



# Somatic embryogenesis from mature split seeds of jaboticaba (*Plinia peruviana* (Poir) Govaerts)

Sheila Susy Silveira<sup>1\*</sup> , Bruno Francisco Sant'Anna-Santos<sup>1</sup>, Juliana Degenhardt-Goldbach<sup>2</sup> and Marguerite Quoirin<sup>1</sup>

<sup>1</sup>Departamento de Botânica, Universidade Federal do Paraná, Avenida Coronel Francisco Heráclito dos Santos, 100, 81531-980, Jardim das Américas, Curitiba, Paraná, Brazil. <sup>2</sup>Empresa Brasileira de Pesquisa Agropecuária, Embrapa Florestas, Colombo, Paraná, Brazil. \*Author for correspondence. E-mail: sheila.susy@gmail.com

**ABSTRACT.** *Plinia peruviana* is a species that is native to Brazil and is important due to the taste and medicinal properties of its fruits. Young leaves and split mature seeds were used as explants to initiate somatic embryogenesis to obtain a large number of plants in a short period of time. Leaf discs were cultured in MS medium containing various concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid) or picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid). In the case of the mature seeds, various concentrations of glutamine, 2,4-D and a combination of auxin and BAP (6-benzylaminopurine) were tested for somatic embryogenesis induction. For somatic embryo maturation, several concentrations of PEG 6000 (polyethylene glycol; up to 90 g L<sup>-1</sup>) were tested. After 60 days of culture using leaf discs, callus formation occurred in all treatments, with the highest averages obtained with 10 µM 2,4-D. However, these calluses did not form somatic embryos. For the cultured seeds, the best treatment was the MS medium with 1,000 mg L<sup>-1</sup> glutamine and 10 µM 2,4-D without BAP. The supplementation of 60 g L<sup>-1</sup> PEG 6000 was sufficient to promote the maturation of the somatic embryos. Histological analyses of the calluses that were formed from leaf discs showed nonembryogenic characteristics. In contrast, the calluses that originated from mature seeds had small and round cells with little vacuolation, which are characteristics of embryogenic structures.

**Keywords:** 2,4-dichlorophenoxyacetic acid; histological analysis; leaf discs; mature seeds; proembryogenic masses.

Received on July 20, 2018.  
Accepted on October 7, 2019.

## Introduction

Jaboticaba (*Plinia peruviana* (Poir) Govaerts) is a species native to Brazil. Its tasty fruits are appreciated *in natura* or as jellies, juices, wines and liqueurs (Duarte & Paull, 2015). Jaboticaba fruits are rich in phenolic compounds and therefore have antioxidant, anti-inflammatory and antidiabetic properties, among others (Souza-Moreira, Moreira, Sacramento, & Pietro, 2008; Wu, Dastmalchi, Long, & Kennelly, 2012; Wu, Long, & Kennelly, 2013).

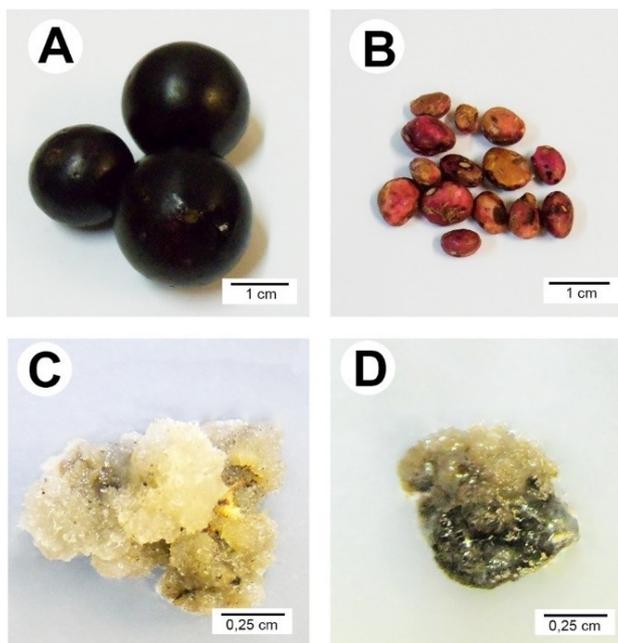
*Plinia peruviana* has high productivity even without management practices, and its main propagation method is through seeds (Cassol, Wagner Júnior, Pirola, Dotto, & Citadin, 2015). However, plantlets exhibit long juvenility periods, which can be up to 15 years long, in addition to high genetic variability (Cassol et al., 2015). In addition, the seeds are classified as recalcitrant, so they lose their viability quickly when stored (Duarte & Paull, 2015). Moreover, the low percentage of rooting of cuttings restricts jaboticaba propagation by this method (Cassol et al., 2015).

Establishing other methods of propagation is necessary to obtain a large number of plants, especially in the case of elite genotypes (Cassol et al., 2015). Among *in vitro* methods, somatic embryogenesis is a universal process and can probably be induced in any plant species if the explant type, culture medium and environmental conditions are optimized (Von Arnold, Sabala, Bozhkov, Dyachok, & Filonova, 2002). This technique has already been applied in species of Myrtaceae such as *Acca sellowiana* (Cruz, Canhoto, & Abreu, 1990; Canhoto & Cruz, 1994; Canhoto & Cruz, 1996; Dal Vesco & Guerra, 2001; Guerra, Dal Vesco, Ducroquet, Nodari, & Dos Reis, 2001), *Psidium guajava* (Rai, Akhtar, & Jaiswal, 2007; Akhtar, 2010; Bajpai, Kalim, Chandra, & Kamle, 2016), *Myrtus communis* (Canhoto, Lopes, & Cruz, 1999) and *Myrciaria aureana* (Motoike, Saraiva, Ventrella, Silva, & Salomão, 2007). Thus, somatic embryogenesis could be applied to overcome the problems of vegetative propagation in jaboticaba. The aim of the present study was to explore the *in vitro* production of somatic embryos of *Plinia peruviana* using explants obtained from leaf discs and mature seeds.

## Material and methods

### Plant material and surface sterilization

The peel and pulp were manually removed from jaboticaba fruits (Figure 1A), as was the seed mucilage (Figure 1B). The seeds were stored in paper bags under refrigeration (4°C) for up to 24h prior to *in vitro* culture. They were surface sterilized with ethanol, 70% (1 min.); sodium hypochlorite, 5% (v/v); and Tween-20®, 0.01% (v/v) (10 min.). Afterwards, the seeds were rinsed three times in sterile distilled water and remained in a 1% (v/v) solution of polyvinylpyrrolidone until *in vitro* inoculation.



**Figure 1.** A - *Plinia peruviana* fruits, B - seeds after the removal of the mucilage, C - callus from a leaf disc that was formed in culture medium containing 5 µM 2,4-D, and D - callus from a leaf disc that was formed in culture medium containing 5 µM picloram.

### Callogenesis from young leaf discs

Surface sterilized seeds (as described above) were cultured in test tubes (15 x 2.5 cm diameter) containing 10 mL WPM/2 (Lloyd & McCown, 1980) culture medium that was supplemented with 0.1% PPM™ (*Plant Preservative Mixture*, Plant Cell Technology Inc., USA) and 6 g L<sup>-1</sup> agar (Vetec®). The pH was adjusted to 5.8, and the culture medium was autoclaved at 120°C (20 min.). The seeds were maintained in a growth room (20°C night and 26 ± 1°C day; photoperiod of 16h) for 120 days. Two-month-old seedlings were used as the source of the explants. Leaf discs (0.5 cm diameter) were cut, maintaining midrib, and placed in Petri dishes (10 cm diameter x 2.5 cm height).

The basal medium “BM” consisted of MS salts and vitamins (Murashige & Skoog, 1962) and was supplemented with 30 g L<sup>-1</sup> sucrose and 6 g L<sup>-1</sup> agar. The pH was adjusted to 5.8 prior to autoclaving. The following compounds were tested:

1) *Different auxins.* Leaf discs were cultured in BM that was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) at 2.5, 5 or 10 µM.

2) *Different concentrations of 2,4-D and two explant positions.* Leaf discs were introduced to BM with different concentrations of 2,4-D (10, 20, 30 or 40 µM). They were positioned with their abaxial or adaxial side facing the culture medium.

### Somatic embryogenesis from mature split seeds

As the seeds of jaboticaba present polyembryony, only the zygotic embryo was used in the experiments. The apomictic embryos were smaller than the zygotic embryos and were discarded due to their high oxidation when cultured *in vitro*. Surface sterilized seeds were longitudinally cut, and the cotyledons were separated, maintaining the embryonic axis. Explants were individually cultured in test tubes (15 x 2.5 cm diameter) containing 10 mL BM. The cultures were maintained in a growth room in the dark (20°C night and 26 ± 1°C day) for 60 days. After one month, explants of all treatments were transferred to fresh identical medium. The following treatments were applied:

1) *Different concentrations of glutamine.* Explants were individually cultured in BM with 10  $\mu\text{M}$  2,4-D and supplemented or not supplemented with 250, 500, 750 or 1,000  $\text{mg L}^{-1}$  L-glutamine (Gln).

2) *Different concentrations of 2,4-D.* Jaboticaba explants were individually cultured in BM with 1,000  $\text{mg L}^{-1}$  Gln and 2,4-D (2.5, 5, 10, 25 or 50  $\mu\text{M}$ ).

3) *Different combinations of BAP and 2,4-D.* Jaboticaba explants were cultured in BM with 1,000  $\text{mg L}^{-1}$  Gln containing 5, 10 or 20  $\mu\text{M}$  2,4-D and combined with 2.5 or 5  $\mu\text{M}$  6-benzylaminopurine (BAP), in addition to a control without BAP.

### Somatic embryo maturation

Somatic embryos (SEs) at the globular stage, which had been developed in BM containing 10  $\mu\text{M}$  2,4-D and 1,000  $\text{mg L}^{-1}$  Gln, were used for the maturation phase of the study. SEs were transferred to BM that was supplemented or not supplemented with 30, 60 or 90  $\text{mg L}^{-1}$  polyethylene glycol 6000 (PEG 6000) for 30 days. The SEs were considered mature when they reached the torpedo or cotyledon stage.

### Statistical analysis

The values are the means of ten replicates. In the experiments with leaf discs, each replicate consisted of a Petri dish containing eight leaf discs (0.5 cm diameter). In the experiments with mature seeds, each replicate consisted of four test tubes containing one explant. In the maturation experiments, each replicate consisted of one glass flask containing five SEs. All experiments were repeated once. The treatment effects were evaluated using ANOVA with Assistat (7.7b). The mean values were compared by Tukey's test ( $p < 0.05$ ).

### Histological analysis

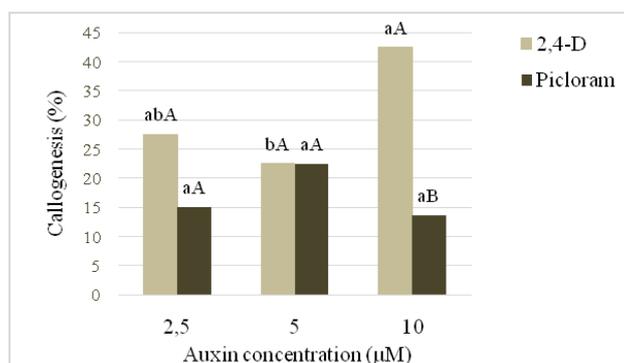
Calluses from the leaf discs were collected from 6-month-old cultures. Proembryogenic masses (PEMs) were collected 60 days after the induction phase in MS medium containing 10  $\mu\text{M}$  2,4-D and 1,000  $\text{mg L}^{-1}$  Gln. Both were fixed in a Karnovsky (1965) solution for 24h and subsequently stored in 70% alcohol. For the light microscope analyses, the samples were embedded in methacrylate resin (Technovit-7100). Sections (5  $\mu\text{m}$ ) were prepared using a rotary microtome (Olympus CUT 4055) and stained with 0.1% toluidine blue in 5% borax. Samples were examined under a microscope with an attached camera (Olympus EX41).

## Results and discussion

### Callogenesis from young leaf discs

#### 1) *Different concentrations of auxins*

Callus formation from leaf discs was initiated after 30 days of culture. After 60 days, approximately 24% of the discs presented calluses, especially at the midrib and cut region. There was no significant difference in callus formation among the picloram concentrations, but differences were observed between the treatments with 2,4-D. The use of 10  $\mu\text{M}$  2,4-D was the most efficient treatment for callogenesis (Figure 2). The calluses exhibited a mucilaginous and compact appearance with variable colors. In the culture medium containing 2,4-D, the calluses showed whitish or light-yellow coloration (Figure 1C), while those obtained in the medium containing picloram were smaller size and showed a dark brown to black coloration (Figure 1D).

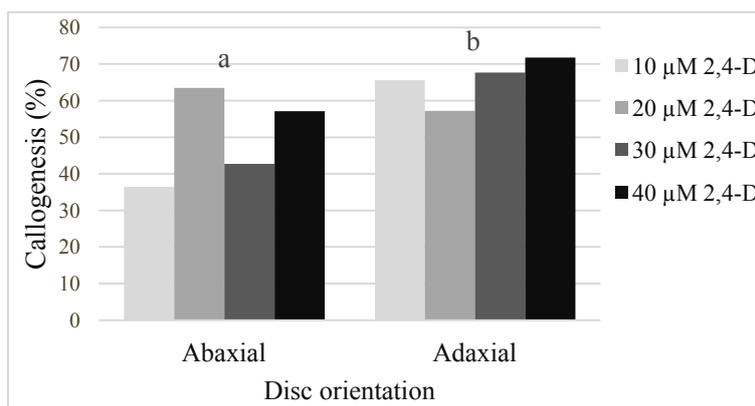


**Figure 2.** Percentage of callus formation from leaf discs of *Plinia peruviana*, according to the type and concentration of the auxin added to the MS culture medium, 60 days after *in vitro* introduction. Means followed by the same lowercase letters for the auxin concentrations and capital letters for the auxin types do not differ by Tukey's test at 5% probability.

In preliminary tests, leaf discs that were introduced to a plant regulator-free medium did not form calluses. For embryogenic culture initiation, normally the explant is cultured on a medium supplemented with auxin (Merkle, Parrott, & Flinn, 1995; Von Arnold, 2008; Nolan & Rose, 2010). The auxin stimulates the appearance of PEMs, which are groups of responsive cells that are competent for the formation of SEs (Nolan & Rose, 2010). Among auxins, 2,4-D is the most efficient for SE induction in most species (Nolan & Rose, 2010; Isah, 2016). However, in contrast to the present work, 2,4-D and picloram resulted in similar percentages of SE induction for *Myrtus communis* (Canhoto et al., 1999). In the case of *Acca sellowiana*, 2,4-D was used by most authors for embryogenesis initiation (Canhoto & Cruz, 1994; 1996; Guerra et al., 2001).

### 2) Effect of different concentrations of 2,4-D and two explant positions

The percentage of callus formation did not differ between the concentrations of 2,4-D. However, in the case of leaf positioning, better results were obtained when the adaxial face was placed in contact with the culture medium than when the abaxial was placed in contact with the culture medium (Figure 3).



**Figure 3.** Callus formation from leaf discs of *Plinia peruviana*, according to the 2,4-D concentration and leaf face in contact with the MS culture medium, 60 days after *in vitro* introduction. Means followed by the same letters do not differ by Tukey's test at 5% probability.

In jaboticaba leaf discs, the use of 10 μM of 2,4-D resulted in callus formation. The increase up to 40 μM did not result in a higher percentage of callogenesis, and no inhibitory effect on callus formation was observed. Typically, concentrations of 1 to 10 μM of 2,4-D are sufficient to stimulate SE formation (Von Arnold, 2008). For zygotic embryos of *Acca sellowiana*, a concentration of 5 μM is commonly used (Cruz et al., 1990; Canhoto & Cruz, 1994; 1996), but 20 μM has been reported for PEM formation for this species (Guerra et al., 2001). For *Myrtus communis*, concentrations above 2 μM 2,4-D have an inhibitory effect on SE formation (Canhoto et al., 1999).

Similar to the present work, when the adaxial side was in contact with the medium, this position, rather than when the abaxial face was in contact with the medium, resulted in higher percentages of callogenesis and embryogenesis for *Quercus rubra* (Rancillac, Klinguer, Klinguer, & Millet, 1996) and *Q. alba* (Corredoira, San-José, & Vieitez, 2012). The explant orientation may be important because nutrient and regulator conduction in leaves is naturally greater from the adaxial to the abaxial face (Corredoira et al., 2012). Palisade parenchyma is predominant at the adaxial surface and is more responsive than other tissues since it is the last tissue to cease growth and cell division (Welandar, 1988).

In most species, embryogenic competence is restricted to certain organs, especially young organs (Fehér, 2005; Corredoira et al., 2012). For jaboticaba, the leaf discs that were obtained from *in vitro*-grown seedlings did not present embryogenic competence, and calluses did not result in SE formation even after one year of culture. Immature zygotic embryos are the most common source of tissue, mainly for woody plant species, due to their embryogenic state (Nolan & Rose, 2010; Isah, 2016). Among members of the Myrtaceae family, the initiation of embryogenic cultures is usually from zygotic embryos or cotyledons (Cruz et al., 1990; Canhoto & Cruz, 1994; 1996; Canhoto et al., 1999; Guerra et al., 2001; Motoike et al., 2007; Akhtar, 2010; Bajpai et al., 2016).

### Somatic embryogenesis from split mature seed explants

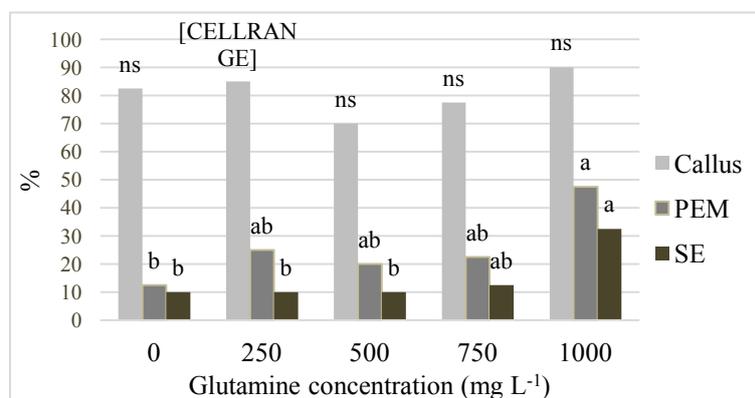
After 30 days of culture, calluses formed on mature split seeds of jaboticaba, and some of them had embryogenic capacity. The PEMs exhibited a friable, yellowish or light brown appearance, while the nonembryogenic structures had a darker and compact appearance and often showed adventitious root formation.

Globular SE formation on the mature seeds was initiated after 21 days of culture. This period was similar to that reported for zygotic embryos of other members of Myrtaceae (Canhoto et al., 1999; Akhtar, 2010). In most cases, the jaboticaba SEs originated from previously formed calluses, notably in the embryonic axis region. The formation of SEs was asynchronous, as it is for other members of the Myrtaceae family (Cruz et al., 1990; Canhoto et al., 1999; Motoike et al., 2007; Akhtar, 2010). Thus, SEs in different developmental stages were observed in the same explant, and they were mainly globular and heart shaped. In some explants, few SEs (1-2) were obtained, while in others, the number exceeded 200. The same result was observed for *Acca sellowiana* (Cruz et al., 1990), *Myrtus communis* (Canhoto et al., 1999), and *Psidium guajava* (Akhtar, 2010). As we used split seeds, only one of the cotyledons remained connected to the embryonic axis, which therefore led to a variation in the response of the explants.

#### 1) Effect of different concentrations of glutamine

After 60 days of culture, 81% of the explants presented calluses, and there were no differences among treatments (Figure 4). Without Gln, 13% of the cultures presented PEMs, while in the treatment using 1,000 mg L<sup>-1</sup> of this amino acid, the percentage of PEMs was 48%. The highest means of SE formation were obtained by using 750 (13%) and 1,000 mg L<sup>-1</sup> (33%) Gln.

As in the present study, Gln showed positive results in inducing SEs of *Acca sellowiana* (Dal Vesco & Guerra, 2001; Guerra, Cangahuala-Inocente, Dal Vesco, Pescador, & Caprestano, 2013) and *Psidium guajava* (Bajpai et al., 2016). Nitrogen is a fundamental nutrient for the efficient production of PEMs, playing an important role as a respiratory substrate and for the synthesis of metabolites (Carlsson, Svennerstam, Moritz, Egertsdotter, & Ganeteg, 2017). Amino acids serve as the primary sources of reduced forms of nitrogen and are beneficial during the induction and development of SEs (Merkle, Parrott, & Flinn, 1995; Dal Vesco & Guerra, 2001; Yang & Zhang, 2010). Gln can stimulate the proliferation and differentiation of SEs in some species of Myrtaceae (Pescador et al., 2012; Cangahuala-Inocente, Silveira, Caprestano, Floh, & Guerra, 2014).



**Figure 4.** Percentages of explants forming callus, proembryogenic masses (PEMs) and somatic embryos (SEs) in *Plinia peruviana* seeds cultured in MS medium that was supplemented with 10 µM 2,4-D for 60 days, as a function of glutamine concentration. Means followed by the same letters in the column do not differ by Tukey's test at 5% probability. ns: not significant.

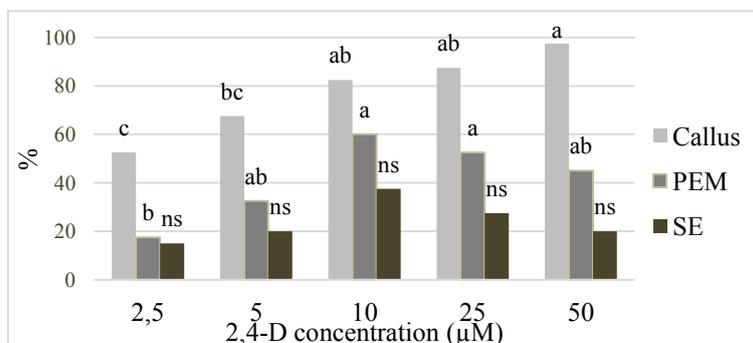
#### 2) Effect of different concentrations of 2,4-D

After 60 days, 68% of the cultures presented callus formation, with higher means in medium containing 10, 25 or 50 µM 2,4-D than in medium containing 2.5 µM 2,4-D (Figure 5). The percentage of PEM formation was 42% on average and did not differ among the concentrations of 2,4-D ≥ 5 µM. SE formation did not differ among the tested treatments (average of 24%).

In preliminary tests, jaboticaba explants that were introduced to culture medium without 2,4-D did not produce calluses or SEs. The control treatment and 2.5 µM 2,4-D treatment resulted in the germination of the embryonic axes that were present in the seeds. The same dependence on auxins for SE induction was observed for zygotic embryos of other Myrtaceae species (Cruz et al., 1990; Canhoto et al., 1999; Akhtar, 2010; Fraga et al., 2012; Guerra et al., 2013).

Auxins are known to mediate the transition from somatic to embryogenic cells by reprogramming genes involved in embryogenesis and are therefore the main regulators used to induce somatic embryogenesis (Quiroz-Figueroa, Rojas-Herrera, Galaz-Avalos, & Loyola-Vargas, 2006; Möller & Weijers, 2009; Yang & Zhang, 2010). 2,4-D is a synthetic auxin used for the initiation of embryogenic cultures in several plant

species (Quiroz-Figueroa et al., 2006; Isah, 2016). The effect of this regulator is related to modifications at the epigenetic level (De-la-Peña, Nic-Can, Galaz-Ávalos, Avilez-Montalvo, & Loyola-Vargas, 2015; Kumar & Van Staden, 2017). These changes include chromatin remodeling (Isah, 2016; Kumar & Van Staden, 2017), histone modifications (Isah, 2016) and an increase in DNA methylation levels (LoSchiavo et al., 1989; Joshi & Kumar, 2013). These processes are essential for the acquisition of embryogenic competence by cells (Bajpai et al., 2016; Kumar & Van Staden, 2017) and consequently for somatic embryogenesis to occur (De-la-Peña et al., 2015).



**Figure 5.** Percentage of explants forming callus, proembryogenic masses (PEMs) and somatic embryos (SEs) in *Plinia peruviana* cotyledons that were cultured in MS medium supplemented with 1,000 mg L<sup>-1</sup> glutamine for 60 days, as a function of 2,4-D concentration. Means followed by the same letters in the column do not differ by Tukey's test at 5% probability. ns: not significant.

In this study, among the treatments used, concentrations equal to or above 5 µM 2,4-D resulted in higher percentages of PEM formation. For Myrtaceae, concentrations up to 5 µM 2,4-D are often more efficient than concentrations above 5 µM in inducing somatic embryogenesis from immature embryos (Cruz et al., 1990). The concentration used in the present study (10 µM) proved to be efficient for SE induction from immature zygotic embryos of *Myrtus communis* (Canhoto et al., 1999) and *Psidium guajava* (Bajpai et al., 2016). However, a minimum concentration of 20 µM was required for the formation of SEs from cotyledons of *Myrciaria aureana* (Motoike et al., 2007).

### 3) Effect of different combinations of 2,4-D and BAP

The interaction between auxin and BAP was evaluated, and after 60 days, 90% of the explants showed callus formation, and 54% exhibited embryogenic characteristics (Table 1). The percentages of PEMs and SEs were increased by the use of 10 or 20 µM 2,4-D. The addition of BAP to the culture medium reduced the percentage of PEMs and SEs that formed, and the highest averages were obtained in the control treatment.

**Table 1.** Proembryogenic mass (PEM) and somatic embryo (SE) formation in *P. peruviana* seed explants cultured in MS medium for 60 days, as a function of 2,4-D and BAP concentrations.

BAP (µM)	% PEM				% SE			
	2,4-D (µM)				2,4-D (µM)			
	5	10	20	Mean	5	10	20	Mean
0	60.0	75.0	65.0	66.6 a	25.0	45.0	57.5	42.5 a
2.5	37.5	65.0	52.5	51.6 ab	22.5	40.0	37.5	33.3 ab
5	35.0	52.5	47.5	45.0 b	20.0	30.0	32.5	27.5 b
Mean	44 b	64.1 a	55.0 ab	54.4	22.5 b	38.3 a	42.5 a	34.4

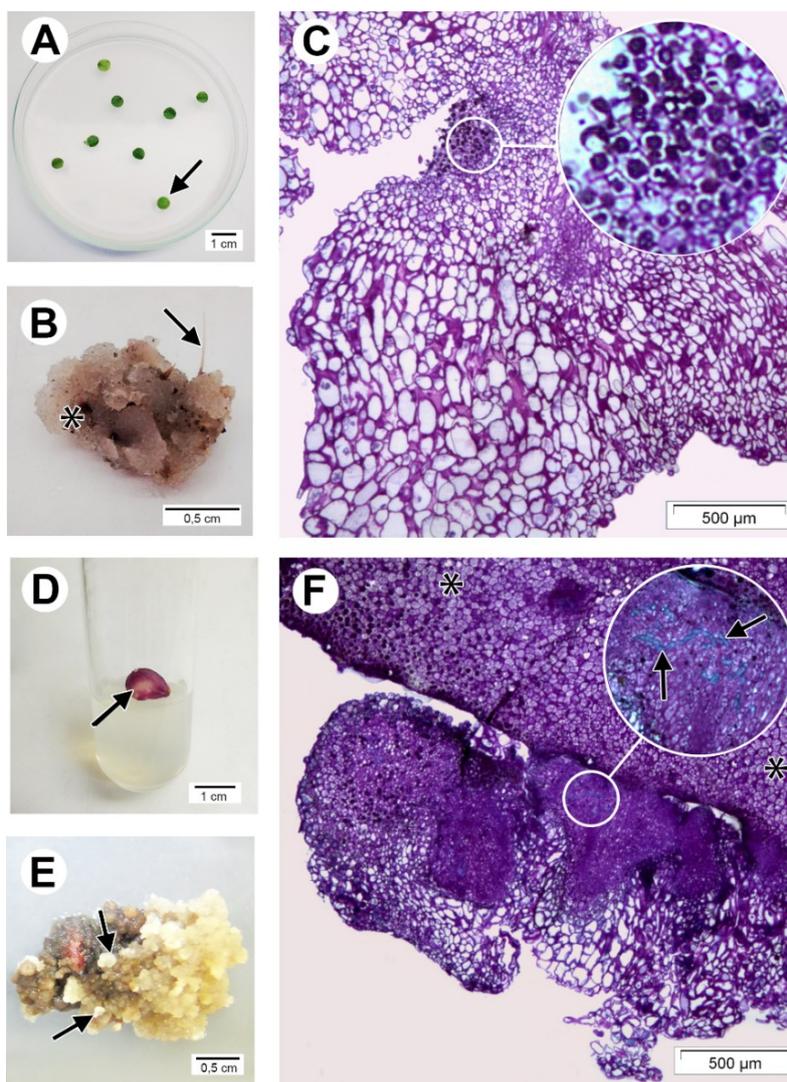
Means followed by same letters in the same column do not differ by Tukey's test at 5% probability.

The participation of regulators other than auxins is important for the hormonal balance that is necessary to achieve somatic embryogenesis (Gutiérrez-Mora, González-Gutiérrez, Rodríguez-Garay, Ascencio-Cabral, & Li-Wei, 2012). When mature zygotic embryos are used as explants, the addition of cytokinin in combination with auxin may be necessary to induce the formation of SEs (Merkle et al., 1995). Cytokinins play an important role in cell division and can stimulate cell proliferation (Gutiérrez-Mora et al., 2012). However, BAP was inefficient in increasing the formation of PEMs or SEs in jaboticaba. For the induction of SEs in most species of Myrtaceae, 2,4-D is used alone (Cruz et al., 1990; Canhoto & Cruz, 1994; Canhoto et al., 1999; Dal Vesco & Guerra, 2001; Guerra et al., 2001; Motoike et al., 2007; Rai et al., 2007; Akhtar, 2010; Bajpai et al., 2016).

### Histological analysis

The calluses that formed on leaf discs (Figure 6A) showed a compact appearance, often followed by adventitious roots (Figure 6B). These calluses showed a nonembryogenic structure with large, disorganized and vacuolated parenchyma cells (Figure 6C). The presence of phenolic compounds in some regions was noticeable (Figure 6C, arrow). These calluses had no embryogenic potential, and there was no SE formation even after 12 months of culture. Large cells with large vacuoles and a low nucleus/cytoplasm ratio are features of nonembryogenic calluses (Shang et al., 2009).

PEMs were formed only in cultures of mature seed explants (Figure 6D). The PEMs showed a friable structure that was a yellowish or brown color (Figure 6E) and was composed of round, compact and organized cells. These cells had a dense cytoplasm and a small degree of vacuolation (Figure 6F). This structure is characteristic of embryogenic cells and is similar to that of meristems and zygotes (Fehér, 2005). PEMs showed vascular tissues without connections to the initial explant. The PEMs showed typical thickening of the cell walls of tracheary cell elements (Figure 6F, arrow).

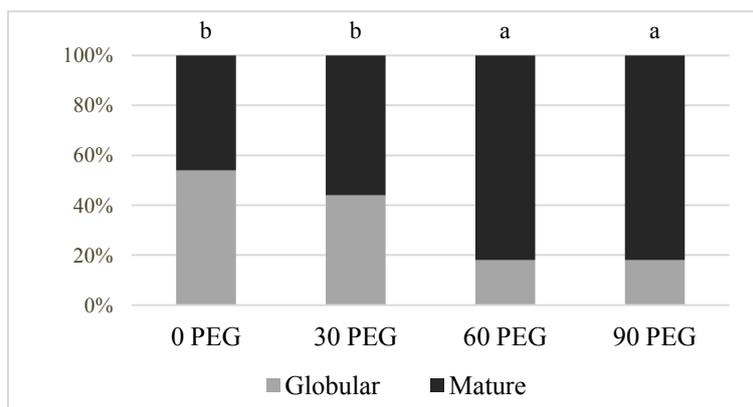


**Figure 6.** Somatic embryogenesis of *Plinia peruviana*. A - culture of leaf discs of jaboticaba (arrow), B - morphological appearance of nonembryogenic calluses formed from leaf discs, showing adventitious roots (arrow) and oxidized portions (asterisk), C - nonembryogenic calluses with cells containing phenolic compounds (detail), D - mature seed of jaboticaba: cotyledon with embryonic axis (arrow), E - morphological appearance of proembryogenic masses formed from cotyledons, with several clusters of somatic embryos (arrows), and F - histological section of proembryogenic masses, with vascular tissue in detail (arrows).

### Somatic embryo maturation

For somatic embryo maturation, we tested various concentrations of PEG 6000. After 30 days, the highest percentages of globular SEs were obtained in PEG-free medium or medium supplemented with 30 g L<sup>-1</sup>. As the PEG concentration was increased to 60 or 90 g L<sup>-1</sup>, an increase in the number of mature embryos was observed (Figure 7). These results indicate that PEG promoted the development of SEs, since it caused a

reduction in the percentage of embryos that remained in the globular stage, i.e., it allowed the SEs to advance to the last stages of development.

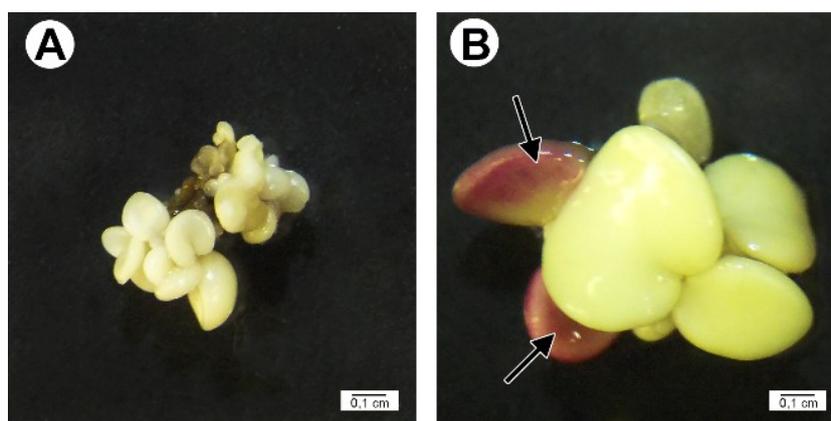


**Figure 7.** Percentages of globular and mature somatic embryos of *Plinia peruviana* 30 days after transfer to maturation medium containing different concentrations (in g L<sup>-1</sup>) of polyethylene glycol 6000 (PEG 6000). Columns followed by the same letter do not differ by Tukey's test at 5% probability.

In some plant species, there high osmolarity is required for the maturation of SEs, as in the case of zygotic embryos (Merkle et al., 1995). Nonpermeating osmotic substances, such as PEG, are high molecular weight molecules that are unable to cross the cell wall and remain in the culture medium (Attree & Fowke, 1993). This type of osmoticum restricts the availability of water, exposing SEs to water stress (Jalali, Sirmandi, & Hatamzadeh, 2017). In this case, the reestablishment of water availability is possible only by increasing the concentrations of solutes inside the cell (Attree & Fowke, 1993). This process leads to an accumulation of reserve compounds, which is similar to the process that occurs in zygotic embryos (Misra, Attree, Leal, & Fowke, 1993).

PEG inhibits the proliferation of globular embryos and stimulates their development and subsequent conversion (Rudiyanto, Efendi, & Ermayanti, 2014). This osmotic agent also promotes the maturation of SEs of some Myrtaceae (Motoike et al., 2007; Bajpai et al., 2016).

Embryos in the control treatment were smaller than SEs that were exposed to PEG (Figure 8A). The PEG treatments also anthocyanins to accumulate in jaboticaba cultures, resulting in purplish SEs (Figure 8B). This increase in anthocyanin levels is related to osmotic stress, which inhibits cell division and stimulates the synthesis of secondary metabolites (Tholakalabavi, Zwiazek, & Thorpe, 1994). The synthesis of anthocyanins and other pigments can be enhanced during the maturation of SEs of species such as *Theobroma cacao* (Pence, 1992) and *Populus deltoides* (Tholakalabavi et al., 1994).



**Figure 8.** Maturation of somatic embryos from mature seeds of *Plinia peruviana*. A - In PEG-free culture medium and B - supplemented with 90 g L<sup>-1</sup> PEG 6000, showing the presence of anthocyanin (arrows).

## Conclusion

This study showed, for the first time, the formation of SEs in *Plinia peruviana* from mature seeds. However, calluses obtained from leaf discs did not have embryogenic potential in the tested treatments. Through histological analyses, it was possible to differentiate calluses and PEMs.

2,4-D is important for somatic embryogenesis of this species, and the addition of Gln to the culture medium may increase the formation of PEMs and SEs. In contrast, BAP reduces the percentage of SE formation. The addition of 60 g L<sup>-1</sup> PEG 6000 to the medium produces good results for maturation of the obtained SEs.

### Acknowledgements

To CAPES for a grant given to SS, to CNPq (Process No. 309323/2014-2) for the research grant and stipend given to MQ and to Eileen Bagyary for the edition of the manuscript.

### References

- Akhtar, N. (2010). Evaluation of the efficiency of somatic embryogenesis in guava (*Psidium guajava* L.). *The Journal of Horticultural Science and Biotechnology*, 85(6), 556-562. DOI: 10.1080/14620316.2010.11512714
- Attree, S. M., & Fowke, L. C. (1993). Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. *Plant Cell, Tissue and Organ Culture*, 35(1), 1-35. DOI: 10.1007/BF00043936
- Bajpai, A., Kalim, S., Chandra, R., & Kamle, M. (2016). Recurrent somatic embryogenesis and plant regeneration in *Psidium guajava* L. *Brazilian Archives of Biology and Technology*, 59(1), 1-12. DOI: 10.1590/1678-4324-2016150170
- Cangahuala-Inocente, G. C., Silveira, V., Caprestano, C. A., Floh, E. I. S., & Guerra, M. P. (2014). Dynamics of physiological and biochemical changes during somatic embryogenesis of *Acca sellowiana*. *In Vitro Cellular & Developmental Biology - Plant*, 50(2), 166-175. DOI: 10.1007/s11627-013-9563-3
- Canhoto, J. M., & Cruz, G. S. (1996). Histodifferentiation of somatic embryos in cotyledons of pineapple guava (*Feijoa sellowiana* Berg). *Protoplasma*, 191(1), 34-45. DOI: 10.1007/BF01280823
- Canhoto, J. M., & Cruz, G. S. (1994). Improvement of somatic embryogenesis in *Feijoa sellowiana* Berg (Myrtaceae) by manipulation of culture media composition. *In Vitro Cellular & Developmental Biology - Plant*, 30(1), 21-25. DOI: 10.1007/BF02632115
- Canhoto, J. M., Lopes, M. L., & Cruz, G. S. (1999). Somatic embryogenesis and plant regeneration in myrtle (Myrtaceae). *Plant Cell, Tissue and Organ Culture*, 57(1), 13-21. DOI: 10.1023/A:1006273128228
- Carlsson, J., Svennerstam, H., Moritz, T., Egertsdotter, U., & Ganeteg, U. (2017). Nitrogen uptake and assimilation in proliferating embryogenic cultures of Norway spruce – Investigating the specific role of glutamine. *PLoS ONE*, 12(8), 1-18. DOI: 10.1371/journal.pone.0181785
- Cassol, D. A., Wagner Júnior, A., Pirola, K., Dotto, M., & Citadin, I. (2015). Embalagem, época e ácido indolbutírico na propagação de jabuticabeira por alporquia. *Revista Brasileira de Fruticultura*, 37(1), 267-272. DOI: 10.1590/0100-2945-011/14
- Corredoira, E., San-José, M. C., & Vieitez, A. M. (2012). Induction of somatic embryogenesis from different explants of shoot cultures derived from young *Quercus alba* trees. *Trees*, 26(3), 881-891. DOI: 10.1007/s00468-011-0662-7
- Cruz, G. S., Canhoto, J. M., & Abreu, M. A. V. (1990). Somatic embryogenesis and plant regeneration from zygotic embryos of *Feijoa sellowiana* Berg. *Plant Science*, 66, 262-270. DOI: 10.1016/0168-9452(90)90212-7
- Dal Vesco, L. L., & Guerra, M. P. (2001). The effectiveness of nitrogen sources in *Feijoa* somatic embryogenesis. *Plant Cell, Tissue and Organ Culture*, 64(1), 19-25. DOI: 10.1023/A:1010635926146
- De-la-Peña, C., Nic-Can, G. I., Galaz-Ávalos, R. M., Avilez-Montalvo, R., & Loyola-Vargas, V. M. (2015). The role of chromatin modifications in somatic embryogenesis in plants. *Frontiers in Plant Science*, 6(635), 1-15. DOI: 10.3389/fpls.2015.00635
- Duarte, O., & Paull, R. E. (2015). Myrtaceae. In O. Duarte, & R. E. Paull (Eds.), *Exotic fruits and nuts of the new world* (p. 51-95). Wallingford, UK: CABI Publishing.
- Fehér, A. (2005). Why somatic plant cells start to form embryos? In A. Mujib, & J. Samaj (Eds.), *Somatic embryogenesis, vol. 2, Plant cell monographs* (p. 85-101). Heidelberg, GE: Springer.
- Fraga, H. P. F., Vieira, L. N., Caprestano, C. A., Steinmacher, D. A., Micke, G. A., Spudeit, D. A., ... Guerra, M. P. (2012). 5-Azacytidine combined with 2,4-D improves somatic embryogenesis of *Acca sellowiana* (O. Berg) Burret by means of changes in global DNA methylation levels. *Plant Cell Reports*, 31(12), 2165-2176. DOI: 10.1007/s00299-012-1327-8

- Guerra, M. P., Cangahuala-Inocente, G. C., Dal Vesco, L. L., Pescador, R., & Caprestano, C. A. (2013). Micropropagation systems in Feijoa (*Acca sellowiana* (O. Berg) Burret). In M. Lambardi, E. A. Ozudogru, & S. M. Jain (Eds.), *Protocols for micropropagation of select economically-important horticultural plants* (p. 45-62). New York, US: Human Press.
- Guerra, M. P., Dal Vesco, L. L., Ducroquet, J. P. H. J., Nodari, R. O., & Dos Reis, M. S. (2001). Somatic embryogenesis in goiabeira serrana: genotype response, auxinic shock and synthetic seeds. *Revista Brasileira de Fisiologia Vegetal*, *13*(2), 117-128. DOI: 10.1590/S0103-31312001000200001
- Gutiérrez-Mora, A., González-Gutiérrez, A. G., Rodríguez-Garay, B., Ascencio-Cabral, A., & Li-Wei, L. (2012). Plant somatic embryogenesis: some useful considerations. In K. Sato (Ed.), *Embryogenesis* (p. 229-248). London: UK, InTech.
- Isah, T. (2016). Induction of somatic embryogenesis in woody plants. *Acta Physiologiae Plantarum*, *38*(5), 1-22. DOI: 10.1007/s11738-016-2134-6
- Jalali, M. A., Sirmandi, H. B., & Hatamzadeh, A. (2017). Effects of carbohydrate source and polyethylene glycol on maturation and germination of somatic embryos in walnut (*Juglans regia* L.). *Journal of Crop Science and Biotechnology*, *20*(1), 29-35. DOI: 10.1007/s12892-016-00089-1
- Joshi, R., & Kumar, P. (2013). Regulation of somatic embryogenesis in crops: a review. *Agricultural Reviews*, *34*(1), 1-20.
- Karnovsky, M. J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *Journal of Cell Biology*, *27*(2), 137-138.
- Kumar, V., & Van Staden, J. (2017). New insights into plant somatic embryogenesis: an epigenetic view. *Acta Physiologiae Plantarum*, *39*(194), 1-17. DOI: 10.1007/s11738-017-2487-5
- Lloyd, G., & McCown, B. H. (1980). Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. *Combined Proceedings International Plant Propagators' Society*, *30*, 421-427.
- LoSchiavo, F., Pitto, L., Giuliano, G., Torti, G., Nuti-Ronchi, V., Marazziti, D., ... Terzi, M. (1989). DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. *Theoretical and Applied Genetics*, *77*, 325-331. DOI: 10.1007/BF00305823
- Merkle, S. A., Parrott, W. A., & Flinn, B. S. (1995). Morphogenic aspects of somatic embryogenesis. In T. A. Thorpe (Ed.), *In vitro embryogenesis in plants* (p. 155-204). Dordrecht, NE: Kluwer Academic Publishers.
- Misra, S., Attree, S. M., Leal, I., & Fowke, L. C. (1993). Effect of abscisic acid, osmoticum and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. *Annals of Botany*, *71*(1), 11-22. DOI: 10.1006/anbo.1993.1002
- Möller, B., & Weijers, D. (2009). Auxin control of embryo patterning. *Cold Spring Harbor Perspectives in Biology*, *1*(5), 1-13. DOI: 10.1101/cshperspect.a001545
- Motoike, S. Y., Saraiva, E. S., Ventrella, M. C., Silva, C. V., & Salomão, L. C. C. (2007). Somatic embryogenesis of *Myrciaria aureana* (Brazilian grape tree). *Plant Cell, Tissue and Organ Culture*, *89*, 75-81. DOI: 10.1007/s11240-007-9210-y
- Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, *15*, 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x
- Nolan, K. E., & Rose, R. J. (2010). Plant regeneration – somatic embryogenesis. In M. R. Davey, & P. Anthony (Eds.), *Plant cell culture* (p. 39-60). Chichester, UK: Wiley-Blackwell.
- Pence, V. C. (1992). Abscisic acid and the maturation of cacao embryos *in vitro*. *Plant Physiology*, *98*(4), 1391-1395. DOI: 10.1104/pp.98.4.1391
- Pescador, R., Kerbaudy, G. B., Fraga, H. P. F., Hamasaki, R. M., Tavares, L. B. B., & Guerra, M. P. (2012). Dynamics of free and <sup>3</sup>H-labelled glutamine concentrations during zygotic and somatic embryogenesis of Feijoa [*Acca sellowiana* (O. Berg.) Burret]. *Journal of Horticultural Science and Biotechnology*, *87*(6), 583-587. DOI: 10.1080/14620316.2012.11512915
- Quiroz-Figueroa, F. R., Rojas-Herrera, R., Galaz-Avalos, R. M., & Loyola-Vargas, V. M. (2006). Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell, Tissue and Organ Culture*, *86*(3), 285-301. DOI: 10.1007/s11240-006-9139-6

- Rai, M. K., Akhtar, N., & Jaiswal, V. S. (2007). Somatic embryogenesis and plant regeneration in *Psidium guajava* L. cv. Banarasi local. *Scientia Horticulturae*, *113*, 129-133. DOI: 10.1016/j.scienta.2007.02.010
- Rancillac, M., Klinguer, A., Klinguer, S., & Millet, B. (1996). Preliminary investigations on somatic embryogenesis from leaf discs of red oak (*Quercus rubra* L.). *Plant Growth Regulation*, *20*(1), 67-73. DOI: 10.1007/BF00024061
- Rudiyanto, R., Efendi, D., & Ermayanti, T. M. (2014). Somatic embryo germination of *Jatropha curcas* L in presence of sucrose and poly ethylene glycol (PEG). *Annales Bogorienses*, *18*(1), 35-43. DOI: 10.14203/ann.bogor.2014.v18.n1.35-43.
- Shang, H., Liu, C., Zhang, C., Li, F., Hong, W., & Li, F. (2009). Histological and ultrastructural observation reveals significant cellular differences between *Agrobacterium* transformed embryogenic and non-embryogenic calli of cotton. *Journal of Integrative Plant Biology*, *51*(5), 456-465. DOI: 10.1111/j.1744-7909.2009.00824.x
- Souza-Moreira, T. M., Moreira, R. D., Sacramento, L. V. S., & Pietro, R. C. L. R. (2008). Histochemical, phytochemical and biological screening of *Plinia cauliflora* (DC.) Kausel, Myrtaceae, leaves. *Revista Brasileira de Farmacognosia*, *20*(1), 48-53. DOI: 10.1590/S0102-695X2010000100011
- Tholakalabavi, A., Zwiazek, J. J., & Thorpe, T. A. (1994). Effect of mannitol and glucose-induced osmotic stress on growth, water relations and solute composition of cell suspension cultures of poplar (*Populus deltoides* var. *occidentalis*) in relation to anthocyanin accumulation. *In Vitro Cellular and Developmental Biology*, *30*, 164-170. DOI: 10.1007/BF02632208
- Von Arnold, S. (2008). Somatic embryogenesis (3rd ed.). In E. F. George, M. A. Hall, & G. J. De Klerk (Eds.), *Plant propagation by tissue culture* (p. 335-354). Dordrecht, NE: Springer.
- Von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., & Filonova, L. (2002). Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture*, *69*(3), 233-249. DOI: 10.1023/A:1015673200621
- Welander, M. (1988). Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. *Journal of Plant Physiology*, *132*(6), 738-744. DOI: 10.1016/S0176-1617(88)80238-4
- Wu, S. B., Dastmalchi, K., Long, C., & Kennelly, E. J. (2012). Metabolite profiling of jaboticaba (*Myrciaria cauliflora*) and other dark-colored fruit juices. *Journal of Agricultural and Food Chemistry*, *60*(30), 7513-7525. DOI: 10.1021/jf301888y
- Wu, S. B., Long, C., & Kennelly, E. J. (2013). Phytochemistry and health benefits of jaboticaba, an emerging fruit crop from Brazil. *Food Research International*, *54*(1), 148-159. DOI: 10.1016/j.foodres.2013.06.021
- Yang, X., & Zhang, X. (2010). Regulation of somatic embryogenesis in higher plants. *Critical Reviews in Plant Science*, *29*(1), 36-57. DOI: 10.1080/07352680903436291