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In vitro establishment and multiplication of Japanese plum cv. América

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The *in vitro* plant tissue culture allows rapid propagation of interest species with high genetic, sanitary and physiological quality, which may contribute to increase the efficiency of plant production system. Therefore, the aim was to develop a protocol for *in vitro* establishment and multiplication of Japanese plum cv. América. The survival of basal buds was 83.33% in the establishment and it was possible to obtain maximum increase of 68.08% of shoot number. During the multiplication, the shoots length and the number of buds per shoots had maximum values of 1.34 cm and 14.16, whit 0.68 and 0.61 mg L^{-1} BA, respectively. In an elongation test, it was verified that using 0-10 mg L^{-1} GA_3 on medium with activated charcoal, there was linear increasing in shoot length from 1.14 to 3.50 cm. It was concluded that for cv. América, more basal than apical buds survive during the establishment. Concentrations between 0.25 through 0.50 mg L^{-1} BA are more suitable for *in vitro* multiplication and the MS medium with 1.0 mg L^{-1} GA_3 and 2.0 g L^{-1} activated charcoal promotes better shoots elongations.

Key words: *Prunus salicina* Lindl.; benzylaminopurine; gibberellic acid; activated charcoal.

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A micropropagação *in vitro* permite a rápida propagação da espécie de interesse com elevada qualidade genética, sanitária e fisiológica, podendo contribuir para melhorar a eficiência do sistema produtivo de mudas. Portanto, o objetivo foi desenvolver um protocolo de estabelecimento e multiplicação *in vitro* de ameixeira japonesa, cv. América. O percentual de sobrevivência das gemas basais foi de 83,33% no estabelecimento e foi possível obter um incremento máximo no número de brotações de 68,08%. Durante a multiplicação, o comprimento das brotações e número de gemas por brotação, apresentaram valores máximos de 1,34 cm e 14,16, nas concentrações de 0,68 e 0,61 mg L^{-1} de BAP, respectivamente. Nos ensaios de alongamento, verificou-se que em concentrações de 0-10 mg L^{-1} de AG_3 e em meio contendo carvão ativado, houve um incremento linear no comprimento das brotações de 1,14 a 3,50 cm. Concluiu-se que para a cv. América a sobrevivência de gemas basais é superior ao de apicais durante o estabelecimento *in vitro*. Concentrações entre 0,25 a 0,50 mg L^{-1} de BAP são mais adequadas para multiplicação *in vitro* e que o meio MS com 1,0 mg L^{-1} de AG_3 e 2,0 g L^{-1} de carvão ativado promove melhor alongamento das brotações.

Palavras-chave: *Prunus salicina* Lindl.; benzilaminopurina; ácido giberélico; carvão ativado.

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INTRODUCTION

The culture of the Japanese plum (*Prunus salicina* Lindl.) stands out in the Brazilian domestic market, mainly in Rio Grande do Sul (RS) and Santa Catarina (SC) where the winter conditions favor its cultivation (Bandeira et al., 2012). Besides adapting well to the edaphoclimatic conditions of the states of RS and SC the América cultivar is considered to be one of those preferred in the Southern brazilian market due to the organoleptic features of its fruit (Bandeira et al., 2011). However, the Japanese plum production is insufficient to supply the market, because of its limited area of cultivation among other factors, such as the susceptibility to leaf scald, caused by the bacterium *Xylella fastidiosa* Wells, which is disseminated mainly by contaminated propagative material (Eidam et al., 2012) and the gametophytic self-incompatibility (Takayama & Isogai, 2005).

The introduction of new species, genetic improvement and the production of healthy plants are factors which can contribute to increase the efficiency of the productive system. Plants in good healthy contribute to a worthy response to the technology applied in the orchard, helping to reduce costs and produce fruit of a high quality and productivity.

The *in vitro* culture enables rapid propagation of elite genotype, being able to produce clonal plants, with high genetic and sanitary quality, when compared with grafting and cutting propagation methods (Radmann et al., 2011). Moreover, the *in vitro* cultivation can be used to aid several other biotechnological studies, such as genetic transformation for features of interest.

The practical use of *in vitro* propagation of woody plants, mainly of the Prunoidea subfamily, requires study and adaptation of culture conditions, which are specific to each genotype, requiring the adjustment of specific protocols (Rodrigues et al., 2003). This technology is affected by several factors, including the explant type, the constitution of the culture medium, as well as the type and concentration of plant growth regulators (Radmann et al., 2011). The first stage of the process is the *in vitro* establishment, which starts selecting the most suitable explants and disinfesting those (Rocha et al., 2007b). In the *in vitro* culture, the nutritive media provide essential substances for growing and developing of the explant (Radmann et al., 2009b).

During the *in vitro* multiplication stage, the shoots formed can have a reduced length, damaging the percentage of rooting when transferred directly to the rooting medium, giving rise to low quality plantlets for the acclimatization phase (Radmann et al., 2011). According to Radmann et al. (2009b) the length of shoots *Prunus* micropropagated varies depending on the species or cultivar. However, growth can be stimulated by adding gibberellic acid (GA_3) (Wagner Júnior et al., 2008) and activated charcoal to the culture medium (Villa et al., 2006), in order to increase their length, due to cells expansion and elongation.

In summary the present study aimed to develop a protocol for *in vitro* establishment and propagation of the Japanese plum (*Prunus salicina* Lindl.) cv. América.

METHODOLOGY

Nodal segments with 0.5-0.7 cm in length, containing an axillary bud, of greenhouse-grown plants were employed as explants. Nodal segments were excised from branches of 10 months old plants of Japanese plum (*P. salicina* Lindl.) cv. América. Branches in full vegetative growth (45-50 cm in length) were collected and their leaves were detached. The first two nodes of the apical part of the branches were removed. These branches were then divided into two segments: from the third to the sixth node, counting from the apex to the base, characterizing the apical explants source and from the seventh to the tenth node, characterizing the basal explants source. The plants were treated with fungicide and bactericide (0.6 mg L^{-1} Benlate and 2.4 mg L^{-1} Agrimicine), every two days, during two weeks before branches harvested for *in vitro* cultures.

The apical and basal explants were immersed in ethylic alcohol (70%) for one minute and in sodium hypochlorite solution (1.5%) for twenty minutes to disinfestation and then rinsed tree times with autoclaved distilled water. Then the explants were inoculated in tubes containing 5 mL of a basic culture medium composed by MS salts (Murashige & Skoog, 1962) supplied with vitamins (1 mg L^{-1} thiamine, 0.5 mg L^{-1} pyridoxine, 0.5 mg L^{-1} nicotinic acid and 2 mg L^{-1} glycine), 30 g L^{-1} sucrose, 100 mg L^{-1} inositol, with the pH adjusted to 5.8 and solidified with agar (7 g L^{-1}), maintained in a growth room for seven days in the dark. Following this process the cultures were exposed to photoperiod of 16h (42 µmol m^{-2} s^{-1}) at 25±2ºC for 30 days. The parameters evaluated were the percentage of explants sprouted, oxidized and contaminated; the length of the initial shoots (cm), and the number of buds formed in each shoot. It was analyzed one hundred repetitions for each type of explant which was represented by a tube containing a nodal segment. The experimental design was unifactorial, entirely randomized and the data obtained was submitted to variance analysis and means test, by Tukey test ($p \leq 0.05$), with Winstat Software (Machado & Conceição, 2007).

For the shoot multiplication experiment, nodal segments of *in vitro* growing shoots with approximately 0.5 cm in length, were inoculated in bottles containing 25 mL of the basic culture medium described before and the treatments composed by different BA concentrations (0.25, 0.50, 0.75, 1.0 mg L^{-1} and a control without BA). The cultures were maintained in a growth room for 40 days, under the conditions described above. The parameters evaluated were the length of the shoots (cm), number of shoots per explants and buds per shoots as well as the dry mass of the shoots (mg). Buds with at least 0.5 cm in length were considered as shoots. The experimental design was unifactorial entirely randomized, with five concentrations of BA, containing eight repetitions, each one composed of a culture bottle with five explants. The data obtained was submitted to variance analysis and polynomial regression (Machado & Conceição, 2007).

As the shoots obtained in the multiplication stage had very short inter-node lengths and small leaves, they were used to run two independent elongation tests. In the first one, shoots (± 0.5 cm in length) cultivated in the

basic culture medium added with 0.3 mg L^{-1} BAP, for 30 days, were transferred to the same basic culture medium supplied with 2.0 g L^{-1} of activated charcoal and different gibberellic acid (GA_3) concentrations (0.25, 0.50, 0.75, 1.0 mg L^{-1} and a control without GA_3). The second test was performed in the same basic culture medium with higher GA_3 concentrations (1, 5, 10 mg L^{-1} and a control without GA_3), in the presence or absence of activated charcoal.

For both tests, the culture bottles were maintained in a growth room for 40 days, in the conditions described above. The parameters evaluated were the length of the shoots (cm), the number of shoots (developed buds at least 0.5 cm in length), the number of buds per explants and the dry mass of the shoots (mg). The experimental design, for both elongation experiments, was entirely randomized containing, in the first test, five repetitions, composed of a culture bottle with five explants and, in the second one, a 4 x 2 factorial design, with four concentrations of GA_3 and mediums with or without activated charcoal, containing four repetitions each represented by a culture bottle with six explants. The data collected was submitted to variance analysis and polynomial regression (Machado & Conceição, 2007). All the experiments were performed in duplicate.

RESULTS AND DISCUSSION

In vitro establishment

There was 76.19% and 83.33% survival of the apical and basal explants, respectively, and these values exceeded those recorded by Silva et al. (2003) in *in vitro* establishment of *Prunus* rootstocks (62% survival for the shoots apex and 58.8% for the lateral shoots).

Regarding the contamination rate, there was a higher percentage of apical explants (6.67% of fungus and 13.33% of bacterium) than basal ones contaminated (3.13% of fungus and 6.25% of bacterium), although there was a larger percentage of oxidation in basal explants (7.29%) compared to apical ones (3.81%). This response may be related to the higher lignification of the basal explants and higher presence of phenolic compounds, bearing in mind that different types of phenol present in the tissues, upon coming into contact with oxygen, are oxidized, leading to darkening and necrosis of the vegetable tissue (Rodrigues et al., 2003).

The contamination percentage observed in the present study exceeded those recorded by Rocha et al. (2007b), who in the *in vitro* establishment of *Prunus* rootstock cv. Tsukuba obtained 3.32% of explants contaminated and was under the percentages recorded by Silva et al. (2003), which varied 14.8% through 29.8% for the rootstocks Capdeboscq, GF677 and VP411. On the other hand, explants of six *Prunus* sp. Rootstock cultivars collected directly in the field presents contamination rates between 50% to 95.8% (Rodrigues et al., 2003).

Depending on the species, in woody fruit plants there are great difficulties for *in vitro* establishment, mainly due to the high percentage of contamination and oxidation of the explants (Chaves et al., 2005). However, the reduction of contaminated explants during this stage can be obtained by maintaining mother plants under controlled conditions, in a greenhouse, executing periodic phytosanitary treatment (Rocha et al., 2007b), as performed in the present study.

The length of the shoots and number of buds were different depending upon the type of explants used, the basal explants presented higher averages than the apical ones (Figure 1). Similar results were found in apple *in vitro* establishment, where the basal explants formed shoots with higher length and more number of shoots in relation to apical explants (Pereira & Fortes, 2001) according to the same authors, basal portions are more thickened showing greater amount of reserves accumulated in their tissues, which are used to support the initial growth for the formation of new shoots. After all, the *in vitro* establishment and initial growth of new shoots from nodal segments can be influenced by several factors such as the bud development stage, its location in the branch, as well as reserve substances and hormonal relationship (Rocha et al., 2005).

Thus, it can be considered that the results obtained are related to the larger quantity of reserves present in basal explants, as compared to apical ones (Pereira & Fortes, 2001), considering that the C/N (carbohydrate/nitrogen) ratio increases from the base to the tip of the branch, in basal buds occurs lower C/N ratio due to the higher concentration of nitrogen compounds which promotes cell division and growth plant organs (Souza et al., 2011).

In vitro multiplication

This is an important stage which aims at cloning the shoots in a large number, maintaining the genetic and sanitary integrity of the vegetable material. In this respect, in the multiplication experiment, there were differences related to the BA concentrations used, for all analyzed variables. A linear increase was found for the number of shoots per explant and dry mass of the shoots, varying from 1.28 to 4.01 and 14.34 to 21.80 mg, respectively (Figure 2A and 2B). This response is justified by the fact that the cytokinins stimulate cellular multiplication and proliferation of axillary buds, causing greater production of the aerial part and, consequently, more dry mass accumulation (Radmann et al., 2009a).

Nevertheless, the length of the shoots and number of buds per shoots have quadratic responses, with maximum values of 1.34 cm and 14.16, in the concentrations of 0.68 and 0.61 mg L^{-1} BA, respectively, with consequent reduction according to the increase in the concentration of cytokinin (Figure 2A and 2B). With these results it was possible to show that for *in vitro* multiplication of cv. América, concentrations of BA above 0.7 mg L^{-1} impair the elongation of the shoots and the forming of new buds per shoot.

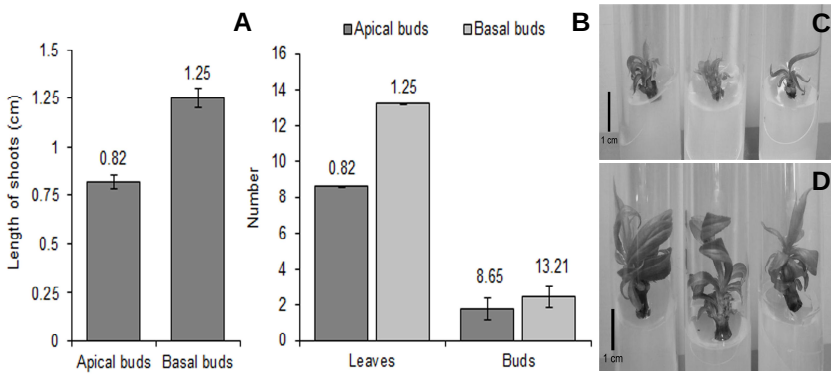

Figure 1 – *Length of shoots (A), number of leaves and buds formed in the shoots of apical and basal explants (B), where the bars represent the average standard error; and aspect of the initial shoots of Japanese plum (Prunus salicina Lindl.) cv. América, from apical buds (C) and basal buds (D), 30 days after in vitro establishment in basic culture medium.*

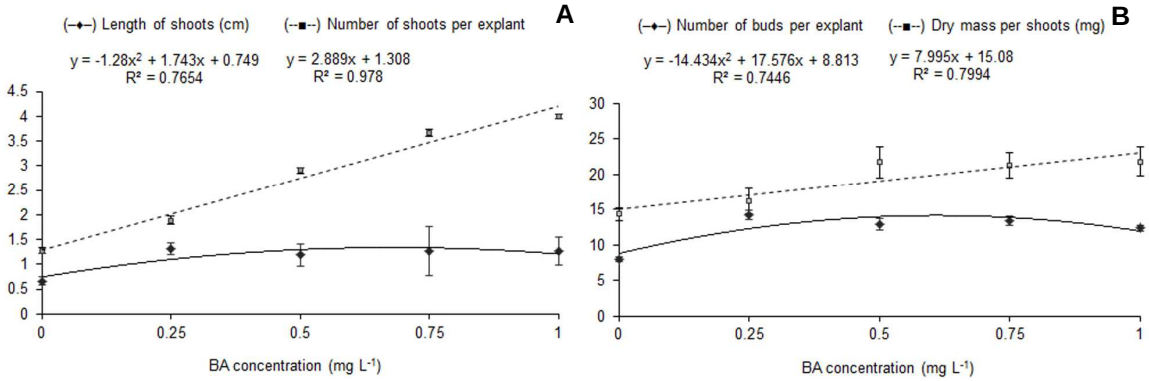

Figure 2 – *Length of the shoots and number of shoots per explant (A); number of buds and dry mass per shoots (B) of Japanese plum (Prunus salicina Lindl.) cv. América, at 40 days of in vitro cultivation related to the addition of different concentrations of BAP (0.25, 0.50, 0.75, 1.0 mg L^{-1} and a control without BA). The bars represent the average standard error.*

The cytokinins can stimulate the multiplication of the shoots until a determined concentration and may become harmful, depending on the studied genotype. This damaging effect was also observed in shoots of *Prunus dulcis* cv. Nonpareil, multiplied *in vitro* at concentrations above 0.67 mg L^{-1} BA (Channutapipat et al., 2003). On the other hand, the results obtained in the present study exceeded those reported by Rocha et al. (2007b), who found that the length of shoots of the 'Tsukuba' rootstock (0.5-0.4 cm) were smaller using concentrations of 0.4 to 0.8 mg L^{-1} BA. Thus, it can be concluded that the best concentration of cytokinin in *in vitro* multiplication medium depends upon the genotype, requiring adjustments of the protocol for each one.

In a concentration equal to or greater than 0.5 mg L^{-1} BA, the explants developed a high number of shoots per explant (2.19) and a high number of buds per shoot (12.88). However, the shoots formed in these conditions have short inter-node lengths, making them difficult to handle and diminishing the survival rate of the shoots of a reduced size, during the successive plantings out.

In general way, the cytokinins lead to the forming of aerial parts, but can damage the development of the cultures when in overdoses, due to the shortening of the inter-node lengths and reduction in the size of the leaves, causing problems in the rooting phase (Chaves et al., 2005; Radmann et al., 2009a; Radmann et al., 2011). Although the goal of *in vitro* multiplication is to produce as many shoots as possible, in the amount of time, the quality and homogeneity of the aerial parts should be taken into consideration (Chaves et al., 2005), since shoots with small elongation can compromise the success of the next steps of plant production, such as the rooting and acclimatization phases.

As in the present study, Pérez-Tornero et al. (2000) also observed that BA provided a high rate of *in vitro* multiplication of plum trees, but with a reduced shoot length. This fact would seem to be a common feature

for many species and cultivars of *Prunus* genus, obtaining, in the majority of the studies, explants smaller than 1.0 cm (Radmann et al., 2009a), considering that to facilitate the rooting of the shoots, they should have a length greater than or equal to 2.0 cm (Silva et al., 2003).

The shoots elongation is inhibited with BA increase in culture medium (Chaves et al., 2005). Even so, it is an essential component of the multiplication medium for *Prunus* spp., being necessary for the survival and ensuing multiplication of the shoots (Wagner Júnior et al., 2003). Moreover, the greater number of shoots per explant means that there is an increase in the demand for mineral salts and vitamins from the culture medium, being able to be a limiting factor for their elongation (Rocha et al., 2007a).

Due to the results obtained in the multiplication experiment, an additional stage - of elongation - became necessary as BA, in the highest concentrations tested, resulted in the forming of shoots with short internode lengths.

Shoots elongation

Two tests were performed to evaluate the elongation, in the first one there were differences only on the variable length of the shoots, showing linear increase accordingly the concentration of GA_3 (Figure 3), which varied from 1.12 to 1.82 cm, representing a raise of approximately 40% in length compared to the control group.

For the others variables analyzed there was no significant difference, in relation to the different concentrations of GA_3 added to the culture medium, showing an average of 9.98 buds and 1.12 shoots per explant, whereas the dry mass of shoots per explant was 20.09 mg.

In the second test, there was a significant interaction between the factors for the variable length of the shoots. A linear increase was found for the shoots cultivated in the medium containing activated charcoal, according to the increase of GA_3 concentration, varying from 1.14 to 3.50 cm, which represents an increase of 67% in the length of the explants. Nevertheless, the shoots that were maintained in the medium without activated charcoal had a quadratic response, reaching 2.41 cm with the addition of 6.74 mg L^{-1} GA_3 (Figure 4A).

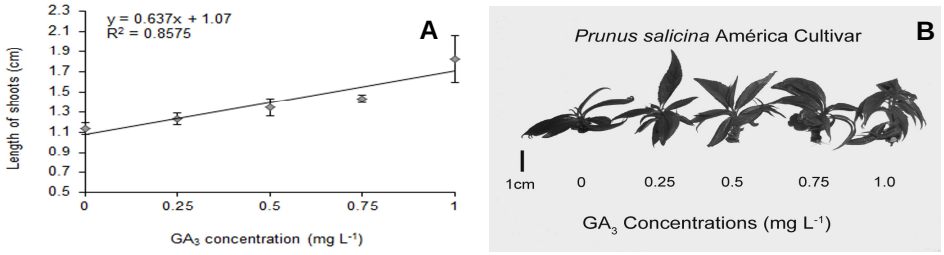

Figure 3 – *Length of shoots (A), where the bars represent the average standard error; and aspect of the shoots (B) of Japanese plum (Prunus salicina Lindl.) cv. América, at 40 days of in vitro elongation in MS medium supplemented with GA_3 (0.25, 0.50, 0.75, 1.0 mg L^{-1} and a control without GA_3) with activated charcoal.*

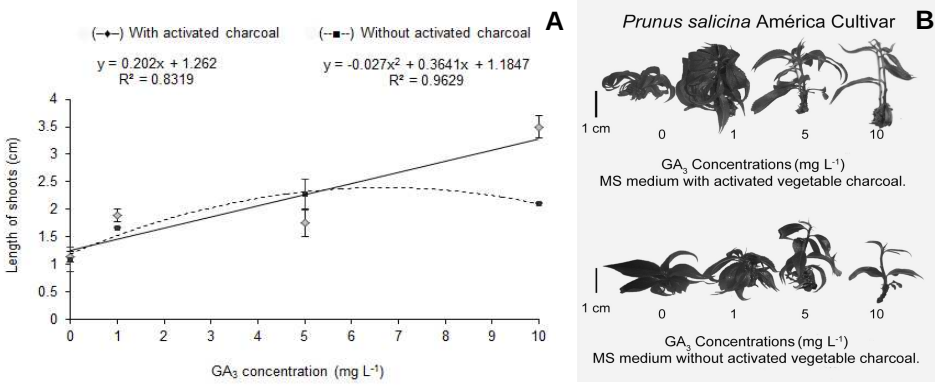

Figure 4 – *Length of shoots (A), where the bars represent the average standard error; and aspect of the shoots (B) of Japanese plum (Prunus salicina Lindl.) cv. América, at 40 days of in vitro elongation in basic culture medium supplemented with GA_3 in concentrations of 1, 5, 10 mg L^{-1} and a control without GA_3, with or without activated charcoal.*

The activated charcoal added to the culture medium functions as an absorber of the products coming from the vegetable metabolism, as well as from the plant growth regulators, minerals and vitamins added to the culture medium (Thomas, 2008), it has the capacity to retain these components, making them gradually available for the shoots, which can explain the greater elongation of the shoots in the medium containing activated charcoal. In contrast, when the shoots were cultivated in the absence of activated charcoal, they reached the maximum length of 2.41 cm, decreasing afterwards, showing that concentrations above 6.74 mg L^{-1} GA_3, in the absence of activated charcoal were harmful for this feature (Figure 4A).

As in the present work, several studies reported the beneficial effect of the activated charcoal upon the elongation of shoots for numerous species of fruit plants, being employed in *in vitro* multiplication of grapevines, plums, raspberrys, strawberrys, apples, pineapples and banana plants (Villa et al., 2006).

In the second elongation test, the number of shoots per explant, buds per shoots, as well as the dry mass of the shoots formed per explant, did not show any differences in relation to the factors analyzed. An average of 1.34 shoots, 10.01 buds and 16.53 mg of dry matter per shoot were recorded.

Even having led to a significant increase in the length of the shoots, concentrations equal to or greater than 5 mg L^{-1} GA_3 were harmful, they caused foliar abscission and with 10 mg L^{-1}, it was also possible to observe apical necrosis of the shoots (Figure 4B). These features were also observed by Mishra et al. (1999), in studies done with *Emblica officinalis* Gaertn., where 3.0 mg L^{-1} GA_3 caused defoliation of shoots, whereas only 1.0 mg L^{-1} led to appropriate elongation of the inter-node lengths. Likewise, Fráguas et al. (2004) reported the prejudicial effect of high concentrations of GA_3 (6 and 8 mg L^{-1}) in shoots of *Ficus carica* L. Despite having provided greater shoot elongation, due to excessive etiolation, which is not a desired effect, resulting in hyperhydricity of shoots, chlorotic leaves elongated and apical necrosis occurred.

Apical necrosis and foliar abscission are effects which can be caused by the excess of ethylene produced and accumulated during *in vitro* cultivation. Their synthesis is stimulated by auxins, the synthesis of which can in turn be stimulated by gibberellins, causing a cascading effect (Nepomuceno et al., 2007).

Another possible cause for the apical necrosis may arise from the poor calcium distribution, as this nutrient has problems with absorption and redistribution in the explant, due to its low mobility (Collier & Huntington, 1983). Moreover, the absorption is made difficult by the lack of roots in the explant and then, since calcium is translocated with water absorption, by the xylem vessels via mass flow, it becomes allocated mainly in the organs of greater transpiration. Once deposited in the mature leaves, it cannot be redistributed to the other parts of the plant, as young leaves (Millaway & Wiersholm, 1979). Considering that the transpiration rate is low in *in vitro* cultivated plants, due to the relatively high humidity inside the bottles, the supply of this mineral element for young tissues may have been inappropriate, and thus may be a factor related to the apical necrosis and foliar abscission (Collier & Tibbitts, 1983).

The results obtained in this study shown that *in vitro* establishment and multiplication can be performed efficiently from axillary buds inoculated in MS medium, after the phytosanitary treatment executed periodically in the greenhouse. The addition of BAP to concentrations between 0.25 and 0.50 mg L^{-1} led to a good rate of multiplication (about 13 buds per shoots and 2.19 shoots per explant), facilitating the handling of the shoots of this cultivar, with minimum elongation to be handled for other subcultures, but not for rooting. Thus, it was necessary to execute the shoots elongation stage with the addition of 1.0 mg L^{-1} AG_3 and 2.0 mg L^{-1} activated charcoal, allowing suitable growth of the shoots for rooting.

The importance to adapt different concentrations of plant growth regulators was demonstrated, as well the possible interactions between them and the salts present in the culture medium, confirming that the micropropagation process depends upon the genotype and/or cultivar (Rocha et al., 2007b).

CONCLUSION

In vitro establishment of basal explants of *P. salicina* cv. América, is greater than apical explants, for its multiplication concentrations between 0.25 and 0.50 mg L^{-1} BA are more appropriate, whereas concentrations above 0.5 mg L^{-1} BA induce a higher multiplication rate but leads to the formation of small shoots which make its use impractical in the next stages of the multiplication process. Furthermore, MS medium supplemented with activated charcoal, with 1.0 mg L^{-1} GA_3, is more suitable for shoots elongation.

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