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Optimal culture conditions for the initial development of *Ilex paraguariensis* A.St.-Hil. explants

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ABSTRACT

In the state of Rio Grande do Sul, Brazil, yerba mate (*Ilex paraguariensis* A. St.-Hil.) is an economically and culturally important species. Because of the difficulty in obtaining plantlets from seeds, micropropagation has emerged as an alternative that could increase production. This work aimed to evaluate the effect that treatment with 6-benzyladenine (BA), at concentrations of 2.2, 8.9, and 20 μM and associated with naphthaleneacetic acid (NAA, 0.5 μM), has on the *in vitro* growth and leaf emission of *I. paraguariensis* nodal segments cultivated in 0.5 X Murashige and Skoog (MS) medium or 0.5 X Woody Plant Medium (WPM). The most efficient treatment was MS medium with 8.9 μM BA+NAA, in which *I. paraguariensis* presented greater shoot elongation and higher leaf counts than in the other treatments. Therefore, use of the MS medium with 8.9 μM BA+NAA is recommended in order to increase efficiency in the initial development of *I. paraguariensis* explants.

Key words: Aquifoliaceae, growth regulators, naphthaleneacetic acid, vegetative micropropagation, yerba mate

Introduction

Ilex paraguariensis A.St.-Hil., popularly known worldwide as yerba mate, is cultivated in tropical and temperate regions. It belongs to the family Aquifoliaceae, which encompasses more than 500 recognized species. Being economically important in a few South American countries, including Brazil, Argentina, and Paraguay (Sansberro *et al*. 1999), this species is cultivated for medicinal and commercial purposes. In Brazil, its potential has been exploited mainly for aesthetic, phytotherapeutic, and culinary purposes (Biasi *et al*. 2009). In the Brazilian state of Rio Grande do Sul, it has not only economic value but also environmental value, because it is a highly cultivable species and an excellent alternative for production on small and large properties (Horner *et al*. 2001).

The *Ilex paraguariensis* preparation used in the production of the drink known as "mate" or "chimarrão" is produced from the grinding of its leaves and branches (Sansberro *et al*. 1999). It is also employed in drinks that stimulate the central nervous system, due to the presence of xanthic bases or alkaloids, such as caffeine and theobromine, which are also common in other cultivated plant species used for the same purpose, such as coffee, Indian tea, cocoa, and guarana (Coelho *et al*. 2001; Brenelli *et al*. 2003). In addition, *I. paraguariensis* contains antioxidant compounds, such as phenolic and tannic acids, and is capable of producing hepatoprotective, diuretic, hypocholesterolemic, anti-inflammatory, and anti-obesity effects (Bracesco *et al*. 2011). Studies of the aqueous extract of *I. paraguariensis* have reported that it can inhibit low-density lipoprotein oxidation in human plasma, suggesting that a compound within the extract increases this antioxidant capacity (Gugliucci & Stahl 1995; Gugliucci 1996).

In Brazil, the low productivity of *Ilex paraguariensis* populations and the existence of a great genetic and physiological variability among plantlets obtained from seeds result from the scarcity of practical selection and improvement programs for the species (Horner *et al*. 2001). Given the difficulty in obtaining plantlets from seeds, micropropagation presents itself as an alternative to the production of selected plants (Zaniolo & Zanette 2001). The main goal of adding growth regulators to tissue culture is to compensate for any deficiencies in endogenous hormones (Silva *et al*. 2006). Plant growth regulators include cytokinins, which are typically added to culture media to promote the formation of adventitious shoots, and auxins, which are added in order to stimulate the formation of primordial roots in tissues presenting rooting predisposition. In addition, the differentiation and growth control of *in vitro* tissue cultures is ascribed to the balance between auxins and cytokinins, which yield better development when used in the

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appropriate proportions (Rezende *et al*. 2011). The nutritive media employed in micropropagation are essential to *in vitro* development and are necessary complements for the organogenesis in plant tissues (Caldas *et al*. 1998). Nevertheless, some species present better development when these media are utilized with half of the salt concentrations, as is the case for grapevine rootstocks (Villa *et al*. 2007).

Over the last decades, tissue culture research has provided important advances in multiplication and development *in vitro*, especially of woody species. In studies of *Ilex dumosa*, the use of auxins and cytokinins has been shown to have a significant effect on the induction of shoots and buds (Luna *et al*. 2003). In addition, the interactions among auxins, cytokinins, and plant cells are quite complex, and additional studies on combinations of growth hormones are therefore necessary for the optimization of *in vitro* multiplication protocols (Jardim *et al*. 2010). Hence, this study attempted to assess the formation and growth of *I. paraguariensis* shoots in different culture media.

Material and Methods

Obtainment of plant material

The *Ilex paraguariensis* matrix plants employed in this experiment were obtained from seeds collected in the municipality of Putinga (28°57'28"S; 52°10'39"W), in the state of Rio Grande do Sul. The seeds were collected from a single individual that produces many buds after annual pruning and was found to have an excellent phytosanitary status. Those seeds were germinated in greenhouse conditions and cultivated for two years, the plants reaching 30-50 cm in height. The individual plants were then separated and isolated for treatment in a place without contact with the external environment, being subsequently utilized as matrix plants in the current study. The *in vitro* work was conducted in the Plant Propagation Lab of the UNIVATES University Center, in the city of Lajeado, also in Rio Grande do Sul, between April and October 2013. All cultures were kept in a growth chamber at 25±3°C, on a 16/8-h light/dark cycle. The luminous intensity was approximately 55 µmol photons m^{-2} s^{-1}, obtained via white fluorescent lamps.

Culture media and plant material disinfestation

Murashige and Skoog (MS) medium and Woody Plant Medium (WPM) were employed (Murashige & Skoog 1962; Lloyd & McCown 1981), both at half of the concentrations of salts (macro and micronutrients), maintaining the amount of vitamins present in the complete medium. To each medium, we added 30.0 g of sucrose, 1.0 g of activated carbon, and 7.0 g of agar (pH 5.7).

Plant material was pulverized with the fungicides captan (Orthocide, 0.16%; Arysta LifeScience, Salto de Pirapora, Brazil) and kasugamycin (Kasumin, 0.10%; Arysta LifeScience), applied during a sequence of six days, with an intermediate interval of two days. The sprouts were cut off the matrix plants in pieces approximately 4.0 cm long, keeping at least one node on each piece. Leaves were removed, and the material was washed under running water for 30 min. It was subsequently immersed for 20 min in a solution containing 0.5% captan and two drops of Tween 20. The explants were triple washed: first in distilled, autoclaved water; then by immersion in 70% ethanol for 60 s; and then again in distilled, autoclaved water. They were subsequently immersed for 20 min in a solution of 1.5% hypochlorite and three drops of Tween 20, after which they were again washed in distilled, autoclaved water.

A total of 372 explants (one for each 22 × 180 mm test tube, sealed with aluminum foil that was held firmly in place with elastic rings) were inoculated vertically, equally divided between the media containing the auxin naphthaleneacetic acid (NAA), at 0.5 µM, together with the cytokinin 6-benzyladenine (BA), at different concentrations: 0.5 X MS+2.2 µM BA; 0.5 X WPM+2.2 µM BA; 0.5 X MS+8.9 µM BA; 0.5 X WPM+8.9 µM BA; 0.5 X MS+20 µM BA; and 0.5 X WPM+20 µM BA.

Inoculation of nodal segments

The parts oxidized by asepsis were removed from the explants, maintaining the size of 1.0 cm. Those explants were inoculated in the culture media containing the various treatments. After 40 days, the number of leaves on each explant was counted and the height of the formed shoot was measured using a caliper. Contaminated or oxidized explants were removed during culture.

Statistical analysis

Comparisons among the treatments in each medium were made with one-way ANOVA, followed by Tukey's test ($p \leq 0.05$). The same treatments in different media were analyzed using Student's t-test ($p \leq 0.05$ and $p \leq 0.01$).

Results and discussion

We found that using BA at a concentration of 20 µM resulted in significantly lower shoot growth in both of the culture media tested (Fig. 1). In studies of *Annona glabra* L., taller shoots were obtained at 2.2 µM of BA (Oliveira *et al*. 2008). The addition of BA has been found to increase the number and length of shoots of *Ilex paraguariensis* explants at concentrations up to 4.4 µM (Horbach *et al*. 2011). Higher concentrations of BA have promoted the development of shoots in coffee cultivars (Jesus *et al*. 2010).

Contrary to what has been reported in previous studies of *Ilex paraguariensis* (Sansberro *et al*. 1999), our explants did not present multiple shoots when treated with BA, even at high concentrations (data not shown). This might

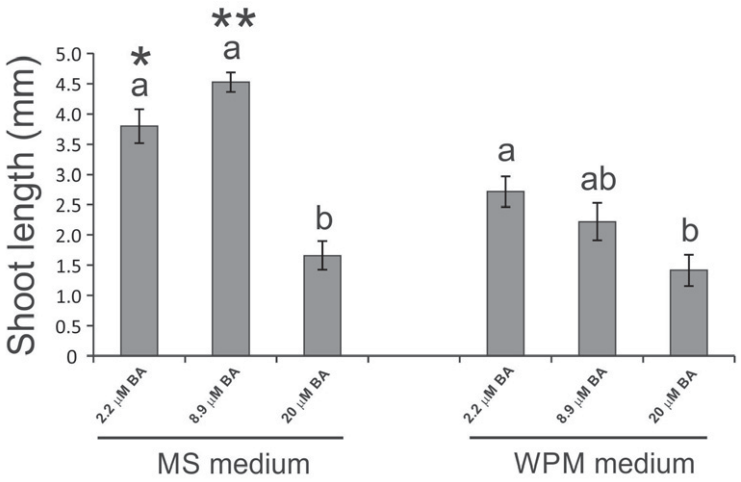

Figure 1. Length (mm) of shoots derived from nodal segments of *Ilex paraguariensis* explants cultured in 0.5 X Murashige and Skoog (MS) medium or 0.5 X Woody Plant Medium (WPM) treated with 0.5 µM naphthaleneacetic acid (NAA) and varying concentrations of 6-benzyladenine (BA).
All values represent the mean±standard error of six replicates. Values followed by different letters denote a statistically significant difference (between treatments) according to Tukey's test ($p \leq 0.05$).
*$p \leq 0.05$ between the two media (Student's t-test).
**p≤0.01 between the two media (Student's t-test).

be attributed to the fact that, after being separated from the matrix plants, some explants autonomously produce endogenous hormones. If the balance ratio between auxins/cytokinins is close to one, the tendency will favor callus formation; however, if it is lower than one, the regeneration of the aerial part will occur (Peres *et al*. 1997).

We also found that the culture medium affects the elongation of the aerial part, with taller shoots being obtained in MS medium than in WPM at two of the three BA concentrations tested (Fig. 1). Sansberro *et al*. (1999) obtained similar results when combining different concentrations of NAA and BA in 0.25 X MS medium. In studies of louro-pardo (*Cordia trichotoma* (Vell.) Arrab. ex Steud.), WPM was found to stimulate better development of gemmae than did MS medium, not only in terms of the number of shoots formed (63.3% of induced gemmae showing developed shoots) but also in terms of their length, which averaged 2.19 cm (Mantovani *et al*. 2001). In experiments involving canjerana (*Cabralea canjerana* (Vell.) Mart.) cultured in WPM and MS medium, the highest nodal segment multiplication rate was observed in MS culture medium supplemented with 2.7 µM BA (Rocha *et al*. 2007). These data underscore the concept that each species requires a certain concentration for *in vitro* cultivation. This is explained by the fact that each species has different demands for agents in the culture medium, and the WPM has a more diluted mineral formulation (lower ionic strength) than does MS. One of the main differences between the two media is in their nitrogen and potassium concentrations, which are lower in WPM (Zaniolo & Zanette 2001).

The number of leaves was also influenced by the BA concentration (Fig. 2). All explants presented more than one leaf, although it was noted that leaf development was inhibited at the highest BA concentration (20 µM) in both culture media. Horner *et al*. (2001) reported that an increase in the concentration of BA had no direct influence on the number of leaves, variations occurring randomly across the different treatments. In the present study, we found that the culture medium can affect leaf development, because the explants cultured in MS medium produced more leaves than did those cultured in WPM, at two of the three BA concentrations tested (Fig. 2). Leitzke *et al*. (2010) demonstrated that the number of leaves on cultivars of blackberry Xavante (*Rubus* sp.) increased in parallel with increases in the concentrations of BA and zeatin in MS medium, indicating a triple interaction between the type of culture medium, the type of cytokinin, and the concentration of growth regulators (Leitzke *et al*. 2010).

Conclusions

The explant development response was directly affected by the culture medium, use of MS medium being associated with greater height and higher leaf counts. Because the treatment 0.5 X MS medium+8.9 µM BA+0.5 µM NAA was associated with the tallest shoots and greatest leaf production, it is the treatment recommended for the optimal development of *Ilex paraguariensis* explants.

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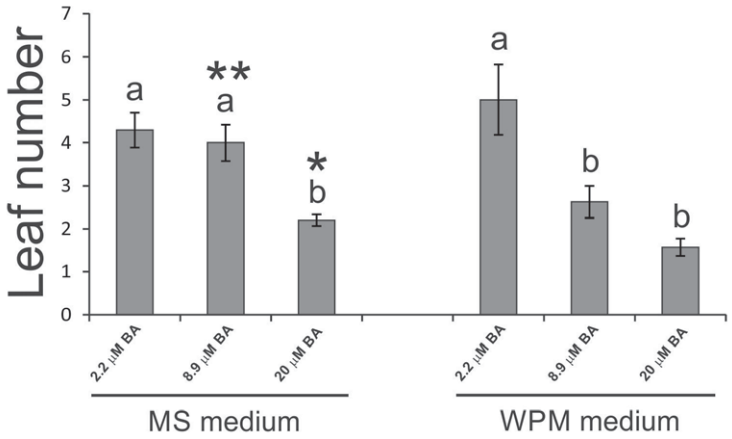

Figure 2. Leaf counts for *Ilex paraguariensis* explants cultured in 0.5 X Murashige and Skoog (MS) medium or 0.5 X Woody Plant Medium (WPM) treated with 0.5 µM naphthaleneacetic acid (NAA) and varying concentrations of 6-benzyladenine (BA).
All values represent the mean±standard error of six replicates. Values followed by different letters denote a statistically significant difference (between treatments) according to Tukey's test ($p \leq 0.05$).
*$p \leq 0.05$ between the two media (Student's t-test).
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