DEVELOPMENT OF A FREEZING METHODOLOGY IN LIQUID NITROGEN OF TREE TOMATO (*Cyphomandra betacea* (Cav.) Sendt) SEEDS

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Abstract

As an alternative way of preserving the agro-biodiversity of tropical fruits, we set up experiments to freeze in liquid nitrogen sexual seeds of tree tomato (*Cyphomandra betacea* (Cav.) Sendt) as a case study. Seeds from three cultivars were obtained from mature fruits, then were sterilized and desiccated in a flow chamber for different periods depending upon the clone. Treated seeds were immersed directly in liquid nitrogen for at least one hour and germinated on a modified Murashige and Skoog basal medium. The average seed germination, after cryopreservation, ranged between 37.4% and 94%. It was possible to recover full plants and transfer them to the greenhouse. The freezing methodology reported here seems to be simple enough to be transferred to national and regional agricultural programs as an alternative method to preserve germplasm.

Keywords: Andean crop, cryopreservation, germplasm conservation, tropical fruits
Abbreviations: LN_2= liquid nitrogen; MC= moisture content; GA_3= 3-gibberellic acid; MS= Murashige and Skoog; TTC= 2,3,5-Triphenyl-2H-tetrazolium chloride; TTG= Tree Tomato Germination medium; masl= meters above sea level

INTRODUCTION

Tree tomato (*Cyphomandra betacea* (Cav.) Sendt) is a of crop Andean origin. Possibly due to consistent market prices, it represents an alternative source of income for many small-to-medium scale farmers in Colombia, especially in the Andean zone (16 to 22°C; 1600 to 2600 masl with annual precipitation between 1300 to 1600 mm). This crop gives them more profit than other traditional crops such as potatoes and vegetables.

Based on the behavior of commercial prices in the 4 principal food-provisioning centers in Colombia (Bogotá, Medellín, Cali and Pereira), during the period 2000-2001, tree tomato maintained best average prices per pound (0.32 US and 0.37 US) compared with potatoes (0.26-0.21 US), kidney beans (0.30-0.32 US), cucumber (0.20-0.21 US), cabbage (0.13-0.12 US) and carrot (0.17-0.20 US; CCI, 2002).

Tree tomato is a hillside, non-mechanized crop that requires intensive pre and post-harvest hand-labor for processing and commercialization, thus generating employment in the region.

Most of them come from local cultivars maintained by farmers in their fields. The agricultural national program *Corporación Colombiana de Investigación Agropecuaria* –CORPOICA maintains approximately 60 accessions in the field and as a seed bank stored at -20°C (Mario Lobo, personal communication). With crop intensification, it is expected that farmers and breeders will concentrated on fewer genotypes with uniform characteristics (i.e. yield, maturity), resulting in fewer varieties under cultivation. It is therefore important to collect the existing wide diversity and establish a conservation strategy to ensure its availability to future users and breeding programs.

Cryopreservation in liquid nitrogen can reduce cost, space, and labor while maintaining genetic integrity. Over long durations, it is the technique of choice to establish a basic seed bank. Cryopreservation of different explants has been adopted to preserve about 30 tropical species (Engelmann, 1991; Shibli *et al.*; 1999; Shibli 2000), including jackfruit (Engelmann, 1995) and cassava (Escobar *et al.*, 1997) among others. Besides, cryopreservation has been applied to preserve sexual seeds of blackpepper, cardamom (Chaudhury, 2000) and *Passiflora spp* (Ospina *et al.*, 2000).

Based on CIAT's experience on cassava cryopreservation (Escobar *et al.*, 1997), keeping in mind that for some crops it is necessary to establish conservation strategies, we select tree tomato as a case study for long-term conservation in LN_2.

MATERIALS AND METHODS

Plant material

Sexual seed of three cultivars of tree tomato, selected and maintained by farmers, known as common red *"Común"*; purple-red *"Mora"* and around-yellow *"Amarillo"* (Garcia and Garcia 2001) were used for cryopreservation testing (Figure 1). Ripen fruits of the three cultivars were obtained (September 2001) in local supermarkets in Palmira, Cali and Medellín. The *Mora* cultivar was the most expensive (about 0,63 US dollars per pound), although the most consumed are *Común* and *Amarillo* (about 0.38 US dollars per pound each).

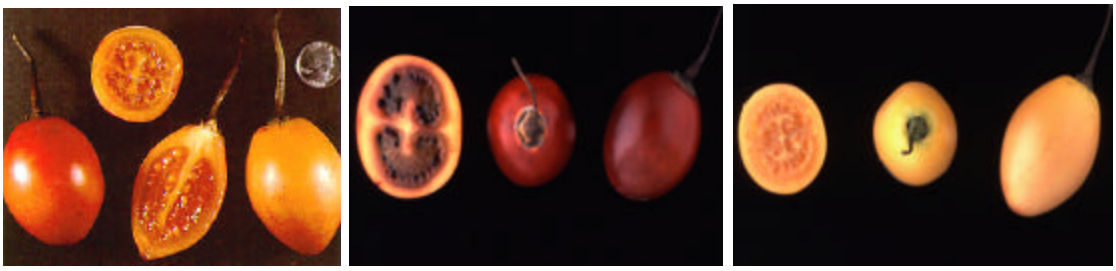

Figure 1. Appearance of tree tomato fruits from cultivars *Común* (left), *Mora* (center) and *Amarillo*.

Healthy fruits were washed thoroughly. Transversal slices were made to collect pulp and seeds, which were soaked in water for one day. The mucilaginous seed cover was removed using a colander. The seeds were sterilized in a flow chamber by immersion in 70% alcohol for 20s, 1% sodium hypochloride for 10 min, and washed three times with distilled sterile water.

Seed moisture content, cryopreservation and plant recovery

Sterilized seeds were dried on open petri dishes in a flow chamber during 1h to 2½h according cultivars. For *Mora* the drying time ranged between 2 to 2½h, while for the others it was 1h. Moisture content (MC) was determined gravimetrically comparing final weight of

freshly extracted (FEW) and flow-chamber-dried seeds (FCW), maintained during 1h in an oven at 130°C (ISTA 1993). It was expressed as percentage of fresh weight ($\%MC = [(FEW - FCW)/FEW]*100$). Three replicates of 5g (818 seeds average) per cultivar were used.

Dried seeds were set into cryo-vials (1.0 internal threads, Nunc®) and immersed directly in LN_2 for at least 1h. Frozen seeds were thawed in a water bath at 37°C for 1 min. Seeds were then placed on baby food jars with 15 ml of *TTG* medium containing MS basal salts (Murashige and Skoog, 1962), MS vitamins, 0.577µM/l GA_3, 58.44mM sucrose, 0.45% agar and pH 5.7-5.8. Germination conditions were on a Percival Scientific™ growth chamber (model CU-32L) at 27±2°C, photoperiod of 12h at 44µmol^{-2} s^{-1}. Evaluation of seed germination was determined 40-50 days after seeds were planted on TTG medium.

A viability test using TTC was done with non-germinated, frozen seeds of *Amarillo* (ISTA, 1999). The color patterns of TTC –stained papaya seeds (*Carica papaya*; Chia and Warren, 1999) were used as standard to determine seed viability. The testa was removed from frozen non-germinated seeds of *Amarillo* using scalpel and forceps, then they were immersed in a 0.5% (w/v) *TTC* solution for 24h, rinsed and evaluated. Seeds were classified as viable or non-viable according to staining pattern.

Shoots from germinated seeds were cut of the seedling and transferred to 17N (Roca, 1984) rooting medium for 20-25 days until roots were strong enough to stand transfer to pots in the greenhouse. Rooting conditions were the same as those used for seed germination, except that the temperature was maintained at 25±1°C. Plants were potted in a 2:1 soil-sand mixture.

Statistical analysis

Five replicas per treatment (frozen and non-frozen) with 20 seeds each were used. Treatments were evaluated on the basis of seed germination percentage and statistical differences between treatments and cultivars were estimated by Chi-square (X^2) tests (S.A.S Institute, 1989).

RESULTS AND DISCUSSION

Seed moisture content

Tree tomato seeds are considered orthodox (Hong *et al.*, 1996). This seed types can be dehydrated extensively without damage to low moisture content and, over a wide range of environments, their longevity increases with decreases in seed storage moisture in a quantifiable and predictable way (Richards 1973). The recommended *MC* for orthodox seeds is among 5-10% depending on the species, cultivars or variety (Hong *et al.*, 1996; Hong and Ellis, 1996). As it is shown in Table 1, MC for all materials reached approximately 7% after different drying periods. Seeds of *Común* and *Amarillo* dried faster than *Mora*. The drying time was shorter for the former two (1h) than for *Mora* (2.5h).

Table 1. Moisture content of tree tomato seeds after different drying periods.

Cultivars	Drying time (h)	Moisture Content	
		Fresh	Dried
Común	1	83.7%	6.9%
Amarillo	1	84.5 %	7.3%
Mora	2.5	86.3%	6.7%

Germination of cryopreserved seeds and plant recovery

Seed germination after freezing depended upon cultivars. For *Común* and *Mora*, more than 80% of the seeds germinated at 40 and 49 days respectively, while 37.4% of seeds of *Amarillo* germinated after 45 days. Non-significant differences in germination were observed between frozen and non-frozen seeds of *Mora* and *Común*, while for *Amarillo* the percentage of germination of frozen seeds were significantly lower, if compared to non-frozen control seeds (Table 2).

Table 2. Comparations of germination percentage in different cultivars until 90% of responses were observed.

Treatments	Cultivars		
	Amarillo	*Mora*	*Común*
Frozen	37.4 %	86 %	94 %
Non frozen	97.5 %	90 %	95 %
Probability (p = 0,001)	***	ns	ns

(***, significant differences; ns= non-significant ; $\propto$ = 95%).

Viability Test (TTC)

Non- germinated frozen seeds of Amarillo were used to monitor the seed viability by TTC staining (viable tissues stain red; Figure 2). The results are summarized in Table 3.

In general, a ripe seed needs water, oxygen and a temperature in a range 5-45 °C to initiate germination. However, if it fails to respond to the environmental conditions and get into a quiescence state characterized by its inability to grow, through continuing its morphological and physiological activities it could be consider under dormancy stage. The duration of this state is extremely variable, it may last only a few days up to several years (Thomson 1979; Parker 1989).

The main purpose of tetrazolium test is to distinguish viable from non-viable seeds. Whether a seed is rated viable or non-viable derives directly from the importance of the different seed tissues responsible for the emergence and development of a normal seedling, which is species specific. Viable seeds are those that show the potential to produce normal seedlings. Such seeds stain completely, or if only partly stained, the staining patterns indicate that the essential structures are viable (ISTA1999).

Non-viable seeds are those that do not meet these requirements and in addition include seeds, which reveal uncharacteristic coloring and/or flaccid essential structures. Seeds with obviously abnormal development of the embryo or other essential structures shall be regarded as non-viable whether stained or not (ISTA 1999).

Our results show that those non-germinated, frozen seeds were viable but unable to initiate the germination process. In most species, dormancy could be due by physical or chemical factors but the chemical phenomena are, not fully understood. Probably the rapid desiccation treatment and freezing steps could induce dormancy. That seems to be blocking physiological process involved in germination after freezing (Thomson 1979). It was to possible recover plants in all cultivars after freezing and to have them established in greenhouse (Figure 3).

Plants coming from frozen and non-frozen seeds looked morphologically similar, e.g., we could not detect differences in growth rate, and in leaf, stem and root shape.

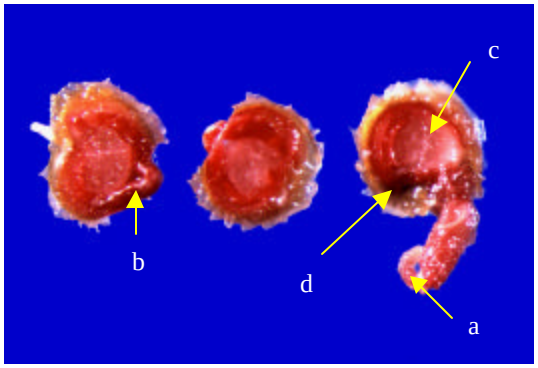

Figure 2. Viable tree tomato, frozen seeds of *Amarillo* cultivars stained with TTC (a, ridicule; b, embryo; c, endosperm; d, cotyledon).

Table 3. Viability test of non-germinated frozen seeds of tree tomato, *Amarillo* cultivars, using TTC

Repetition	n	Viable Seeds	Viability (%)
R1	18	15	83.33
R2	17	15	88.24
R3	16	11	68.75
R4	18	14	77.77
R5	18	15	83.33
Average	-----	-----	80.46

n= number of frozen seed tested

Figure 3. Recovery plants of Tree Tomato cultivars: left, *Mora*; center, *Amarillo*; right, *Común* after freezing steps growing in the greenhouse.

This report could be considered as the first one on tree tomato seed cryopreservation. Being conveniently applied to regional and national agricultural programs to support germplasm conservation due to the simple and low-cost method established in this study.

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