

## INDUCTION OF SOMATIC EMBRYOGENESIS IN IMMATURE SEEDS OF GUAVA TREE cv. PALUMA<sup>1</sup>

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**ABSTRACT** - The biotechnological techniques may help solve many problems of guava culture, such as the high perishability of fruits. Somatic embryogenesis can generate highly multiplicative cell cultures and with high regenerative potential, serving as basis for genetic transformation. The aim of this work was to obtain somatic embryogenesis of guava (*Psidium guajava* L.) cv. Paluma. Immature seeds were used, and they were inoculated in MS environment containing 400 mg L<sup>-1</sup> of L-glutamine, 100 mg L<sup>-1</sup> myo-inositol, 60 g L<sup>-1</sup> sucrose, 100 mg L<sup>-1</sup> ascorbic acid and supplemented with different types and concentrations of growth regulators. Embryogenic callus appeared after 37 days of culture in an environment containing 1.0 mg L<sup>-1</sup> 2,4-D + 2.0 mg L<sup>-1</sup> 2-ip, in 7% of the explants. After 65 days of culture, the treatment containing 0.5 mg L<sup>-1</sup> CPA showed 20% of explants with direct embryos, while the treatment with 1 mg L<sup>-1</sup> had 14% of explants with direct embryos and 7% of explants with embryogenic callus. In 66.6% of embryos regenerated with 0.5 mg L<sup>-1</sup> CPA there was the formation of secondary embryos. The use of IASP and BAP, aiming embryogenesis proliferation, led to an increase in the cellular proliferation, but calli apparently lost their embryogenic potential.

**Index terms:** tissue culture; growth regulators, Myrtaceae.

## INDUÇÃO DE EMBRIOGÊNESE SOMÁTICA EM SEMENTES IMATURAS DE GOIABEIRA cv. PALUMA

**RESUMO** - As técnicas biotecnológicas podem ajudar a solucionar alguns problemas do cultivo da goiabeira, como o alto perecimento dos frutos. A embriogênese somática pode gerar culturas de células altamente multiplicativas e com alto poder regenerativo, sendo base para processos de transformação genética. O objetivo deste trabalho foi obter embriogênese somática de goiabeira (*Psidium guajava* L.) cv. Paluma. Foram utilizadas sementes imaturas, que foram inoculadas em meio MS contendo 400 mg L<sup>-1</sup> de L-glutamina, 100 mg L<sup>-1</sup> de mioinositol, 60 g L<sup>-1</sup> de sacarose, 100 mg L<sup>-1</sup> de ácido ascórbico, suplementado com diferentes tipos e concentrações de reguladores de crescimento. Calo embriogênico surgiu após 37 dias de cultivo em meio contendo 1,0 mg L<sup>-1</sup> de 2,4-D + 2,0 mg L<sup>-1</sup> de 2-ip, em 7% dos explantes. Após 65 dias de cultivo, o tratamento com 0,5 mg L<sup>-1</sup> de CPA apresentou 20% dos explantes com embriões diretos, enquanto o tratamento com 1 mg L<sup>-1</sup> teve 14% dos explantes com embriões diretos e 7% dos explantes com calo embriogênico. Em 66,6% dos embriões regenerados com 0,5 mg L<sup>-1</sup> de CPA, houve formação de embriões secundários. O uso de IASP e BAP, visando à proliferação da embriogênese, levou a aumento de proliferação celular, mas os calos aparentemente perderam o potencial embriogênico.

**Termos para indexação:** cultura de tecidos; reguladores de crescimento; Myrtaceae.

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## INTRODUCTION

The culture of guava tree (*Psidium guajava* L.) is highly lucrative, because its fruit can be commercialized for fresh consumption and can also be processed, thus rapidly aggregating value. It is a source of jobs, since the culture requires tree care and permanent dedication, involving pruning branches and foliage, as well as proper storage of the fruits.

Guava is considered one of the most nutritionally complete fruits because it presents high contents of proteins, fibers, sugars, vitamins, lycopene and mineral salts. It is estimated that it contains four times more vitamin C than an orange and twice as much lycopene as a tomato (Piedade Neto, 2003). That, combined with the fact that it is as an exotic fruit, presents a great potential for export, either to be consumed as a table fruit or as an industrialized product.

As it can be seen, guava tree is a cultivated tree with great potential for the Brazilian agrobusiness. However, its culture presents a number of problems that might limit its expansion. The most important is the high perishability of the fruits during the post harvest phase. Because it is a climacteric fruit, guava presents a high respiratory rate, high ethylene production and high susceptibility to this hormone. That makes the fruit very fragile and easily damaged while being handled, transported and stored, incurring substantial losses of production. Of all the Brazilian cultivars, Paluma is currently the most popular guava tree grown in Brazil. This cultivar is very productive and yields large red fruits that may be used as both table consumption and industrialization.

Biotechnological techniques may serve as a way to obtain genetically improved plants for several characteristics, including tolerance to ethylene. The best known example is the "long life" tomato, in which genes from the ethylene pathway had been used in the antisense orientation (Hamilton et al., 1990; Kramer & Redenbaugh, 1994). However, a prerequisite for the transformation techniques or obtainment of mutant plants is the regeneration via tissue culture. Among the tissue culture techniques, somatic embryogenesis involves the regeneration of embryos from somatic tissues. The major benefit from somatic embryogenesis is that, unlike organogenesis, the regeneration of somatic embryos may occur from single cells (Litz & Gray, 1995). That should avoid the occurrence of chimera, being this a more efficient way of regeneration of the transformed plants. Besides, in many plants, in which somatic

embryogenesis was obtained, secondary embryogenesis was often present, a phenomenon in which new embryos emerges from somatic embryos (Raemarkers et al., 1995). Secondary somatic embryogenesis has many uses in biotechnology, such as the mass propagation of new plants, cryopreservation, genetic transformation and the induction of mutation (Litz & Gray, 1995; Raemarkers et al., 1995).

Previous studies have described regeneration systems from somatic embryogenesis of guava tree (Vilchez et al., 2002; Chandra et al., 2004; Raí et al., 2007), however for cultivars from India and one from Venezuela. Thus, the aim of this study was to obtain somatic embryogenesis from a Brazilian cultivar of guava tree.

## MATERIAL AND METHODS

The study was done at the Laboratory of Cell Culture and Plant Tissue of the Department of Plant Sciences of the Federal University of Viçosa at Viçosa, MG, Brazil.

### *Induction of somatic embryogenesis with different growth regulators*

Immature fruits were collected from guava trees cv. Paluma in an open pollination system approximately 50 days after the anthesis. The fruits were washed with tap water and liquid soap and disinfected as follow: 10 minutes in fungicide 2.5%, under agitation; 1 minute in absolute ethanol and 30 minutes in sodium hypochlorite solution 2.5%, under agitation. Between each phase, the fruits were rinsed three times with autoclaved distilled water. Immature seeds were removed from the fruits using tweezers and a scalpel and then inoculated in 25 x 150mm test tubes containing 10 mL of basal medium (BM) supplemented with different growth regulators (Table 1). The BM was composed of salt and vitamins from MS (Murashige & Skoog, 1969), 400 mg L<sup>-1</sup> of L-glutamine, 100 mg L<sup>-1</sup> myo-inositol, 60 g L<sup>-1</sup> sucrose, 100 mg L<sup>-1</sup> ascorbic acid (added after autoclaving the medium) and solidified with 2.5g L<sup>-1</sup> of Gelrite® (Sigma, Brazil). The means were poured into the autoclaved tubes in a flux chamber, after adding ascorbic acid. Forty seeds were inoculated for treatment. The tubes were kept in the dark at 25 ± 2°C.

After 30 days, the explants were transferred to a similar medium with the same composition, aiming the maintenance and multiplication of the callus. The explants were inoculated in 90 x 15 mm polystyrene Petri dishes containing 30 mL of culture medium.

The medium was autoclaved in Erlenmeyer flasks and poured into dishes in laminar flow chamber. The pH of the media was checked to  $5.7 \pm 0.01$ , and the media were autoclaved at  $121^\circ\text{C}$  and 1.5 atm for 20 minutes. The dishes were kept in the dark at  $25 \pm 2^\circ\text{C}$ .

#### *Multiplication of embryogenic calli*

After 60 days, the embryogenic calli from the  $T_{18}$  treatment were transferred to Petri dishes containing 30 mL of BM with  $1 \text{ mg L}^{-1}$  of 2,4-D and  $3.8 \text{ mg L}^{-1}$  of IASP, aiming the multiplication of the embryogenic calli. Ninety days later, the embryogenic calli and somatic embryos obtained in  $T_{10}$  and  $T_{11}$  were inoculated in Petri dishes containing 30 mL of MB with  $2 \text{ mg L}^{-1}$  of CPA e  $2 \text{ mg L}^{-1}$  of IASP. The media were autoclaved in Erlenmeyer flasks and poured into dishes in laminar flow chamber. The media pH was checked to  $5.7 \pm 0.01$ , and the media were autoclaved at  $121^\circ\text{C}$  and 1.5 atm for 20 minutes. The dishes were kept under a photoperiod of 16 hours/light and  $30 \text{ mMol m}^{-2} \text{ s}^{-1}$  at  $25 \pm 2^\circ\text{C}$ .

## RESULTS AND DISCUSSION

After 15 days of culture, all treatments led to callus formation. In general, the callus was formed on the surface of the immature seed. However, the only treatments that induced somatic embryogenesis in immature seeds of guavas cv. Paluma were those containing  $1.0 \text{ mg L}^{-1}$  of 2,4-D +  $2.0 \text{ mg L}^{-1}$  of 2-ip ( $T_{18}$ ),  $0.5 \text{ mg L}^{-1}$  of CPA ( $T_{10}$ ) and  $1 \text{ mg L}^{-1}$  of CPA ( $T_{11}$ ). In these treatments, the embryogenic callus was formed in the zygotic embryos which started the germination in the culture medium. Even though the three treatments had led to the formation of embryogenic callus, the explants responded differently. The treatment with  $1.0 \text{ mg L}^{-1}$  2,4-D +  $2.0 \text{ mg L}^{-1}$  2-ip provided a faster response, since seven days after changing the medium, or on the 37th day of culture, some explants presented callus with yellowish coloration, similar to embryogenic callus (Figure 1A). The frequency of embryogenic callus induction in the explants was 7%, similar to the one obtained by Vilchez et al. (2002). Rai et al. (2007) obtained from 7 to 10% of somatic embryogenesis when zygotic embryos of guava were submitted to constant exposition of 2,4-D.

The guava explants responses to the CPA were longer. After 65 days of culture, 20% of the treated explants with  $0.5 \text{ mg L}^{-1}$  of CPA presented somatic embryogenesis which surged without

visible formation of callus (Figure 1B). For the treatment with  $1.0 \text{ mg L}^{-1}$  of CPA, it presented 14% of explants with direct embryos and 7% of explants with embryogenic callus. The somatic embryos were solid and had a milky white coloration, as well as the embryogenic callus. In 66.6% of the regenerated embryos with  $0.5 \text{ mg L}^{-1}$  of CPA, there was formation of secondary embryos.

Contrary to reports in which somatic embryogenic callus of guava was obtained (Vilchez et al., 2002; Chandra et al., 2004; Rai et al., 2007), the 2,4-D alone did not induce embryogenic calli in the Paluma cultivar. That differentiated response is probably related with the interaction between treatments and the genotype of the cultivar Paluma. Only when combined with 2-ip, the 2,4-D induced somatic embryogenesis. Besides, the concentration of 2-ip was twice the concentration of 2,4-D. There are other examples of somatic embryogenesis induction in Myrtaceae species when cytokinins and auxins were used (Parra & Amo-Marco, 1998; Stefanello et al. 2005). According to Gaj et al. (2004), when cytokinin induces somatic embryogenesis, it is always combined with an auxin.

Treatment with CPA was more successful, since there was the formation of somatic embryo in a more advanced stage, while the 2,4-D only led to the formation of embryogenic callus. CPA is an herbicide of chemical structure similar to 2,4-D, and it is used less often in tissue culture. This auxin was used successfully in the somatic embryogenesis of *Pisum sativum* (Loiseau et al., 1995) and *Brassica nigra* (Narasimhulu et al., 1992).

The high concentration of sucrose (6%) was the same used by Chandra et al. (2004) in the guava embryogenesis. Rai et al. (2007) confirmed that lower concentration of sucrose did not lead to somatic embryogenesis induction in guava zygotic embryos. The high concentration of sucrose and other polysaccharides in the medium induces an osmotic effect, creating stress that can lead to embryogenesis induction, and, besides, generates energy to the process (Agarwal et al., 2004).

The subculture of embryogenic callus and somatic embryos in MS medium with IASP or BAP, aiming the proliferation of embryogenesis, also led to cell proliferation, but with apparent loss of potential embryogenic.

In recent reports, guava somatic embryos developed in a medium free of growth regulators (Rai et al., 2007), or the complete process of embryogenesis was obtained in the same medium of induction (Chandra et al., 2004). That indicates that the simple reduction of medium auxins is sufficient to complete

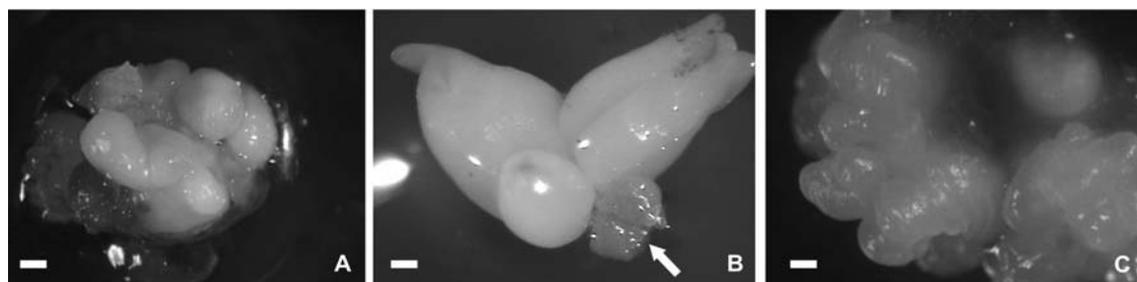
the guava embryo development, which is particular of somatic embryogenic processes, as postulated by Komanine et al. (1992).

This study aimed the multiplication of embryogenic callus, due to low rates of induction. However, the multiplication of calli was not successful, due to tissue oxidation. A problem of

guava *in vitro* culture is the high phenolization of tissues, which was circumvented by the use of high concentration of ascorbic acid ( $100 \text{ mg L}^{-1}$ ). However, during the multiplication phase, even with ascorbic acid there was tissue oxidation, besides inordinate growth. Thus, more tests are necessary to obtain the complete regeneration of somatic embryos of guava cv. Paluma.

**TABLE 1-** Growth regulators used to induce somatic embryogenesis in immature guava seeds.

Treatment	Growth Regulators
T <sub>1</sub>	$0.5 \text{ mg L}^{-1}$ zeatin
T <sub>2</sub>	$1.0 \text{ mg L}^{-1}$ zeatin
T <sub>3</sub>	$2.0 \text{ mg L}^{-1}$ zeatin
T <sub>4</sub>	$0.5 \text{ mg L}^{-1}$ 2-ip
T <sub>5</sub>	$1.0 \text{ mg L}^{-1}$ 2-ip
T <sub>6</sub>	$2.0 \text{ mg L}^{-1}$ 2-ip
T <sub>7</sub>	$0.5 \text{ mg L}^{-1}$ 2,4-D
T <sub>8</sub>	$1.0 \text{ mg L}^{-1}$ 2,4-D
T <sub>9</sub>	$2.0 \text{ mg L}^{-1}$ 2,4-D
T <sub>10</sub>	$0.5 \text{ mg L}^{-1}$ CPA
T <sub>11</sub>	$1.0 \text{ mg L}^{-1}$ CPA
T <sub>12</sub>	$2.0 \text{ mg L}^{-1}$ CPA
T <sub>13</sub>	$0.5 \text{ mg L}^{-1}$ 2,4-D + $0.5 \text{ mg L}^{-1}$ 2-ip
T <sub>14</sub>	$0.5 \text{ mg L}^{-1}$ 2,4-D + $1.0 \text{ mg L}^{-1}$ 2-ip
T <sub>15</sub>	$0.5 \text{ mg L}^{-1}$ 2,4-D + $2.0 \text{ mg L}^{-1}$ 2-ip
T <sub>16</sub>	$1.0 \text{ mg L}^{-1}$ 2,4-D + $0.5 \text{ mg L}^{-1}$ 2-ip
T <sub>17</sub>	$1.0 \text{ mg L}^{-1}$ 2,4-D + $1.0 \text{ mg L}^{-1}$ 2-ip
T <sub>18</sub>	$1.0 \text{ mg L}^{-1}$ 2,4-D + $2.0 \text{ mg L}^{-1}$ 2-ip
T <sub>19</sub>	$2.0 \text{ mg L}^{-1}$ 2,4-D + $0.5 \text{ mg L}^{-1}$ 2-ip
T <sub>20</sub>	$2.0 \text{ mg L}^{-1}$ 2,4-D + $1.0 \text{ mg L}^{-1}$ 2-ip
T <sub>21</sub>	$2.0 \text{ mg L}^{-1}$ 2,4-D + $2.0 \text{ mg L}^{-1}$ 2-ip



**FIGURE 1-** Embryogenic response in immature seeds of guava cv. Paluma. A: Embryogenic callus induced with  $1.0 \text{ mg L}^{-1}$  2,4-D +  $2.0 \text{ mg L}^{-1}$  2-ip (Bar = 1 mm). B: Somatic embryos induced with  $0.5 \text{ mg L}^{-1}$  of CPA (Bar = 1 mm). Arrow indicates beginning of secondary embryogenesis. C: Embryogenic callus induced with  $1.0 \text{ mg L}^{-1}$  of CPA (Bar = 0.5 mm).

## CONCLUSIONS

1-Immature seeds of guava cv. Paluma have potential for somatic embryogenesis induction.

2-2,4-D can induce somatic embryogenesis in guava cv. Paluma when associated to 2-ip.

3-CPA is better than 2,4-D + 2-ip in the induction of somatic embryogenesis of guava cv. Paluma.

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