period of time with standard LS solution (control). The shoot tips were exposed for different times to the vitrification solution (PVS2) and then frozen (Table 3).

Table III - The effect of pre-treatment in an LS solution with and without Tween-20 0.5% (v/v) followed by different PVS exposure times on the maintenance of green tissue, growth with more the two leaves, roots and callus formation of cassava (cv. CM 507-37) shoot tips without and with LN exposure.

	Exposure	Green (%)		>2 Leaves (%)		Roots (%)		Callus (%)	
Treatment	time	- Tween	+ Tween	- Tween	+ Tween	- Tween	+ Tween	- Tween	+ Tween
PVS2	20	97.8	95.5	86.7	73.3	17.8	13.3	26.7	40
Control	30	100	93.3	77.8	60	28.9	11.1	48.9	62.3
	40	97.8	82.3	37.8	35.6	13.3	8.9	51.1	80.1
PVS2	20	28.9	82.2	4.4	46.7	4.4	0	28.9	37.8
+	30	23.3	64.4	2.2	24.5	5.5	8.9	47.8	60
LN_2	40	44.5	31.1	11.1	8.9	8.9	6.7	75.6	80.1

As indicated by the results in Table 3, treatment with Tween provided better exposure of the shoot tips on LS and PVS2 solution resulting in a significant increase in the survival of shoot tips after LN. Interestingly shoot tips treated with the LS solution containing Tween-20 0.5% (v/v), needed a relatively short exposure time to PVS2 (20 minute) for maximum survival and regrowth of the shoot tips post LN exposure. In contrast, when the shoot tips were treated with the control LS solution a longer exposure time (30-40 minutes) survival increased, confirming the results shown in the original reports.

Both, Tween and PVS2 can cause toxicity in certain plant tissues (89). Short treatments, enough for the acquisition of adequate pre-treatment conditions for vitrification and freezing in LN are needed. We found that the action of the surfactant Tween-20 led to a better contact of the shoot tips with these solutions, favoring increased cryopreservation. Using this detergent, we obtained the best regrowth with 20 minutes exposure to PVS. Without Tween 20, a longer exposure to PVS2 was needed and even that did not yield results comparable with the Tween 20 treatment. These results could clearly be visualized after 30 days (Figure 3).

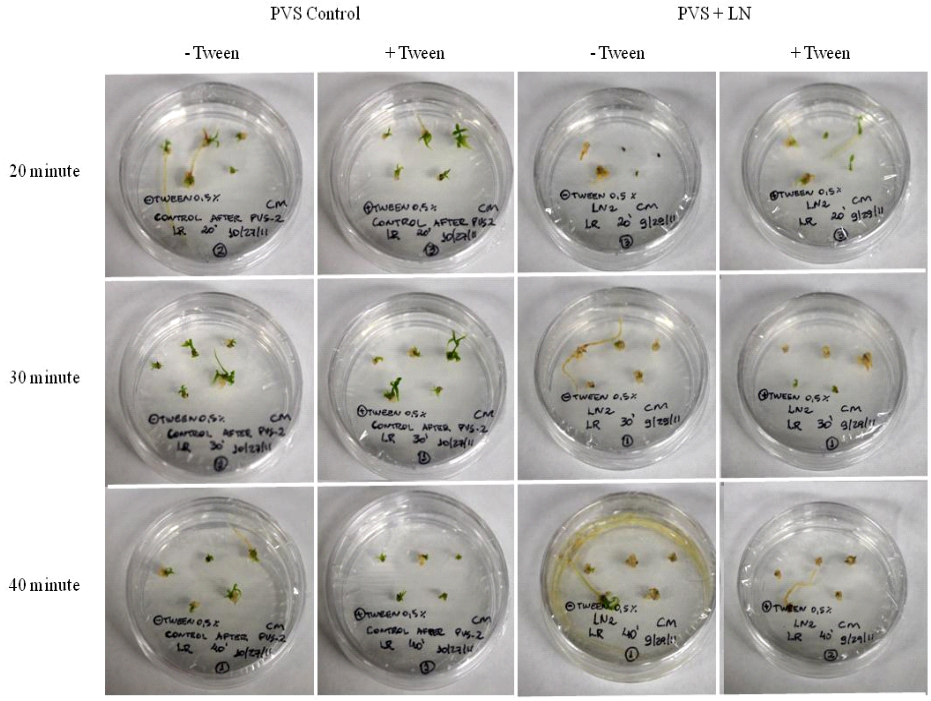

Figure 3: Recovery of cassava plantlets from control and cryopreserved shoot tips. One month-old cassava plantlets obtained from non-cryopreserved control shoot tips (PVS control) and cryopreserved shoot tips (PVS + LN). The shoot tips were pre-treated with LS solution with and without 0.5% Tween-20 and then exposed to PVS2 for varying times.

CONCLUSION

In this study, we report a modified cryopreservation method for cassava shoot tips using a pre-treatment with a LS solution containing 0.5% (v/v) Tween-20 coupled with recovery on the medium of Charoensub *et al*. (2003) (15). The results showed that this modified LS solution containing Tween-20 was superior to standard for regrowth of shoots after LN exposure. Additionally, the medium recommended by Charoensub *et al*. (2003) was superior to CIAT $12A_3$ for recovery shoot tips after LN exposure. However, further adjustments in the medium and growth regulators concentrations should be made to reduce the formation of callus after LN exposure.

The cryopreserved shoot tips produce plantlets phenotypically similar to non-treated controls plants. However, a high rate of callus formation iwas problematic as a callus phase prior to shoot tip formation is not desirable because callus formation could increase the frequency of genetic variants.

Using the methodology described here, we repeatedly obtained >50% shoot growth and complete plant formation for CM 507-37 cassava cultivar.

Post-LN survival rate was increased by utilization of 20 minutes pre-treatment with loading solution supplemented with Tween-20 0.5% (v/v). This result confirms previous studies which reported 20 minutes pre-treatment at room temperature adequate for most plant tissues. Moreover, it also shows that for this cultivar, 20 minutes of exposure to PVS2 solution is sufficient to cryoprotect the shoot tips pre-treated with LS solution containing Tween.

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