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Micropropagation's Complete Protocol of Red Araçá (*Psidium cattleianum*, Myrtaceae) from Germinated Seeds *in vitro*

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Abstract

Red Araçá's (*Psidium cattleianum*) micropropagation processes have shown enormous potential both in terms of research and as a sustainable native resource to be used in the areas of food production, ecology, and pharmacology. Currently, however, despite that potential, research efforts involving this myrtaceae, native to the Brazilian Atlantic Forest, have been scarce. With that in mind, this study set out to establish micropropagation techniques that would allow the development of a feasible protocol to be used with Red Araçá, achieving its mircropropagation from *in vitro* germinated seeds. Different types of explants were tested for *in vitro* establishment. For the multiplication of nodal segments, different concentrations of BAP and IAA combinations were tested in an MS medium. Using the same medium, different concentrations of ampicillin were applied in order to determine its influence on the decontamination of the apical segments. The BAP and IAA combinations were also used to test their effects on the *in vitro* explants' development and rooting. During pre-acclimatization, survival of in vitro rooted plants was tested in a nebulizer chamber, using a commercial substrate and that same substrate mixed with washed sand (1:1). In essence, it was indeed possible to develop a complete protocol for the micropropagation of the Red Araçá from seedlings obtained by *in vitro* germination. The *in vitro* introduction of the Red Araçá was rather efficient, independently of the type of explants used. As the BAP and IAA concentrations increased, so did the *in vitro* seedlings' development (7 leaves explant^{-1}) and rooting (67%). Additionally, the *in vitro* rooted plants exhibited a high rate of survival (80%) in the pre-acclimatization phase, independently of the substrate used.

Keywords: red strawberry guava, Araza, Atlantic Forest, benzylaminopurine, *in vitro* explants, apical segments

1. Introduction

Red Araçá (*Psidium cattleianum* Sabine) is a species native to the Mata Atlântica (Atlantic Forest), a Brazilian ecosystem *hotspot* (RBMA, 2017; Myers et al., 2000), and can be found throughout the Brazilian territory (Vibrans, 2013), as well as in other ecosystems in the world (Tng et al., 2015).

Akin to other myrtaceae, the Red Araçá's research potential is of great importance to the food production, ecology, and pharmacology fields of study (Raseira, Antunes, Trevisan, & Gonçalves, 2004; Franzon, Campos, Proença, & Sousa-Silva, 2009; Kinupp, 2011). Because its fruit is juicy and contains large quantities of nutrients (Galho et al., 2007), the food industry can process it or use it *in natura*. Also, its leaves and fruit contain different

bioactive compounds (Silva, Rodrigues, Mercadante, & De Rosso, 2014), essential oils and volatile substances that play an important role in pharmacology (Marin et al., 2008). Additionally, its capacity to attract pollinators (Gressler, Pizo, & Morelatto, 2006) and produce food for the native fauna (H. G. Baker, I. Baker, & Hodges, 1998; Kuhlmann, 1975) makes it an attractive species to be used as a secondary tree in reforestation efforts of degraded areas (Embrapa, 2006).

The array of potential exhibited by the Red Araçá has drawn the attention of the Brazilian Ministry of the Environment, which included it in their list of food producing native species with immediate or future potential uses in tropical and subtropical ecosystems (Kinupp, 2011). It has also been identified as an important species in the apiculture industry (Falkenberg & Simões, 2011). In spite of its importance and potential in the above mentioned fields, research efforts involving the biological diversity, conservation, and *in vitro* propagation of this species are rather scarce (Pasqual, Chagas, Soares, & Rodrigues, 2012).

Germination is the classic propagation method for this species (Lorenzi, 2008; Raseira, Antunes, Trevisan, & Gonçalves, 2004). However, its seeds' rocklike consistency (*testa petrea*) and integumental impermeability (Da Silva, Gualtieri, Paula, & Paoli, 2013) makes the germination of the Red Araçá less homogeneous, reducing its physiological quality during storage (Cisneiro et al., 2003). This has led recent studies to concentrate on optimizing the *in vitro* production of the species through micropropagation processes (Ribeiro, Souza, Donini, & Schuch, 2007), using material obtained from an adult plant or from *in vitro* germination (Freire, Gardin, Baratto, & Vieira, 2017).

Research efforts involving the different micropropagation phases of the *P. cattleianum* are still rare in many aspects. Some studies involving *in vitro* introduction and multiplication have already been developed, and yielded evidence of problems with phenolic oxidation and high rates of microbial contamination (Freire, Oliveira, & Vieira, 2014; Rodríguez, 2013).

Furthermore, complete protocols for the *in vitro* propagation of other species of the *Psidium* genus, such as the Red Guava (*P. guajava*) (Liu & Yang, 2011), are already available and have been widely used in several fields in Brazil and around the world (IEA, 2017). All of this has made it quite clear that there is a need for further studies involving *P. cattleianum* micropropagation techniques, in order to better understand this genetic resource native to the Atlantic Forest. The purpose of this study, therefore, was to devise micropropagation techniques for the *in vitro* introduction, establishment, multiplication, rooting and pre-acclimatization of *P. cattleianum*, and develop a feasible protocol related to the specie.

2. Materials and Methods

This study used different experiments to test the establishment, multiplication, rooting and pre-acclimatization of Red Araçá from seedlings obtained by *in vitro* germination (see Figure 1).

2.1 General Conditions for the Experiments

The germination, establishment, multiplication and rooting experiments with Red Araçá were all performed at the Plant Tissue Cultures Laboratory belonging to the Experimental Station of the Agricultural Research and Rural Extension Company from Santa Catarina (Epagri), in the City of Caçador. There, the experiments were assembled in a plant growth chamber, set at a temperature of 25±2 ºC, where glass flasks (230 mL capacity) or test tubes (25 × 150 mm) were exposed to a 16-hour photoperiod, with a luminous intensity of 75 μmol m^{-2} s^{-1}, obtained using cool white fluorescent bulbs.

An MS medium was used (Murashige & Skoog, 1962), with all its mineral salts and vitamins, and supplemented by 30 g L^{-1} of sucrose and phytohormones, both dependent on each experiment. The pH factor was always adjusted to 5.8±0.05 before adding the solidifying agent [6 g L^{-1} of agar (Merck®)].

Different assessments of the explants' characteristics were performed after each experiment. Bacterial and filamentous fungal contaminations were quantified according to Scherwinski-Pereira (2010). Partially or totally darkened explants were considered oxidized, whereas the explants that were greenish in color or totally green, and had developed at least one bud or leaf were considered to be established. Rooting was assessed by visually analyzing the growth of adventitious roots, according to Grattapaglia and Machado (1998). The number of completely formed and expanded leaves were counted. The growth of the *in vitro* explants was assessed using the relative growth rate (RGR) established by Briggs, Kid, and West (1920), according to Equation 1:

$$TCR = [(LnC2 - LnC1)/(t2 - t1)] \quad (1)$$

where, *Ln* is the natural or Naperian logarithm for the length values obtained from the different assessments; and *C*2 and *C*1 represent the growth (in mm) recorded at the specific times *t*2 (final) and *t*1 (initial).

Results were assessed by a '*standard statistical test*' using analyses of variance (ANOVA) and accordingly verifying normality and homoscedasticity prerequisites based on the Shapiro-Wilk Normality Test and Bartlett's Test. Subsequently, the means were separated using the Tukey or Scott-Knott Tests ($p < 0.05$) for the qualitative variables and a study of regression for quantitative variables ($p < 0.05$). Software applications used included Microsoft Excel® 2013, R® v. 3.2.3, Assistat® 7.7 beta (Silva & Azevedo, 2009) and GraphPad Prism® v. 5.00. Different data analyses and data transformations are described within each experiment, when appropriate.

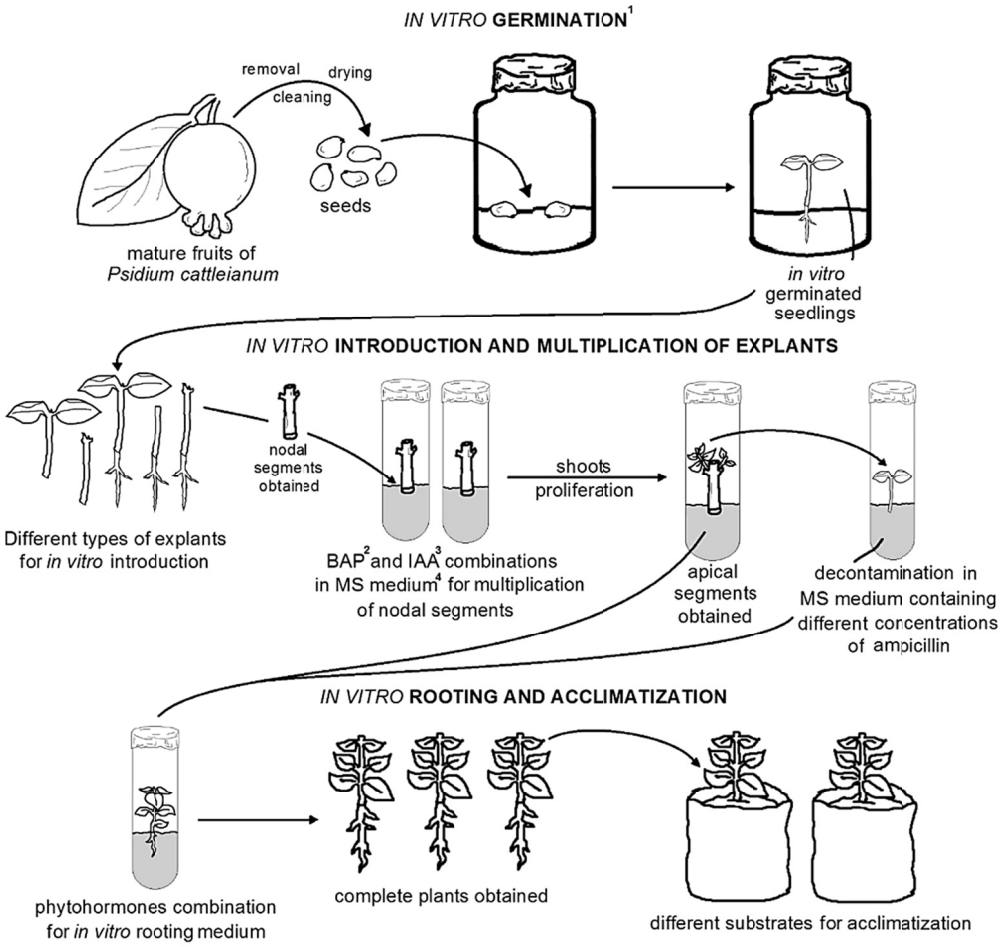

Figure 1. Flow diagram showing the different phases and experiments performed in order to develop an *in vitro* propagation protocol for the Red Araçá (*Psidium cattleianum*)

Note. [1]Accordint to Freire, Gardin, Baratto, and Vieira (2017). [2]Benzylaminopurine. [3]Indoleacetic acid. [4]MS Medium (Murashige & Skoog, 1962).

2.2 In Vitro Establishment of Red Araçá Seedlings

The process used to obtain the *in vitro* germinated seedlings was performed according to Freire, Gardin, Baratto, and Vieira (2017), and the experiment design was completely randomized, with five repetitions of five explants each. The five different treatments shown in Figure 2 were studied. The explants were established in an MS medium, supplemented by 2.0 mg L^{-1} of Benzylaminopurine (BAP) and 2.0 g L^{-1} of powdered activated carbon. They were then kept in the dark for the first five days, and under plant growth chamber conditions for the rest of the experiment. The following variables were studied: bacterial and fungal contamination percentages, oxidation percentage and establishment percentage, all on the 30th day after introduction into the culture medium.

Responses for the bacterial contamination, fungal contamination and oxidation variables were almost all zero; the establishment variable was, therefore, the only variable that was verified with an analysis of variance (ANOVA).

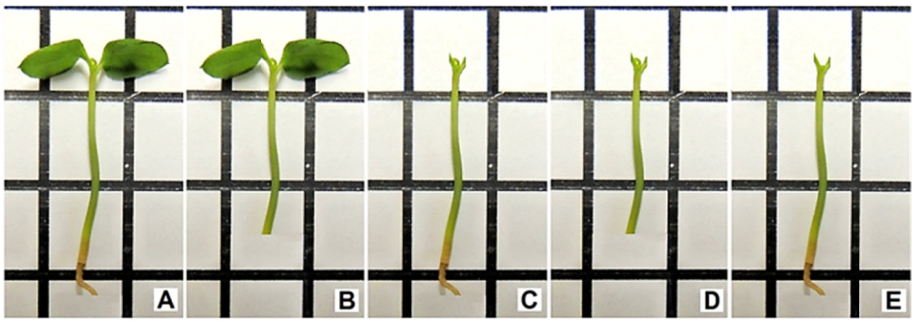

Figure 2. Types of explants in the micropropagation of *Psidium cattleianum* (Red Araçá) derived from *in vitro* germinated seedlings

Note. A, explants containing paracotyledons and 4-6 mm radicle roots; B, explants containing paracotyledons, without radicle roots; C, explants containing apical follicles and 4-6 mm radicle roots; D, explants containing only apical follicles, without radicle roots; and E, explants without apical buds, but with 4-6 mm radicle roots. Squares were dimensioned at 1 cm by 1 cm. Epagri's Plant Tissue Cultures Laboratory in Caçador.

2.3 Growth Phytohormones during the Morphogenesis of Nodal Shoot Segments

After *in vitro* establishment, different concentrations of BAP and Indoleacetic Acid (IAA) were tested in the multiplication and rooting phases. The experiment design was completely randomized, with five explants per repetition, seven repetitions per treatment and five different treatments, totaling 175 explants. The BAP and IAA concentrations used for the treatments were mixed in a 3:1 proportion (in mg L^{-1}) as follows: 1 - BAP (0), IAA (0); 2 - BAP (0.3), IAA (0.1); 3 - BAP (0.6), IAA (0.2); 4 - BAP (0.9), IAA (0.3); and 5 - BAP (1.5), IAA (0.5). The above treatments were applied to the nodal segments with lengths of 7 to 9 mm and containing 2 lateral buds, which were introduced into test tubes containing 8 mL of MS medium, supplemented by 250 mg L^{-1} of polyvinylpyrrolidone (PVP Vetec®) and 2 g L^{-1} of powdered activated carbon.

On the 15th and 45th days after the *in vitro* introduction, the bacterial contamination percentage, oxidation percentage and the number of buds per explant were assessed. Also assessed after the 45th day of *in vitro* introduction were the number of leaves per explant and the percentage of established explants.

2.5 Different Concentrations of Antibiotics in the Culture Medium

Some of the explants developed buds but, at the same time, showed bacterial contamination at its base. To avoid discarding these explants and also to find out if they could be decontaminated, a separate experiment was set up by adding antibiotics to the culture medium. Again, the experiment design was completely randomized, with two treatments (with ampicillin concentrations of 250 mg L^{-1} and 500 mg L^{-1} in the medium) comprised of 4 repetitions of 5 explants each. The MS medium used was supplemented by 1.5 mg L^{-1} of BAP, 0.5 mg L^{-1} of Naphthaleneacetic Acid (NAA), 0.5 g L^{-1} of powdered activated carbon, and 250 mg L^{-1} of PVP (Vetec®). The ampicillin was added to the post-autoclaved medium inside a laminar air flow cabinet, using solutions that were sterilized by microfiltration (with a Millex®-HV 0.45 μm microfilter). Subsequently, 10 mL of the culture medium was poured into each test tube.

Apical segments between 6-11 mm in length, and contaminated with bacterial colonies of the same aspect (circular-punctiform) and characteristics (rosy coloration containing similar DNA coding for the 16S rRNA region) were individually introduced into test tubes.

Evaluations were performed on the 15th and 35th day after the explants were introduced into the medium. The response variables assessed were as follows: bacterial and fungal contamination percentages; oxidation percentage; rooting percentage; number of fully formed leaves produced; and relative growth rate (RGR in mm mm^{-1} day^{-1}).

2.5 Growth Phytohormones during the Morphogenesis of Apical Segments

Non-contaminated buds obtained through *in vitro* explant regeneration were submitted to three treatments, comprised of 5 repetitions, with 3 explants each, in a completely randomized design.

The MS medium used was supplemented by 0.5 g L^{-1} of powdered activated carbon, 250 mg L^{-1} of PVP (Vetec®) and 125 mg L^{-1} of ampicillin (sterilized in a Millex®-HV 0.45 μm microfilter). The experiment used the following three different concentrations of phytohormones as its variables, which were added to the MS medium

prior to autoclaving: T1, 1 mg L^{-1} of BAP; T2, 2 mg L^{-1} of BAP; and T3, 2 mg L^{-1} of BAP and 0.5 mg L^{-1} of IAA. The culture medium (10 mL) was mixed into each of the test tubes. Apical segments between 8-12 mm in length were individually introduced into recipients containing the medium.

The following variables were collected on the 18th and 40th days: bacterial and fungal percentages; oxidation percentage; rooting percentage; number of fully generated leaves; and relative growth rate (RGR, in mm mm^{-1} day^{-1}).

2.6 Pre-Acclimatization of Micropropagated Red Araçá Plants

This experiment was performed at Epagri's Laboratory for Genetic Improvement of Temperate Climate Fruit Bearing Plants, in the City of Caçador.

In vitro multiplied and rooted Red Araçá plants were used, more specifically the ones that had between 8-10 fully formed leaves and a weighed average length of 17.40 mm. The experiment design was completely randomized, with 11 repetitions of one plant each, per treatment. Two types of substrate were tested in the transplantation: S1, a commercial substrate from Maxfértil® (with pine bark, ashes, vermiculite, sawdust and biostabilizers; electrical conductivity of 0.50±0.30 mS cm^{-1}; pH_{water} of 6.00±0.5 (at 25 °C); maximum humidity of 58.00%; water retention capacity of 90.00%; and density of 310 kg m^{-3}); and S2, the same commercial substrate from Maxfértil® mixed with sterilized washed sand, in a 1:1 mass proportion.

The plants were washed with distilled water and transferred to 17 × 17 × 22 cm plastic bags, containing the above mentioned substrates, already moistened with 100 mL of mineral water. All plants were kept in a climate controlled incubator chamber with a nebulizer control system (relative humidity between 85% and 97%), and a temperature of 25±1.0 °C (see Figure 3).

On the 20th and 42nd days after transplantation, survival of the plants was assessed, with the ones that showed development, i.e. were soft, had a greenish coloration, and produced and maintained leaves, being considered as survivors. The data was analyzed using binomial distribution and the Chi-square test (5% probability), taking the substrate and assessment date factors into consideration.

During the experiment, the plants were periodically irrigated (an average of 12 mL day^{-1}) and, ten days after the transplantation, 50 mL $plant^{-1}$ of MS medium was added (note that the medium had no sucrose or agar, and its pH was adjusted to 6.0±0.05).

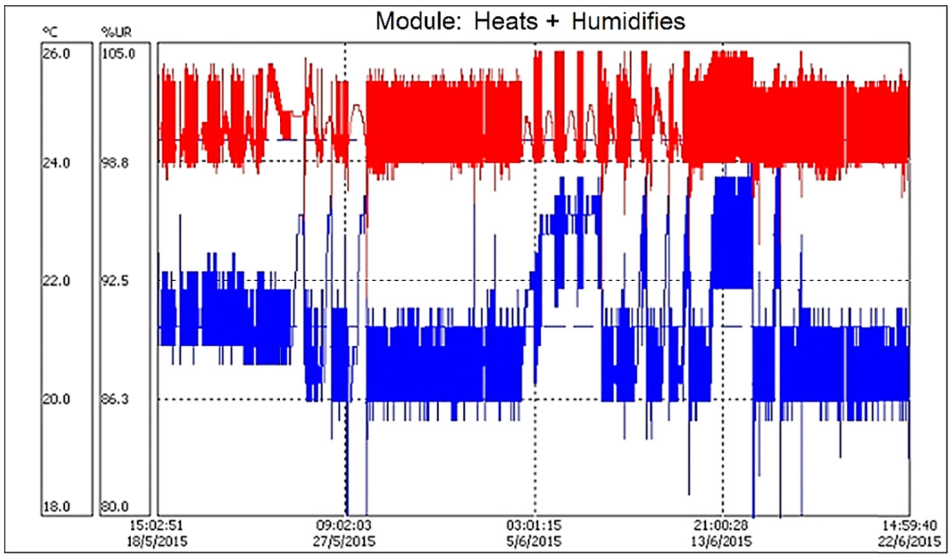

Figure 3. Nebulizer chamber's temperature and humidity conditions during the pre-acclimatization experiment using micropropagated *Psidium cattleianum* plants

Note. Epagri's Laboratory for Genetic Improvement of Temperate Climate Fruit Bearing Plants, in the City of Caçador.

3. Results and Discussion

3.1 In Vitro Establishment of Red Araçá Seedlings

Table 1 shows results for bacterial and fungal percentages, oxidation percentages and establishment percentages. As can be seen, results didn't vary for the different types of explant ($p > 0.05$), but were efficient in establishing the species *in vitro*.

Table 1. Bacterial and fungal contamination percentages, oxidation percentages, and establishment percentages for Red Araçá explants, 30 days after *in vitro* introduction of seedlings obtained by germination

Type of explant	Bact.[2] (%)	Fung.[3] (%)	Oxid.[4] (%)	Estab.[5] (%)
(A) Containing paracotyledons and radicle[1]	0	0	8	92[ns]
(B) Containing paracotyledons, without radicle	0	0	4	96
(C) Containing only apical follicles and radicle	0	0	0	96
(D) Containing only apical follicles, without radicle	8	0	0	92
(E) Without apical buds, but with radicle	0	0	4	96
CV (%)	-	-	-	11.51

Note. [1]Radicle root between 4-6 mm; [2]Bacterial contamination; [3]Fungal contamination; [4]Explant oxidation; [5]*In vitro* explant establishment. [ns] Not significant, based on the analysis of variance's F test.

Table 1 shows that only treatment D had bacterial contamination (8%) and not one of the types of explant tested had fungal contamination. All types of explant also exhibited low percentages of oxidation, and an average of about 95% establishment.

Differences between the types of explant were expected, especially considering the variations in size, and the presence or absence of paracotyledons and radicle roots. Such factors can influence, for example, the quantity of nutritional reserves present in the explants, which could affect *in vitro* establishment (Hartmann, Kester, & Davies Junior, 1990). Grattapaglia and Machado (1998) also determined that bigger explants with leaves have a better possibility of *in vitro* development. By the same token, Souza et al. (2007) used different sized explants (0.5 cm; 1.0 cm; 1.5 cm) during the *in vitro* introduction of the "pitangueira" plant (*Eugenia uniflora*, Myrtaceae), and determined that the bigger explants showed significantly higher results than their smaller counterparts, with approximately 83% having been established by the 45th day of cultivation.

Previous studies had already tested different methods of *in vitro* introduction for the *P. cattleianum*, but the results were less than satisfactory. Freire, Oliveira, and Vieira (2014) tested different asepsis methods (with ethanol 70% v/v and sodium hypochlorite 1.5%) on *P. cattleianum* explants obtained from stock plants that had gone through previous fungal treatment. These authors had problems with bacterial contamination (average of 50%, with 95% for the control group) and fungal contamination (average of 13.34%, with 55% for the control group). Similar data was observed by Souza, Schuch, and Silva (2006) who, when testing the introduction of *P. cattleianum* cv. "Irapuã" using semi-lignified and herbaceous shoots, found that the average percentage of bacterial and fungal contaminations were 89.68% and 46.85%, respectively.

Furthermore, the *in vitro* establishment of native Myrtaceae trees, such as the Red Araçá, also poses problems due to oxidation processes that take place in explants obtained from stock plants (Pasqual, Chagas, Soares, & Rodrigues, 2012). The high concentration of phenolic compounds in these species and the use of different disinfectants during the decontamination process increase oxidation and decrease *in vitro* survival rates (Grattapaglia & Machado, 1998). In general, these negative factors impair or make the subsequent phases of micropropagation unfeasible for different myrtaceae, as has been determined for the "cerejeira-do-mato" (*Eugenia involucrata* or cherry of the Rio Grande) (Golle, Reiniger, Belle, & Curti, 2013) and for the "pitangueira" (*Eugenia uniflora*) (Lattuada, 2010), for example.

Contrary to those efforts, in this study, the use of explants derived from *in vitro* germinated seedlings has proven a rather feasible technique to study the micropropagation capabilities of the Red Araçá. As a matter of fact, the percentages related to bacterial contamination and oxidation were virtually null, and no explant fungal contamination was detected after the 30th day of *in vitro* introduction (Table 1). Such data is of great importance, especially considering the fact that the Red Araçá is a myrtaceae tree with a high incidence of contamination and oxidation during *in vitro* introduction (George, Hall, & De Klerk, 2008).

When studying the micropropagation of Myrtaceae species, other authors have also used explants obtained from *in vitro* germinated seedlings. Souza (2010), for example, yielded high *in vitro* establishment and multiplication rates with "guabijuzeiro" (*Myrcianthes pungens*) explants obtained from *in vitro* germinated seeds. Similarly, Nascimento (2006) germinated "uvaieira" (*Eugenia pyriformis*) seeds and reported rather high establishment, multiplication, and rooting percentages for that species' explants.

3.2 Growth Phytohormones during the Morphogenesis of Nodal Shoot Segments

Table 2 shows results for *Psidium cattleianum* explants after 15 days *in vitro*.

Table 2. Bacterial contamination percentages, oxidation percentages, and number of buds per explant for *Psidium cattleianum* nodal segments cultivated in different concentrations of BAP and IAA, after 15 days *in vitro*

BAP (mg L^{-1})	IAA (mg L^{-1})	Bacterial Contamination (%)	Buds explant^{-1}	Oxidation (%)
0	0	77.14 a	0.51^{ns}	22.86^{ns}
0.3	0.1	97.14 a	0.31	17.14
0.6	0.2	74.29 a	0.29	28.57
0.9	0.3	85.71 a	0.14	34.29
1.5	0.5	28.57 b	0.57	20.00
CV (%)		24.80	81.99	71.05

Note. Averages appended by the same lower case letter within a column do not differ statistically from each other according to the Scott-Knott test, within a 5% significance level. ^{ns} Not significant.

The nodal segments used in this experiment were excised from *in vitro* introduced explants that remained over 90 days without visible contamination. Despite that, after the 12th day of the *in vitro* experiment, many of the segments (Table 2) showed bacterial contamination with the same appearance and coloration as was observed at the base of the explants, eventually developing towards the outside of the culture medium within the test tubes. Thus, a high bacterial contamination percentage ($p < 0.0001$) was observed (see Table 2), with the exception of the trial involving treatment T5 (1.5 mg L^{-1} of BAP + 0.5 mg L^{-1} of IAA), which yielded a value (28.57%) significantly lower than the rest.

Comparable to what was seen in this *P. cattleianum* study, other research efforts, such as the ones involving the *in vitro* establishment of "cerejeira-do-mato" (*Eugenia involucrata*) (Paim, 2011) and "camu-camuzeiro" (*Myrciaria dubia*) (Araújo, 2012), have detected the proliferation of bacterial contamination after a few weeks *in vitro*. According to these authors, such contaminations were very possibly due to the presence of endophytic microorganisms.

As stated by Camara, Willadino, and Albuquerque (2010), many *in vitro* plant cultures can synchronously develop bacterial growths during several of their micropropagation phases. This can occur even if the cultures were apparently pure after several sub-cultivations, in general due to the presence of endophytic or fastidious microorganisms. Therefore, the bacterial contaminations that occurred during this study are believed to have evolved from latent endophytic microorganisms or low concentrations of these microorganisms that are present in the *P. cattleianum* tissues that later proliferate in the culture medium.

On the 15th day after the *in vitro* introduction of the explants, the number of buds ($p = 0.0724$) and oxidation percentage ($p = 0.3804$) showed no significant effects for the different treatments, with values ranging from 0.14 and 0.57 for buds per explant, and from 17.14% and 34.29% for oxidation (Table 2). An assessment on the 45th day of contact with the culture medium, however, showed a significant effect on the bacterial contamination percentages ($p < 0.0001$), oxidation percentages ($p = 0.0020$) and *in vitro* establishment of the explants ($p = 0.0012$), as can be seen on Figure 4.

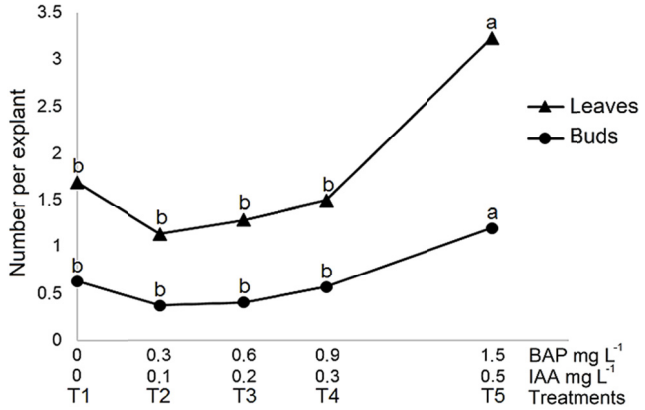

Figure 4. Bacterial contamination percentages, oxidation percentages, and *in vitro* establishment percentages for Red Araçá (*Psidium cattleianum*) nodal segments after 45 days of cultivation in an MS culture medium, with different concentrations of BAP and IAA

Note. Averages indicated by the same letter in the same variable do not differ among themselves by the Scott-Knott test at 5% significance level.

After 45 days *in vitro*, no significant differences were detected in terms of bacterial contamination percentages, oxidation percentages and establishment rates for treatments T1 thru T4 (up to 0.9 mg L^{-1} of BAP and 0.3 mg L^{-1} of IAA). These treatments, however, were quite different from the T5 treatment for all assessed variables. One can also note a tendency for bacterial contamination percentages and oxidation percentages to decrease, and establishment rates to increase with an increase of phytohormone concentrations in the culture medium. The results show that the addition of the highest tested dosage (T5, or 1.5 mg L^{-1} of BAP and 0.5 mg L^{-1} of IAA) to the culture medium is the best treatment for the *in vitro* multiplication of *P. cattleianum*, evidenced by its lowest bacterial contamination percentage (42.86%) and oxidation percentage (31.43%), as well as the highest percentage of *in vitro* nodal segments establishment (51.43%) (Figure 4).

Data gathered by Rodríguez (2013), Souza, Fior, Souza, and Schwarz (2011), and Lattuada (2010) are different than what was observed during this study. Rodríguez (2013) tested different combinations of BAP (between 0 mg L^{-1} and 1.5 mg L^{-1}) and NAA (between 0 mg L^{-1} and 0.5 mg L^{-1}) with *P. cattleianum* apical segments. As reported by Rodriguez, an increase in the concentration of the above mentioned compounds resulted in negative effects, such as a high mortality rate (average of 70%) and an increase in the malformation of the explants. Souza, Fior, Souza, and Schwarz (2011) studied the effects of increasing concentrations of BAP (between 0 mg L^{-1} and 2 mg L^{-1}), determining that higher concentrations of the compound caused an increase in oxidation and a decrease in establishment rates of "guabijuzeiro" (*Myrcianthes pungens*, Myrtaceae) apical segments. Lattuada (2010) also detected phytotoxic effects during *in vitro* multiplication of "pitangueira" (*Eugenia uniflora*, Myrtaceae) nodal segments, which the author related to the high doses of BAP used. This last author determined that the explants' survival rate decreased with increasing concentrations of BAP (between 0 mg L^{-1} and 0.5 mg L^{-1}).

As was the case with bacterial contamination percentages, oxidation percentages, and *in vitro* establishment percentages, results for the *P. cattleianum* number of buds per explant ($p = 0.0006$) and number of leaves per explant ($p = 0.0052$) also showed significant variations for the different BAP and IAA treatments (Figure 5).

In essence, increases in BAP and IAA concentrations yielded an increase in number of leaves and number of buds per explant (Figure 5). The highest concentration of BAP and IAA (T5) resulted in the highest explant *in vitro* development, with 3.23 leaves and 1.20 buds per explant, quite different from the other treatments.

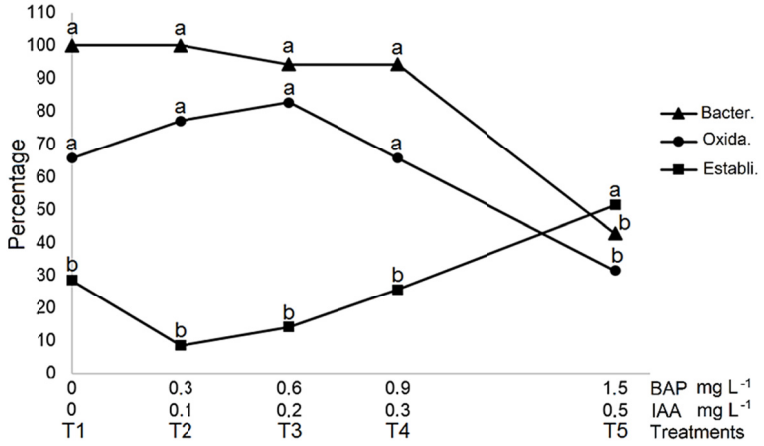

Figure 5. Number of leaves and number of buds per explant for Red Araçá (*Psidium cattleianum*) nodal segments after 45 days of *in vitro* cultivation in an MS culture medium, with different concentrations of BAP and IAA

Note. Averages indicated by the same letter in the same variable do not differ among themselves by the Scott-Knott test at 5%.

As reported by Grattapaglia and Machado (1998), and George, Hall, and De Klerk (2008), concentrations of cytokinins and auxins in the culture medium are among the factors that most affect *in vitro* explant regeneration and multiplication, which are influenced by the concentrations and the types of phytohormones used.

Concentrations of BAP similar to the ones used in this experiment also resulted in positive *in vitro* responses from other species. In a study with cedar (*Cedrela fissilis*), Amaral (2006), for example, concluded that a dosage of 5 μM of BAP (approximately 1.125 mg L^{-1}) yielded the best results in terms of bud production, regardless of the presence or absence of 0.5 μM of IAA or 0.5 μM of Indolebutyric Acid (IBA). That same concentration of BAP was also efficient for bud growth on "pitangueira" (*E. uniflora*) explants in the absence of auxins (Souza, Schuch, Donini, & Ribeiro, 2008).

For myrtaceae explants, however, higher concentrations of phytohormones have proven to be phytotoxic. Rai, Jaiswal, and Jaiswal (2009), for example, have determined that an increase of BAP concentration, up to 1.0 mg L^{-1}, also increases the number of buds during *in vitro* multiplication of the guava tree (*Psidium guajava*). When a higher concentration (2 mg L^{-1}) was used, a sharp decrease in the formation of buds was observed. Similarly, Ribeiro, Souza, Donini, and Schuch (2007) determined that a 5 μM dosage of BAP in a WPM medium yielded a higher number of leaves and buds per explant of *P. cattleianum* nodal segments than when cytokinin was absent; they also noticed, however, that those same variables decreased when 10 μM of BAP was added to the medium.

3.3 Different Concentrations of Antibiotics in the Culture Medium

Table 3 shows the results for all assessed variables after the addition of ampicillin to the culture medium.

Table 3. Bacterial and fungal contamination percentages, oxidation percentages, rooting percentages, number of leaves, and relative growth rate (RGR) for contaminated *Psidium cattleianum* explants after the 15th and 45th day of *in vitro* cultivation in an MS medium, with different concentrations of ampicillin

Ampicillin (mg L^{-1})	Bacterial Contamination (%)		
	15th day	45th day	Average
250	0.00	15.00	7.50 a
500	0.00	0.00	0.00 b
Average	0.00^{ns}	7.50^{ns}	
Ampicillin (mg L^{-1})	Fungal Contamination (%)		
	15th day	45th day	Average
250	0.00	0.00	0.00^{ns}
500	0.00	0.00	0.00^{ns}
Average	0.00^{ns}	0.00^{ns}	
Ampicillin (mg L^{-1})	Oxidation (%)		
	15th day	45th day	Average
250	0.00	10.00	5.00 b
500	15.00	25.00	20.00 a
Average	7.50^{ns}	17.50^{ns}	
Ampicillin (mg L^{-1})	Rooting (%)		
	15th day	45th day	Average
250	0.00	10.00	5.00^{ns}
500	5.00	20.00	12.50^{ns}
Average	2.50 b	15.00 a	
Ampicillin (mg L^{-1})	Number of Leaves explant^{-1}		
	15th day	45th day	Average
250	5.05	6.75	5.90^{ns}
500	4.75	5.50	5.12^{ns}
Average	4.90^{ns}	6.12^{ns}	
Ampicillin (mg L^{-1})	Relative Growth Rate (mm mm^{-1} day^{-1} explant^{-1})		
	15th day	45th day	Average
250	0.017	0.012	0.014^{ns}
500	0.019	0.011	0.015^{ns}
Average	0.018^{ns}	0.011^{ns}	

Note. For each variable, recorded values appended by the same letter, lower case within a row and upper case within a column, do not differ statistically from each other according to the Tukey test, within a 5% significance level. ^{ns} Not significant.

The concentrations of ampicillin added to the medium for the purposes of this study efficiently suppressed contaminations ($p = 0.0240$) and were not phytotoxic to the explants. Throughout the experiment, no bacterial contamination was detected when the medium was treated with 500 mg L^{-1} of ampicillin, with its contamination percentage being statistically inferior ($p = 0.0240$) to that obtained when treated with 250 mg L^{-1} of ampicillin (Table 3).

After 45 days of being kept in a medium with 500 mg L^{-1} of ampicillin, the explants went through a development phase that made them grow in length (RGR = 0.011 mm mm day^{-1}) and generated leaves (5.50 per explant), with 20% of them experiencing rooting, even though the medium was not specific for that process (Table 3). This was evidence that the cultivation of *P. cattleianum* explants, supposedly contaminated with bacteria, in a medium containing 500 mg L^{-1} of ampicillin resulted in a culture that was free of microorganism, also making its development feasible.

Pasqual, Dutra, Araújo, and Pereira (2010) reported that, when using culture tissue techniques, contaminated seedlings and explants are generally discarded; that, however, shouldn't always be the case, since there are microorganism controlling techniques that can be used. Pereira, Mattos, and Fortes (2003), for example,

determined that ampicillin, chloramphenicol, streptomycin, and tetracycline (between 32 mg L^{-1} and 256 mg L^{-1}) efficiently inhibited the growth of endophytic bacteria or contaminants in potato tissue cultures (*Solanum tuberosum*). Similarly, Leifert et al. (1991) cultivated the *Delphinium* sp. and also obtained cultures that were free of pathogens and showed *in vitro* growth increases when adding different antibiotics (gentamicin, streptomycin, carbenicillin, cephalothin, rifampicin and polymyxin) to their MS culture medium.

Besides having been quite effective in decontaminating the Red Araçá in this study, other studies and their results have also attested to the effectiveness of adding ampicillin in the culture medium. Araújo (2012), for example, tested different concentrations (100 mg L^{-1}, 200 mg L^{-1}, 300 mg L^{-1}, 400 mg L^{-1}, 500 mg L^{-1} and 600 mg L^{-1}) of ampicillin in the culture medium for the *in vitro* establishment of the "camu-camuzeiro" (*Myrciaria dubia*, Myrtaceae), which, after 30 days, yielded an establishment rate of 50% and 0% bacterial and fungal contaminations. Additionally, Pereira and Fortes (2003) reported that culture mediums with concentrations of up to 1,024 mg L^{-1} of ampicillin are not phytotoxic to the *in vitro* cultivation of potatoes (*Solanum tuberosum*) because it decontaminates the explants and does not prevent their development.

3.4 Growth Phytohormones during the Morphogenesis of Apical Segments

Treatments of the full strength MS medium with different concentrations of BAP and IAA phytohormones did not significantly affect oxidation percentages ($p = 0.4242$), rooting percentages ($p = 0.3910$), number of leaves per explant ($p = 0.2537$) and relative growth rate per explant (RGR) ($p = 0.4679$) (Table 4). Significant differences were only detected between dates of assessment for the variables rooting percentages ($p = 0.0004$) and number of leaves per explant ($p = 0.0002$), both being higher on the 40th day of *in vitro* cultivation (Table 4).

Table 4. Oxidation percentages, rooting percentages, number of leaves and relative growth rates (RGR) for Red Araçá (*Psidium cattleianum*) apical segments after the 18th and 40th day of *in vitro* cultivation in an MS medium containing different concentrations of phytohormones

Treatment (mg L^{-1})	Oxidation (%)		
	18th day	40th day	Average
(T1) 1.0 de BAP	6.67	13.33	10.00^{ns}
(T2) 2.0 de BAP	0.00	6.67	3.33^{ns}
(T3) 2.0 de BAP e 0.5 de IAA	0.00	6.67	3.33^{ns}
Average	2.22^{ns}	8.89^{ns}	
Treatment (mg L^{-1})	Rooting (%)		
	18th day	40th day	Average
(T1) 1.0 de BAP	20.00	53.33	36.67^{ns}
(T2) 2.0 de BAP	0.00	66.67	33.33^{ns}
(T3) 2.0 de BAP e 0.5 de IAA	6.67	33.33	20.00^{ns}
Average	8.89 b	51.11 a	
Treatment (mg L^{-1})	Number of Leaves explant^{-1}		
	18th day	40th day	Average
(T1) 1.0 de BAP	4.60	5.80	5.20^{ns}
(T2) 2.0 de BAP	4.60	6.67	5.64^{ns}
(T3) 2.0 de BAP e 0.5 de IAA	5.07	6.93	6.00^{ns}
Average	4.75 b	6.47 a	
Treatment (mg L^{-1})	Relative Growth Rate (mm mm^{-1} day^{-1} explant^{-1})		
	18th day	40th day	Average
(T1) 1.0 de BAP	0.0156	0.0131	0.0145^{ns}
(T2) 2.0 de BAP	0.0157	0.0124	0.0140^{ns}
(T3) 2.0 de BAP e 0.5 de IAA	0.0177	0.0152	0.0164^{ns}
Average	0.0163^{ns}	0.0136^{ns}	

Note. For each variable, recorded values appended by the same letter, lower case within a row and upper case within a column, do not differ statistically from each other according to the Tukey test, within a 5% significance level. ^{ns} Not significant.

The results of experiments performed in this study with respect to Red Araçá's *in vitro* rooting percentage are unprecedented in the existing literature. *In vitro* cultivated Red Araçá apical segments showed no restrictions as to the rhizogenesis process (Figure 6A), with an average rooting of 51.11% after the 4th day (Table 4). The highest rooting percentage, 66.67%, was attained with treatment T2, even in the presence of high osmotic pressure and a high concentration of cytokinin (2 mg L^{-1} of BAP) in the medium (full strength MS) (Table 4). On the 18th day *in vitro*, complete (rooted) plants had already been obtained (Figure 6A); and, between the 40th and the 60th day, a root system was already in place, with secondary root growths (Figure 6E). This rooting data is entirely relevant for research efforts involving *P. cattleianum* micropropagation, especially considering that one of the greatest difficulties mentioned in the existing literature about micropropagation protocols for species of trees, including the Myrtaceae, is the inefficiency of *in vitro* formation of adventitious roots (George, Hall, & De Klerk, 2008; Grattapaglia & Machado, 1998).

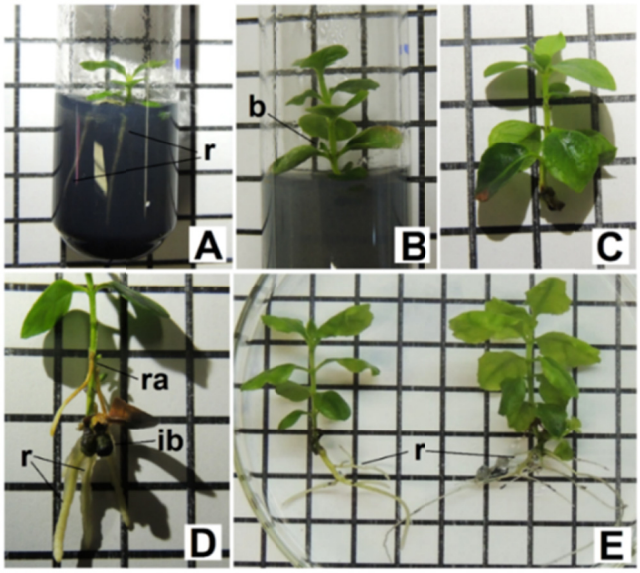

Figure 6. *In vitro* micropropagated *Psidium cattleianum* apical segments in MS medium containing BAP and IAA

Note. A, explant containing basal roots (r) after 18 days *in vitro*; B, explant with over two centimeters in length and lateral bud development (b) after 40 days *in vitro*; C, general appearance of non-rooted buds attained after 40 days; D, explant showing basal roots (r), adventitious roots at the stem's internodes (ra) and basal intumescence (ib); E, general appearance of fully formed plants attained after 60 days *in vitro*, with a root system and secondary roots. Squares were dimensioned at 1 cm by 1 cm.

Souza (2010), for example, reported an average rooting of only 3.5% for "guabijuzeiro" (*Myrcianthes pungens*) explants, when testing different concentrations (0.2 mg L^{-1}; 0.4 mg L^{-1} and 0.6 mg L^{-1}) of NAA and IBA auxins in a WPM medium. According to the author, a high percentage of callogenesis at the base of the explants was one of the factors that contributed to the resulting low rhizogenesis. Similarly, Lattuada (2010) tested different dosages of NAA (between 0 mg L^{-1} and 0.5 mg L^{-1}) in a WPM medium and assessed the *in vitro* formation of roots in "pitangueira" (*Eugenia uniflora*) explants, recording a maximum rooting percentage of approximately 10% when using an NAA concentration of 0.24 mg L^{-1}.

3.5 Pre-Acclimatization of Micropropagated Red Araçá Plants

On the 20th and 42nd days, no significant difference in survival percentages ($p = 0.5571$) was observed for the types of substrate used in the pre-acclimatization phase (Figure 7). Though not significant, it was determined that substrate S1 (Maxfértil®, a commercial substrate) yielded higher survival rates for both the 20th and the 42nd days, with 81.82% and 72.73%, respectively (Figure 7). It was also noticeable that substrate combinations can influence, or not, the acclimatization of micropropagated plants, depending on the species being studied and several other factors, such as phytohormonal variations, relative humidity, and luminous intensity (Pospíšilová, Synková, Haisel, & Semorádová, 2007).

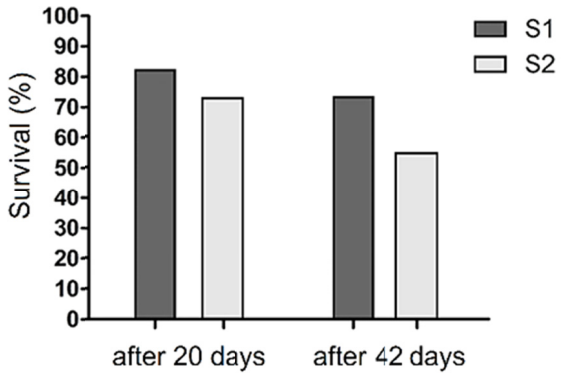

Figure 7. Survival percentage of micropropagated *Psidium cattleianum* plants in different substrates, during pre-acclimatization

Note. S1, commercial substrate from Maxfértil®; S2, commercial substrate from Maxfértil® mixed with sterilized washed sand, in a 1:1 mass proportion. Not significant by Chi-square test at 5% significance level.

Similar survival percentages were observed during the acclimatization of other micropropagated myrtaceae. Sospedra et al. (2003), for example, recorded an average survival rate of 75% for micropropagated "araçá-rasteiro" (*Psidium salutare*) plants. By the same token, Nascimento et al. (2008) reported an average survival rate of 77% for micropropagated "uvaieira" (*Eugenia pyriformis*) plants.

Up to the time of this study, there was absolutely no data related to acclimatization survival percentages of micropropagated *P. cattleianum* plants. The unprecedented data gathered here is evidence that transplantation of this species can indeed be done with the substrates used in this experiment, coupled with the use of a nebulizer chamber, or comparable equipment that can sustain high relative humidity (between 88% and 95%).

During the experiment, all surviving plants were soft and green, and developed new leaves within two to three weeks after being transplanted into the substrate (Figure 8F), while the non-surviving plants showed water stress, followed by foliar senescence and abscission. Among other factors, the water stress is believed to have been caused by stomas malformation or dysfunction (Costa, Pasqual, Scherwinski, & Castro, 2009), a thin or inexistent layer of protective wax, or even by an *in vitro* developed vascular system that was disconnected or less efficient (George, Hall, & De Klerk, 2008).

After a period in the nebulizer chamber, the pre-acclimatized plants were transferred to field conditions and monitored. Those plants formed well developed leaves and showed normal stem development, whose appearance after 180 days in the acclimatization substrate can be seen in Figure 8G.

This study made it possible to develop a complete protocol for the micropropagation of the Red Araçá (*Psidium cattleianum*) from seedlings obtained by *in vitro* germination, as can be seen in Figure 8. Results ascertained that approximately 95% of the introduced explants were actually established *in vitro* and showed contamination levels that were almost null, regardless of the type of explant used. The experiments also determined that explants can multiply in different concentrations of cytokinins and auxins, exhibiting *in vitro* rhizogenic capacity with low restrictiveness, which translated to approximately 70% rooting. These factors made it possible for the seedlings to completely regenerate from nodal and apical segments. Additionally, unprecedented results were obtained during pre-acclimatization, with a survival rate of approximately 80%.

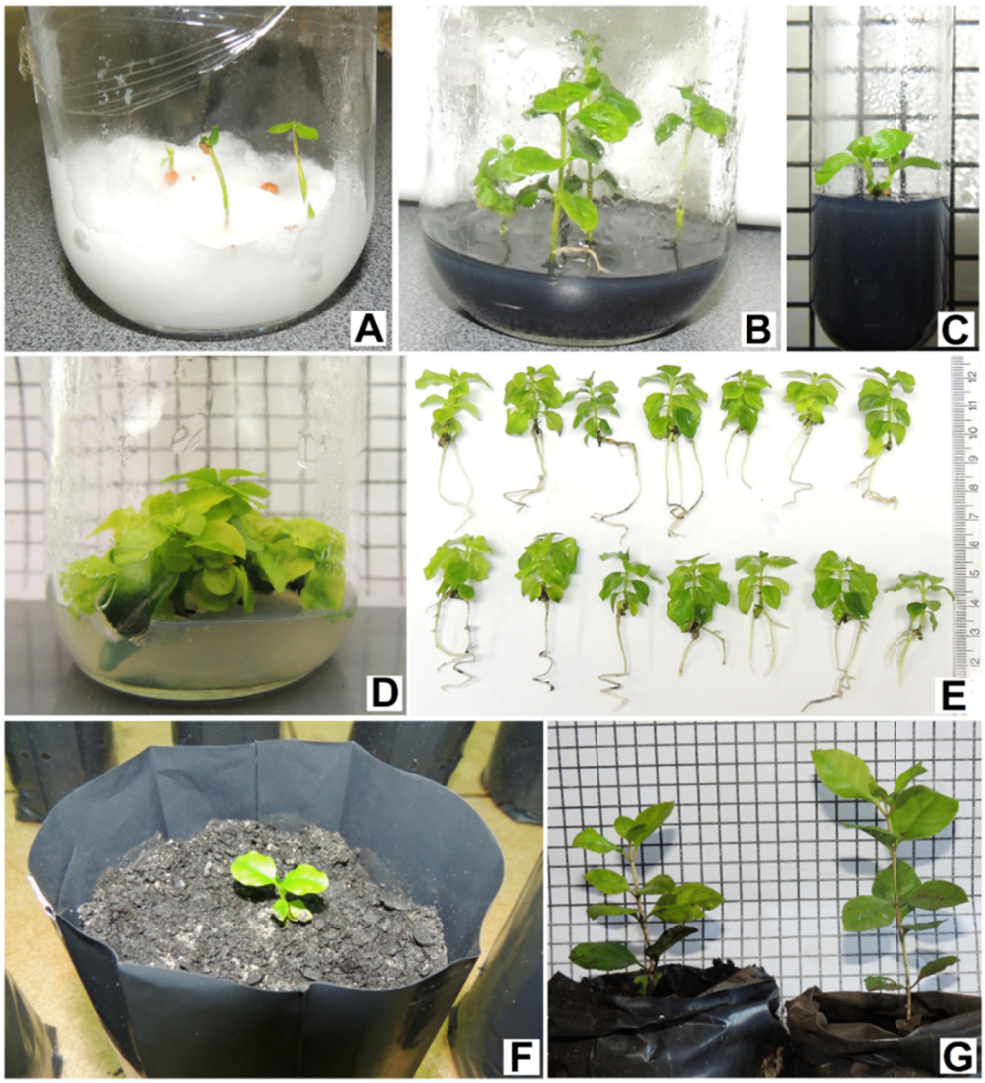

Figure 8. Red Araçá (*Psidium cattleianum*) *in vitro* propagation phases

Note. A, seedlings obtained from *in vitro* germination in hydrophilic cotton; B, plants elongated in MS medium; C, internode with bud growths in a medium containing ampicillin; D, bud multiplication in a medium containing BAP and IAA; E, rooted plants; F, plant in the process of pre-acclimatization, after 20 days in the substrate; G, acclimatized plants, after 180 days in the substrate. Squares were dimensioned at 1 cm by 1 cm. Epagri's Plant Tissue Cultures Laboratory in the City of Caçador.

4. Conclusions

When taking into account all the conditions for the development of the experiments in this study, the main conclusions were as follows:

- *In vitro* introduction of Red Araçá from seedlings obtained through germination is efficient, regardless of the presence or absence of paracotyledons and radicle roots in the explants;
- Even after 90 days of *in vitro* introduction, Red Araçá explants, though showing no signs of contaminants, can develop bacterial contaminations in the multiplication phase, possibly due to the presence of endophytic or fastidious microorganisms;
- Increasing concentrations of BAP and IAA in the MS medium enhance the development of nodal shoot segments;
- An MS medium with 500 mg L^{-1} of ampicillin can be used to decontaminate Red Araçá apical segments without phytotoxic effects and maintaining the explants' *in vitro* development and rooting rates;

- Even in mediums containing high osmotic pressure and cytokinins, *in vitro* Red Araçá apical segments still develop adventitious roots;
- It is possible to obtain complete (rooted) *in vitro* plants from apical segments in a full strength MS medium treated with different combinations of BAP and IAA;
- Pre-acclimatization of *in vitro* multiplied and rooted Red Araçá plants is efficient when using a commercial substrate, or a combination of commercial substrate and sand;
- In essence, it was indeed possible to develop a complete protocol for the micropropagation of the Red Araçá (establishment, multiplication, rooting, and acclimatization) from seedlings obtained by *in vitro* germination, just as it could be done for other Myrtaceae.

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