IN VITRO CONSERVATION OF DIPLOID BANANA ACCESSIONS

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ABSTRACT: A protocol for the *in vitro* conservation of diploid banana accessions based on lower temperature for culture environment was developed. Twenty four genotypes, four clones per genotype were studied. After disinfection, shoot tips (0.5 cm diameter x 0.5 cm height) were established *in vitro* and submitted to multiplication induced by benzylaminopurine. Twelve explants (0.6 cm diameter x 1.2 cm height) from each accession were transferred to the MS medium supplemented with 20 g L^{-1} sucrose and 2 g L^{-1} Phytagel, without growth regulators, under light intensity of 2000 lux, 16-hour photoperiod, at temperatures of: 17 ± 2°C, 22 ± 2°C and 26 ± 2°C. The development of plantlets was evaluated during 450 days and the survived plantlets were acclimated under greenhouse conditions. There were no differences among genotypes concerning the behavior during *in vitro* conservation; all genotypes showed potential to be conserved for a long time. The interval for transference should be 180, 360 and 450 days under the mean temperatures of 26, 22 and 17°C, respectively.
Key words: *Musa* spp., germplasm, *in vitro* conservation

CONSERVAÇÃO *IN VITRO* DE ACESSOS DIPLÓIDES DE BANANEIRA

RESUMO: Foi desenvolvido um protocolo para conservação *in vitro* de acessos diplóides de bananeira, baseando-se na redução da temperatura da sala de cultivo. Foram utilizadas como matrizes, quatro mudas do tipo chifrinho de 24 genótipos. Após desinfestação, ápices caulinares (0,5 cm diâmetro x 0,5 cm altura) foram introduzidos *in vitro* e submetidos à multiplicação induzida por benzilaminopurina. Após esta fase, 12 explantes (0,6 cm de diâmetro por 1,2 cm de altura) de cada acesso foram introduzidos em meio de conservação (MS contendo 20 g L^{-1} de sacarose e 2 g L^{-1} de Phytagel), sem fitorreguladores, a qual foi realizada sob intensidade luminosa de 2000 lux, fotoperíodo de 16 horas, sob temperaturas de: 17 ± 2°C, 22 ± 2°C ou 26 ± 2°C. O desenvolvimento das plântulas foi analisado durante 450 dias e as plântulas sobreviventes foram aclimatadas em casa-de-vegetação. Não foram observadas diferenças entre os acessos quanto ao seu comportamento *in vitro*; todos apresentaram potencial para serem conservados por longos períodos. O intervalo entre as transferências deve ser de 180, 360 e 450 dias, sendo que as temperaturas médias devem ser mantidas a 26, 22 e 17°C, respectivamente.
Palavras-chave: *Musa* spp., germoplasma, conservação *in vitro*

INTRODUCTION

Banana crop involves billions of dollars per year all over the world and more than 35 millions of people depend on this agricultural activity (Arias, 1993).

One of the Brazilian Embrapa Research Centers (CNPMF) is dedicated to tropical fruit research. It is located in Cruz das Almas, BA and has a banana breeding program that consists in obtaining more productive and pest, and disease tolerant tetraploid cultivars which yield fruits with better quality, by selecting among bred diploid and commercial triploid progenies.

The active bank of banana germplasm at CNPMF has 280 classified accessions in which 232 are already characterized and evaluated, using 107 agricultural botanical descriptors. Among these accessions, 86% are cultivars and 14% are native species.

The preservation of banana germplasm can be done by means of collection in the field, seed bank (diploids) and in laboratories under reduced growth conditions or cryopreservation (Escalant, 1993). The *in vitro* banana collections under minimal growth conditions show, as advantage, the easiness for exchanging germplasm compared with the field collections. This advantage is due to the reduced weight and volume of *in vitro* plants, minimal possibility of disease dissemination and more pronounced control of environmental conditions. Moreover, the accessions can be rapidly multiplied and the whole process demands small space and labor (De Langue, 1984; Jarret, 1986; Vuylsteke, 1989). The seed conservation in banana is limited by the fact that various diploids do not yield fertile seeds (Williams, 1987). Although Withers (1990) and Panis (1995) described protocols for cryopreservation of banana suspension cells, a system to reduce risks of somaclonal variation was not yet developed.

The *in vitro* conservation by minimal growth rate of the plantlets can be done by addition of osmotic stabilizers such as sorbitol, manitol or sucrose in culture medium (Zamora et al., 1986), and/or by reducing temperature of the culture environment (Vuylsteke, 1989).

Temperatures between 15 and 21°C in conservation chambers are frequently utilized to reduce the growth rate (Banerjee & De Langhe, 1985; Jarret et al., 1986; Van Den Houwe et al., 1995).

However, it is necessary for each species, even for each accession to define factors such as composition of culture medium, conservation chamber temperature and period of transference to maximize the efficiency of conservation (Withers, 1990). The conserved material also needs to be monitored concerning its genetic stability, viability and phytosanitary state.

The aim of the present work was to develop a protocol for *in vitro* conservation of diploid banana (AA) accessions based on the reduction of the temperature of the conservation chamber.

MATERIAL AND METHODS

Twenty four accessions of diploid bananas were studied (TABLE 1) and, four clones per genotype were used. The suckers were washed with tap water and cut into explants of 1.2 cm diameter and 3.0 cm height. The explants were surface sterilized in 70% ethanol (v/v) for 3 minutes and in 6% calcium hypochloride solution for 20 minutes. They were rinsed three times in sterile distilled water, and reduced to 0.5 cm diameter and 0.5 cm height.

TABLE 1 - Type and origin of banana diploid accessions (AA).

Accession	Type	Origin
F3P4	Partenocarpic hybrid	Equator
Jaran	Cultivar	Indonesia
Khae	Wild	Thailand
Khai	Cultivar	Thailand
Lidi	Cultivar	Honduras
M-53	Partenocarpic hybrid	Equator
M-61	Partenocarpic hybrid	Equator
Malaccensis	Wild material	Honduras
Malbut	Cultivar	New Guinea
Mambe Thu	Cultivar	New Guinea
Monyet	Wild material	Indonesia
NBC-20	Cultivar	New Guinea
Niyarma Yik	Cultivar	New Guinea
Ouro	Cultivar	Brazil
Pa Phatthalung	Wild material	Thailand
PA Rayong	Wild material	Thailand
PA Songkla	Wild material	France
Raja Uter	Cultivar	Indonesia
S/N. 2	Cultivar	New Guinea
Sowmuk	Cultivar	New Guinea
Thong Dokmak	Cultivar	Thailand
Tjau Lagada	Cultivar	Honduras
Tongat 1	Cultivar	Honduras
Tuugia	Cultivar	Hawaii

Explants were cultured on MS (Murashige & Skoog, 1962) medium supplemented with 2 mg L^{-1}BA (benzylaminopurine), 30 g L^{-1} sucrose and 2 g L^{-1} Phytagel, and the pH was adjusted to 5.7 before autoclavage (cultures were maintained for 4 weeks under 2000 lux, 16 h photoperiod and temperature of 27°C ± 2°C). Explants were then subcultured three times every four weeks in the MS medium supplemented with 3 mg L^{-1} BA, 30 g L^{-1} sucrose and 2 g L^{-1} Phytagel, under the same physical conditions. Twelve rooted plantlets of each accession were individualized. The roots and pseudostems were pruned off, leaving the explants with 0.6 cm diameter and 1.2 cm height. Each explant was transferred to a test tube (25 x 150 mm) containing 20 mL of the MS medium supplemented with 20 g L^{-1} sucrose and 2 g L^{-1} Phytagel, devoid of growth regulators. Conservation was done under 2000 lux light intensity, 16 h photoperiod and at three temperatures: 17 ± 2, 22 ± 2 and 26 ± 2°C. Four plantlets per treatment were used. The survival rate, plantlet height, tissue oxidation level and root formation were evaluated each 90 days. After 450 days, the surviving plantlets were acclimated under greenhouse conditions and transferred to the field.

RESULTS AND DISCUSSION

Explant contamination concerning the 24 accessions was on the average 13% at the establishment, 7% at the multiplication stage and less than 1% during the conservation period. Similar results were obtained at the multiplication of several banana cultivars (Sandoval et al., 1991; Leifert et al., 1994). The use of Phytagel instead of agar was favorable, since the development of bacteria and fungi was quickly identified in the cultures. The losses by microbial contamination were reduced during conservation.

Average number of multiplied explants varied from 2 to 3.4 plantlets per subculture, depending on the accession. The multiplication rates reported in this kind of work varies from 2 to 10 plantlets per subculture in several diploid, triploid and tetraploid banana accessions (Banerjee & De Langhe, 1985; Vuylsteke & De Langhe, 1985; Wong, 1986). Although significant differences occur as function of the genotype, the propagation method used in the present work was reasonable to multiply the accessions studied.

High survival rates of the explants were observed, even at a lower conservation temperature (TABLE 2). According to Banerjee & De Langhe (1985), physiological damage happens at temperatures lower than 10°C and *in vitro* growth of banana plantlet stopped at temperature lower than 12°C. Although none of plants of the accessions S/N.2, Thong Dokmak and Tuugia survived under mean temperatures of 17°C, it can not be concluded that they are sensitive to low temperature because of the small number of replicates used.

As was expected, the *in vitro* plantlet growth was higher with the increase of the temperature of the

TABLE 2 - Effect of temperature on banana diploid accessions (AA) survival along 450 days of culture.

Accession	% Survival														
Number of days	90			180			270			360			450		
Temperature[1]	17	22	26	17	22	26	17	22	26	17	22	26	17	22	26
F3P4	100	0	100	100	0	100	100	0	75	100	0	0	100	0	0
Jaran	100	100	100	100	100	100	75	50	50	75	50	50	75	25	0
Khae	50	100	100	50	100	100	50	50	0	50	50	0	50	25	0
Khai	50	50	50	50	50	50	50	50	50	50	50	50	50	50	0
Lidi	100	100	50	100	100	50	100	50	0	100	50	0	100	50	0
M-53	100	100	50	75	100	50	50	75	25	50	75	25	50	25	0
M-61	100	100	100	100	100	100	100	100	100	100	100	100	75	75	25
Malaccensis	100	100	50	100	100	0	100	100	0	100	100	0	75	100	0
Malbut	100	100	100	50	100	100	50	100	50	50	100	50	50	75	0
Mambe Thu	100	100	100	100	100	100	100	100	100	100	100	100	75	75	0
Monyet	100	100	100	100	100	100	100	100	25	100	100	25	100	100	0
NBC-20	100	100	50	75	50	50	50	50	50	50	50	0	50	50	0
Niyarma Yik	100	100	100	100	100	100	100	100	50	100	100	50	100	75	25
Ouro	100	100	100	100	100	100	100	100	100	100	100	50	100	75	0
PA Rayong	100	100	100	100	100	75	100	100	25	100	100	25	100	100	0
PA Songkla	100	100	100	100	100	100	75	100	100	75	100	100	75	50	25
Phatthalung	100	50	100	100	50	100	100	50	100	100	50	100	100	50	25
Raja Uter	100	100	0	25	100	0	0	100	0	0	100	0	0	75	0
S/N.2	50	100	100	0	50	100	0	50	75	0	50	50	0	25	0
Sowmuk	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0
Thong Dokmak	100	100	100	0	100	50	0	100	0	0	100	0	0	50	0
Tjau Lagada	100	100	50	100	100	50	100	100	50	100	100	0	100	50	0
Tongat 1	75	75	75	50	50	50	50	50	0	50	50	0	50	25	0
Tuugia	50	100	100	0	100	100	0	50	50	0	50	50	0	50	25
Mean	91±19	91±24	82±28	74±36	85±28	76±33	69±38	76±29	49±38	69±38	76±29	39±38	66±36	57±28	5±10

[1]Mean temperature during the period of conservation.

conservation chamber (TABLE 3). At the first 180 days, the plantlets which were maintained at 26°C showed leaf and root growth three to four times higher on the average than the observed under 17°C. At the end of 180 days, all plantlets cultured at 26°C reached to the top test tub (12 cm). This *in vitro* growth was only observed after 360 days at the treatments of 17°C and 22°C (TABLE 3).

During the period of conservation, no differences in development among the accessions were observed. However, Banerjee & De Langhe (1985) verified that the cultivars of plantain Asamiensa Agbagba and Ntanga (AAB) and Bluggoe (ABB) were more tolerant to low temperature than the cultivars of Dwarf Cavendish and Pisang Nangka (AAA). Zamora et al. (1989) verified that genotypes of the AA and AAA groups had higher survival rates than those of the ABB and BBB groups under the same conditions. These results indicate that further research is necessary.

At first 90 days, the plantlets had vigorous green leaves below 26°C and yellow-green leaves below 17°C. Later on, the leaves of the conserved plantlets became more yellowish proportionally to the increase of temperature. After 180 days, the plantlets at 26°C started showing signals of senescence. At 270 days, the leaves showed necrotic spots and high browning grade. Cases of death were more common at this time. Therefore, for diploid (AA) bananas conserved at 26°C, the subcultures should be done every 180 days. Zamora et al. (1986) verified that the *in vitro* conservation of banana species could be carried at multiplication temperatures, but the subcultures should be done every two months, requiring more labor, reagents and increasing risks of contamination losses. The time when the *in vitro* material should be replicated depends on different factors such as survival rate, viability and genetic stability.

Even after 360 days of conservation, the plantlets showed strong green color, low browning level, normal root development when grown at 17°C or below. Although they had reached the total height of the test tubes, the plants were perfectly healthy to be kept under the same conditions without replicating. The plantlets grown under mean temperature of 22°C at this day showed an intermediate behavior requiring replication. Van Den Houwe et al. (1995), working with 41 banana clones maintained at

TABLE 3 - Effect of temperature on banana diploid plantlets (AA) height along 450 days of culture.

Accession	Mean height (cm)														
Number of days	90			180			270			360			450		
Temperature[1]	17	22	26	17	22	26	17	22	26	17	22	26	17	22	26
F3P4	0.2	---[2]	3.2	1.0	---	10.3	3.5	---	12.0	10.3	---	---	11.5	---	---
Jaran	0.4	0.2	2.5	2.3	3.5	11.5	6.2	12.0	12.0	12.0	12.0	12.0	12.0	12.0	---
Khae	1.5	1.1	2.8	3.4	4.0	12.0	6.3	8.5	---	12.0	12.0	---	12.0	12.0	---
Khai	0.4	0.2	2.5	3.3	2.8	11.4	8.1	6.2	12.0	12.0	12.0	12.0	12.0	12.0	---
Lidi	0.5	1.0	2.8	2.1	2.8	11.0	5.1	9.7	---	12.0	12.0	---	12.0	12.0	---
M-53	0.3	0.3	4.5	6.0	5.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	---
M-61	0.2	1.7	6.1	2.3	3.6	12.0	7.6	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Malaccensis	0.9	1.5	2.0	8.0	3.0	12.0	12.0	8.0	---	12.0	12.0	---	12.0	12.0	---
Malbut	0.7	1.1	1.9	5.1	5.3	12.0	10.4	12.0	12.0	12.0	12.0	12.0	12.0	12.0	---
Mambe Thu	0.3	0.9	2.5	4.3	3.9	12.0	10.7	11.5	12.0	12.0	12.0	12.0	12.0	12.0	---
Monyet	1.2	1.3	2.3	2.1	3.7	12.0	5.3	9.0	12.0	11.2	11.1	12.0	11.6	11.8	---
NBC-20	0.3	1.0	4.0	4.5	4.4	12.0	12.0	12.0	12.0	12.0	12.0	---	12.0	12.0	---
Niyarma Yik	0.6	0.9	2.5	4.3	4.5	12.0	9.3	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Ouro	0.3	0.8	4.6	1.5	2.6	10.5	4.0	8.3	12.0	12.0	12.0	12.0	12.0	12.0	---
PA Rayong	0.6	2.2	3.0	4.5	3.5	11.1	9.5	12.0	12.0	12.0	12.0	12.0	12.0	12.0	---
PA Songkla	0.5	1.2	3.4	5.1	4.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Phatthalung	0.6	1.8	3.5	4.2	4.3	12.0	10.2	10.2	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Raja Uter	0.2	0.2	---	2.3	3.0	---	---	12.0	---	---	12.0	---	---	12.0	---
S/N.2	0.3	0.6	3.0	---	4.0	12.0	---	9.3	12.0	---	12.0	12.0	---	12.0	---
Sowmuk	1.1	1.9	3.5	4.0	4.1	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	---
Thong Dokmak	0.3	0.4	3.5	---	5.3	12.0	---	10.2	---	---	12.0	---	---	12.0	---
Tjau Lagada	0.4	0.2	2.6	2.0	2.1	---	6.1	9.3	9.3	12.0	12.0	---	12.0	12.0	---
Tongat 1	0.2	0.2	1.5	2.4	3.6	12.0	6.6	12.0	---	12.0	12.0	---	12.0	12.0	---
Tuugia	0.1	0.2	2.8	---	4.1	12.0	---	8.3	12.0	---	12.0	12.0	---	12.0	12.0
Mean[3]	0.5±0.4	0.9±0.6	3.1±1.0	3.6±1.7	3.8±0.8	11.7±0.5	8.4±2.9	10.4±1.8	11.9±0.6	11.9±0.4	11.9±0.2	12.0±0.0	12.0±0.1	12.0±0.0	12.0±0.0

[1]Mean temperature during the period of conservation. [2]No survival. [3]Survived plantlets only.

temperature of 22°C, suggest replicates every 220 days. In this work, it was verified that, for plantlets kept at mean temperature of 17°C, replicates every 450 days are recommended. This result is similar to that obtained by De Langhe (1984), Banerjee & De Langhe (1985) and Van Den Houwe et al. (1995), who recommended replicates between 330 and 540 days at temperatures ranging from 13 to 17°C.

The use of low temperatures without adjustments in the chemical components of the culture medium or modification in other physical culture conditions was sufficient to reduce the *in vitro* growth of the plantlets. Other changes could also be evaluated like light intensity (Banerjee & De Langhe, 1985) and hormonal balance (Zamora et al., 1989). In the present work, the conservation of plantlets was done under light intensity of 2000 lux. However, several authors have reported that the reduction of light intensity increases viability of crops at low temperatures, since the process of tissue deterioration is reduced (Centro Internacional de Agricultura Tropical, 1984).

The banana diploid plantlets were easily acclimated. No somaclonal variants were observed under greenhouse conditions. One possible reason is that genotypes used were diploids with low number of subcultures. However, the possible presence of these variants must be suitably monitored in the field, since banana plants multiplied *in vitro* have showed somaclonal variation ranging from 0 to 69% (Vuylsteke et al., 1991).

CONCLUSION

• The evaluated diploid accessions (AA) show potential to be conserved *in vitro* for a long time.

• The *in vitro* behavior of studied diploids (AA) is similar during conservation.

• The use of low temperatures, without adjustments in chemical components of the culture medium and/or in other physical culture conditions, is sufficient to reduce the *in vitro* development of banana diploid plantlets (AA).

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