# PLANT REGENERATION FROM LONG-TERM CALLUS CULTURE OF AAA-GROUP DESSERT BANANA[1]

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ABSTRACT - The banana plant is one of the most widely cultivated crops in the world. However, banana breeding has been a slow process, due to the low seed set and low germination rates. Selection of useful somaclonal variations and genetic transformation in cells or calluses are promising techniques to accelerate the breeding process. Therefore, callus culture was carried out, aiming the establishment of one protocol for plant regeneration, to be used in banana breeding program. Leaf sheath disks of 'Nanicão' banana (*Musa* sp., AAA group, Cavendish subgroup) were cultured on a Murashige and Skoog (MS) basal medium supplemented with activated charcoal (0.2 %), MES (2 [N-morpholino] ethanesulfonic acid) (15.3 mM), arginine (300 mM), Picloram (414 μM) and 2iP (2-isopentenyl adenine) (492 μM). Globular calluses developed on the leaf tissue were subcultured in the same medium, acquiring a friable and translucid appearance after one and a half year of culture. The friable calluses were transferred to the medium without growth regulators and arginine, and supplemented with casein hydrolysate (0.05%), where they formed embryo-like structures after transference to light. From these structures, shoots with roots were obtained and plantlets developed. The plant regeneration protocol shown here may be useful to banana breeding via somaclonal variation.

Index terms: *Musa*, tissue culture.

REGENERAÇÃO DE PLANTAS DE BANANEIRA DO GRUPO AAA A PARTIR DE CALOS CULTIVADOS DURANTE LONGO PERÍODO

RESUMO - A bananeira é uma das plantas mais cultivadas no mundo. Porém, o melhoramento genético de bananeira tem sido um processo vagaroso, em virtude das baixas taxas de formação e germinação de sementes. São técnicas promissoras a seleção de variações somaclonais úteis e a transformação genética em células e calos, para acelerar o processo de melhoramento. Realizou-se a cultura de calos, com o objetivo de estabelecer um protocolo de regeneração de plantas, para ser usado no programa de melhoramento genético de bananeira. Discos de bainha foliar da banana cv. Nanicão (*Musa* sp., grupo AAA, subgrupo Cavendish) foram cultivados no meio básico de Murashige e Skoog (MS) suplementado com carvão ativado (0,2%), MES (ácido 2 [N-morfolino] etanesulfônico) (15,3 mM), arginina (300 mM), Picloram (414 μM) e 2ip (2-isopentenil adenina) (492 μM). Calos globulares surgidos nos tecidos foliares foram subcultivados no mesmo meio, e obteve-se uma aparência friável e translúcida após um ano e meio de cultura. Os calos friáveis foram transferidos para meio sem reguladores de crescimento e arginina, e suplementado com caseina hidrolisada (0,05%), onde formaram estruturas semelhantes a embriões após transferência à luz. A partir destas estruturas, foram obtidos brotos com raízes, dos quais se originaram plântulas. O protocolo da regeneração de plantas apresentado aqui poderá ser útil para o melhoramento genético de bananeira via variação somaclonal.

Termos para indexação: *Musa*, cultura de tecidos.

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## INTRODUCTION

Banana production is estimated at over 70 million tons in the world, representing significant portion of the total diet of millions of people (International Network for the Improvement of Banana and Plantain, 1993).

*Musa* cultivars of economic interest are among the most sterile cultivated plants, and it creates a barrier for the conventional cross-breeding programs (Shepherd, 1987).

The new developed techniques of *in vitro* culture have made possible a fast vegetative multiplication (Krikorian & Cronauer, 1984), germplasm storage or exchange (Williams, 1987) and production of virus free plantlets (Berg & Bustamante, 1974). In the breeding programs, the *in vitro* culture technique of embryo

rescue may increase the amount of germinated seeds. Cell and protoplast cultures and long-term culture of dedifferentiated callus offer also new sources of genetic variation (Novak, 1992).

Initial works in banana callogenesis did not accomplish organogenesis (Mohan Ram & Steward, 1964; Srinivasa Rao et al., 1982). Embryo-like structures which developed only roots were obtained by Cronauer & Krikorian (1983), Jarret et al. (1985), and Banerjee et al. (1987). There are, recently, a few reports showing banana plant regeneration through a callus or cell suspension phase, from actively proliferating shoot tip culture (Novak et al.,1989; Dhed'A et al., 1991) and from zygotic embryos (Cronauer-Mitra & Krikorian, 1988; Escalant & Teisson, 1989). However, the success was concentrated only in varieties of diploid (AA group or wild relatives) or cooking (ABB group) bananas. Further studies are needed, using dessert (AAA and AAB group) bananas which major cultivars of many countries, particularly of Brazil, are classified in.

Prata and Nanicão are the most widely cultivated varieties in Brazil. Prata (AAB group) can produce a few fertile seeds when it is pollinated by pollens of fertile diploid variety (Dantas et al.,1993). However, Nanicão (AAA group) is highly sterile and does not produce seeds by pollination. Somaclonal variation through cell or callus cultures could be an important source for genetic improvement of this variety.

The present work describes the production of banana plants from a 18-month old callus of *in vitro* cultivated leaf tissue of a Brazilian dessert banana, aiming to obtain one protocol for plant regeneration, to be used in the banana breeding program.

## MATERIAL AND METHODS

Shoot-tips were excised from suckers of Nanicão cultivar (*Musa* sp., AAA group, Cavendish subgroup). They were surface disinfected for 1 minute in 70% ethanol followed by immersion in 1% sodium hypochlorite for 20 minutes, both solutions containing a few drops of tween 80. They were rinsed in sterile distilled water for three times, and the outermost leaves were removed until a size of approximately 1 cm.

The shoot-tip explants were cultured in a MS basal medium (Murashige & Skoog, 1962) supplemented with 22.2 µM BAP (6-benzylamino purine), with or without 0.5% (w/v) agar. The cultures were maintained in a sixteen-hour photoperiod (25 $\mu Mm^{-2}s^{-1}$) by cool-white fluorescent tubes at 28°C and were subcultured every eight weeks alternating between the mentioned semi-solid and liquid medium, until the leaf sheaths reached two to five cm in length.

Leaf sheath disks of 2 mm wide (Fig. 1) were cut and inoculated with their bases in contact with the MS basal medium supplemented with various concentrations of Picloram (4-amino-3,5,6-trichloropicolinic acid) and 2iP (2-isopentenyl adenine) (Table 1 and 2), 0.7% (w/v) agar, 0.2% (w/v) activated charcoal, 15.3 mM MES (2 [N-morpholino] ethanesulfonic acid) and 300 mM arginine. These cultures were incubated in darkness at 28°C. Thirty explants per treatment were served to analyze effects of concentrations of the growth regulators. After two months of culture, tissue growth and callus induction were evaluated by weighing ten samples per treatment and classified in four levels, as shown in Table 1 and 2. Aiming to utilize in following callus-growth experiments, another 20 explants per treatment were classified into the four levels by visual observation without weight measurement.

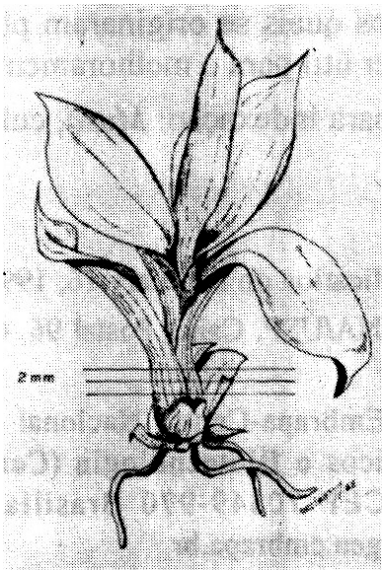


**FIG. 1. Schematic drawing of a 'Nanicão' plantlet, showing the place where the leaf sheath disks were excised.**

**TABLE 1. Effect of low concentrations of Picloram and 2ip on leaf sheath disks of Nanicão banana after 2 month of culture[1].**

| 2ip (μM ) | Picloram (μM)[2] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.00 | | 0.04 | | 0.41 | | 2.07 | | 4.14 | |
| | T | C | T | C | T | C | T | C | T | C |
| 0.00 | + | - | - | - | ++ | - | ++ | - | +++ | - |
| 0.49 | ++ | - | + | - | + | - | +++ | - | +++ | - |
| 4.92 | + | - | +++ | - | +++ | - | + | - | +++ | - |
| 49.20 | + | - | +++ | - | +++ | - | ++ | - | ++ | - |
| 492.00 | +++ | - | +++ | - | ++ | - | + | - | - | - |

[1] The growth of the explants were shown as: -, smaller than 10 mg; +, 10-200 mg; ++, 200-400 mg; +++, bigger than 400 mg fresh weight.
[2] T: tissue growth; C: callus induction.

**TABLE 2. Callus induction by high concentrations of Picloram and 2ip on leaf sheath disks of Nanicão banana[1].**

| 2ip (μM) | Picloram (μM) | | | | |
|---|---|---|---|---|---|
| | 4.14 | 20.70 | 41.40 | 200.70 | 414.00 |
| 0.00 | - | - | - | + | ++ |
| 4.92 | - | - | - | - | ++ |
| 49.20 | - | - | - | - | ++ |
| 492.00 | - | - | - | + | +++ |

[1] The induced calluses were shown as: -, smaller than 10 mg; +, 10-100 mg; ++, 100-300 mg; +++, bigger than 300 mg fresh weight.

The globular calluses formed on the tissue were isolated and transferred to the fresh medium every two months. When they changed to friable calluses, they were transferred onto the medium which differed from the above mentioned medium by the absence of growth regulators and arginine, and the addition of 0.05% (w/v) of casein hydrolysate and 0.5% (w/v) agar.

When the calluses became yellowish and less friable, they were transferred to the same medium without activated charcoal, and placed under a sixteen-hour photoperiod (25 $\mu Mm^{-2}s^{-1}$) at 28°C. All media were adjusted to pH 5.8 with KOH (1 N) prior to autoclaving at 120°C per 20 minutes and distributed in Petri dishes (90 x 10 mm) for callus culture.

## RESULTS AND DISCUSSION

In the first experiment, relatively low concentrations (0.04 - 4.14 μM) of the auxin were used, and no callus formation was observed (Table 1). Based on these results, the second experiment was carried out analyzing higher concentrations (4.14 - 414 μM). Callus induction was observed in the medium containing 200.7 μM or higher concentrations of Picloram (Table 2). The 2ip slightly improved callus induction when added at a concentration of 492 μM. The calluses were formed on the leaf sheath disks after 9-10 weeks in culture (Fig. 2). They were of a white-cream color and had a globular form.

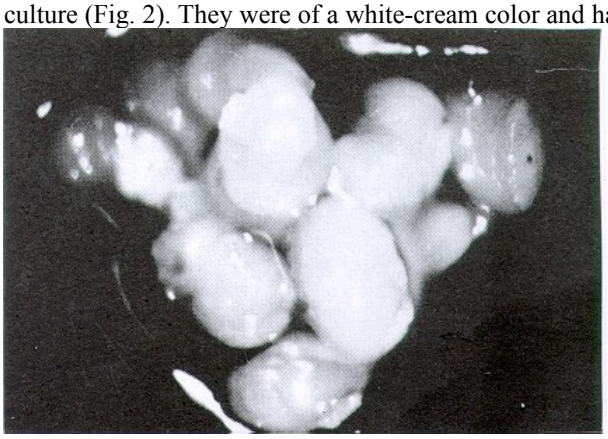

**FIG. 2. Globular callus isolated from the leaf sheath disks.**

The most critical step was a culture initiation, due to a high polyphenol oxidation of the tissue surface, that may kill the banana explant. The presence of activated charcoal and high concentration of Picloram in the culture medium, and the maintenance of the culture in darkness reduced noticeably the tissue browning and allowed the globular calluses to emerge from the initial explant. Many previous papers cited the successful effect of activated charcoal and darkness on the tissue browning prevention (Asahi, 1989), but no report is available about the Picloram effect. Auxin was commonly used to induce banana calluses as concentrations of 5 to 30 μM in the medium without activated charcoal (Novak et al., 1989; Dhed'a et al., 1991). Under these conditions, however, the leaf disks of Nanicão cultivar did not induce calluses and turned to brown. Activated charcoal was needed to reduce browning. To replace auxin adhered by the activated charcoal and to supply enough quantity for callus induction, an extremely high concentration (414 μM) of Picloram was required. The Picloram should activate basal metabolism of the cells and consequently reduce secondary metabolism such as polyphenol production. This may be another reason for the tissue browning reduced by the high Picloram concentration.

The calluses were separated from the initial explant and were subcultured every two months. The addition of arginine into the medium was beneficial to the growth of the culture in comparison with the medium without this amino acid. The globular calluses were formed from the leaf vessels.

The calluses became friable and translucent after 18 months in culture (Fig. 3). At this phase, they were transferred onto the medium without growth regulators and with lower agar concentration (0.5% w/v), where they reorganized less friable and yellowish calluses within ten weeks. When the culture was transferred to light condition, tiny embryo-like protuberances were formed (Fig. 4). Each structure developed into a plant or gave rise to new protuberances (Fig.5). Further cycle of proliferation and differentiation of protuberances resulted in a formation of more plantlets (Fig. 6). Thus, the continuous proliferation of protuberances and plants was obtained on the same medium without any growth regulators.

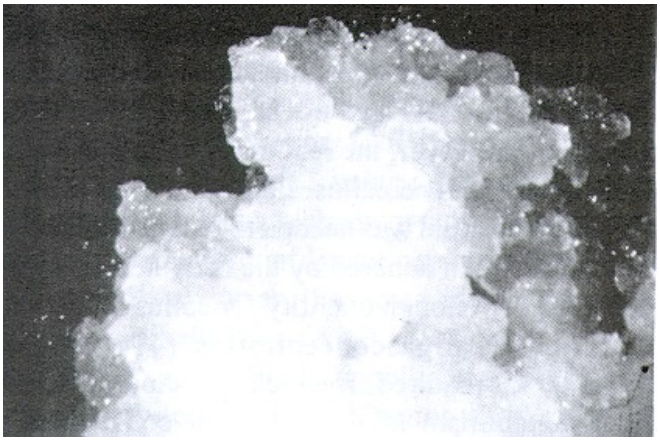

**FIG. 3. Friable and translucent callus obtained from the globular form of callus.**

**FIG. 4. Embryo-like protuberances (arrow).**

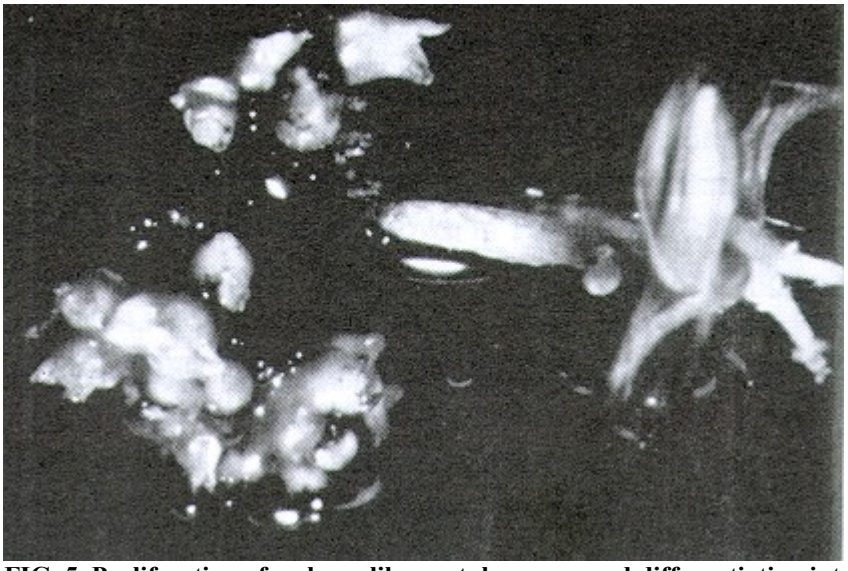

**FIG. 5. Proliferation of embryo-like protuberances and differentiation into plants.**

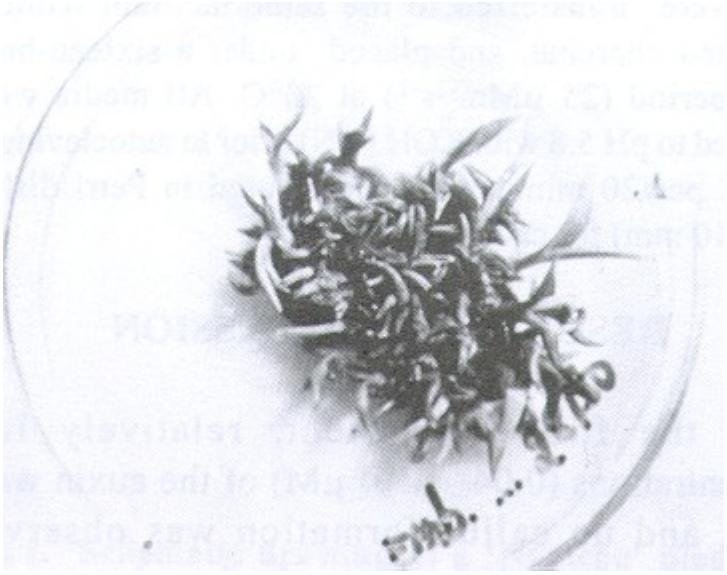

**FIG. 6. Mass proliferation of plantlets from proembryo-like structures.**

Two markedly different types of calluses have been obtained from the foliar explant to final plants. First type is a globular, opaque, and compact callus. It can form a second type of callus which is highly friable and translucent. Only the latter has generated plants.

There is no information upon the exact ontogenesis of these plants from calluses. However, it may be suggested they were originated by somatic embryogenesis, because the sequential steps carried out here are similar to those of other monocot species related previously as somatic embryos (Ahee et al., 1981; Vasil et al., 1984; Cheng & Raghavan, 1985; Teixeira et al., 1993).

The long-term callus culture (18 months) did not affect the plant regeneration capacity of the cells as it was also shown by Vasil et al. (1984) on *Zea mays*. Approximately one thousand plants were regenerated and planted in the field. Thus, the present paper propose one protocol of plant regeneration in Nanicão banana for somaclonal variant acquisitions with potential use in banana plant breeding.

## CONCLUSIONS

1. An extremely high concentration of auxin (414 μM Picloram) is needed to induce embryogenic globular calluses from leaf sheath disks of Nanicão cultivar in banana.

2. The 18-month old calluses of Nanicão cultivar still maintain the plant regeneration capacities.

3. The plant regeneration technique developed here is useful for somaclonal variation studies in banana plant breeding.

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