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Establishment and initial development *in vitro* of Yellow Ipe

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Abstract. Micropropagation protocol success clearly depends on the establishment and initial development *in vitro*. Following steps can only be performed after obtaining of aseptic cultures endowed with good plant vigor. This study aimed to evaluate different culture medium and agar concentrations in the establishment and early development *in vitro* yellow ipe. For both experiments the source of explants were shoot apical segments, obtained *in vitro* seed germination. For the first experiment was used a ½ WPM (diluted to 50% normal salt concentration) and different concentrations of agarose (4, 6, 8 and 10 g L^{-1}). For the experiment of culture nutrient medium treatments were: control (agar and water); MS medium; ½ and ¼ MS; WPM; ½ and ¼ WPM. After 30 days of culture were performed evaluations. For the agar concentration experiment evaluated the percentage of contamination, establishment, *in vitro* root formation and callus, number of roots and leaves. For the experiment of culture medium evaluated the percentage of establishment and *in vitro* formation of roots, number of roots and leaves. With increasing concentrations of agar added to the culture medium, it was found that there is a significant increase in the formation and in the number of roots per plant yellow ipe. The WPM and the diluted ½ and ¼ provided greater root formation and with better training, and more training leaves. We conclude that higher agar concentration, the lower the callus formation and the WPM and their dilutions are effective in establishing and initial *in vitro* development of yellow ipe.
Keywords: *Handroanthus chrysotrichus*, micropropagation, native tree species.

Introduction

The yellow ipe (*Handroanthus chrysotrichus* Mart. Ex A. DC. Mattos) is commonly used in urban forestry and landscape projects, mainly due to the beauty of its flowers and canopy size. Wood is moderately heavy, tough and long lasting, which makes high quality timber production. It also can be used in reforestation of degraded areas, riparian forests and permanent preservation areas (Lorenzi, 1992).

Propagation of this species is made by seeds, nevertheless seeds have germination and conservation problems (Oliveira et al., 2005). One of the alternative to improve vegetative propagation of yellow ipe is micropropagation technique that has been a useful tool for obtaining clones in many forest species of economic importance.

Micropropagation protocol success clearly depends on the phases of establishment and initial development *in vitro*, once the following stages can only be performed after obtaining of aseptic cultures with good vegetative vigor (George & Debergh, 2008).

Micropropagation comprising the phases of establishment, proliferation elongation *in vitro*, rooting *in vitro* or *ex vitro*, and acclimation (George & Debergh, 2008; Xavier et al, 2007). *In vitro* establishment starts with the management of the stock plant and the selection of the most suitable explants to use in micropropagation technique; this stage finishes with obtaining a pieces of tissue, free from obvious contamination and sufficiently adapted to the culture conditions *in vitro* (Grattapaglia & Machado, 1998).

Culture medium used in the micropropagation has essential substances to growth tissues; it supplies the metabolic, energy and structural requirement of tissues (Caldas et al., 1998). Salts and sugars which form the culture medium have not only a purely nutritional effect, but they also influence cellular growth and morphogenesis by osmotic properties (George, 1993).

Among the culture medium most employed in culture of plant tissues are MS medium (MURASHIGE, SKOOG, 1962) and "Woody Plant Medium" - WPM (Lloyd & Mccown, 1981). The WPM promotes good results in woody species propagation (Amaral, 2006; Perez-Parrón et al., 1994), because of its lower concentration of nitrogen and potassium,

besides lower total ionic strength (Harry & Thorpe, 1994). Apart from the composition of culture medium, its salts concentration in each stage of micropropagation have been studied (Forges, 2004; Souza et al., 2003). Appropriate salts concentration depends of species used (Guerra & Nodari, 2006).

In the culture medium, another important factor in establishing *in vitro* is the gelling agent. It is commonly used semi solid jellified with agar medium. The agar advantage is the ability to form a colloid when added to water; the medium become liquid at 100 °C, solid at 45 °C and keep stable at incubation temperatures. Due to the presence of organic and inorganic inhibitors associated with agar (Pierik, 1987), both the quality and the agar concentration affect the chemical characteristics of the culture medium and consequently the response of the explants *in vitro* (Romberge & Tabor, 1971).

Thus, this study aimed to evaluate different culture medium and agar concentrations in the establishment and initial development *in vitro* of yellow ipe apical stem segments.

Methods

Seeds used in the assay were purchased commercially and collected in eight matrix trees located in the São Paulo - Brazil. Initially, seeds were subjected to a pretreatment carried out by soaking in 100 g of a solution containing 1 g of Benomyl® (Benzimidazol) (w/v) for 24 hours. After this period, seeds were washed in running tap water for 10 minutes and rinsed in sterile water. Aseptically, the seeds were surface-sterilized in 70% (v/v) ethanol for 30 seconds followed by 2 % sodium hypochlorite (NaOCl) (v/v) for 15 minutes, and then rinsed three times with in sterile water.

Seeds were inoculated in flask containing 30 ml of WPM culture medium with half of original composition of salts, and more vitamins, sucrose (30 g L^{-1}), agar (7 g L^{-1}) and pH was adjusted to 5.7. The culture medium were previously sterilized for 15 minutes at 121 °C. Four seeds were placed in each flask.

After 30 days of *in vitro* culture, shoot apical segments with approximately 1 cm of length was isolated from seedlings and established in aseptic condition in two different experiments.

Experiment 1 - Agar Concentration

The treatments consisted of WPM medium of half original composition of salts gelled with different agar concentrations (4, 6, 8 and 10 g L^{-1}).

Experiment 2 - Culture medium

For this experiment seven different culture medium were used: control compound agar (1.0% (w/v)) and water; MS (full); ½ MS (50% of MS normal concentration of salts); ¼ MS (25% of MS normal concentration of salts); WPM (full); ½ WPM (50% of WPM normal concentration of salts); ¼ WPM (25% of WPM normal concentration of salts). Furthermore, vitamins, sucrose (30 g L^{-1}) and agar (10 g L^{-1}) added to the nutritive medium (MS, WPM and dilutions).

For both experiments, three apical shoot segments were transferred to 150 ml flasks containing 30 ml of each medium. The treatments consisting of 10 replications in a completely randomized design.

All the cultures were incubated at temperature of 25 ° C with a 16 hours photoperiod at an light intensity of 20 uM m^{-2} s^{-1} provided by cool white fluorescent lamps.

After 30 days of cultivation *in vitro*, the percentage of establishment (development of new leaf primordia), formation of roots, number of rooted plant, number of the roots and number of leaves were evaluated for both experiment. In addition the percentage of contamination and callus formation for the agar concentration experiment were evaluated.

Data were processed as necessary for the function $\sqrt{x+0,5}$, with "x" the observed value, and subjected to analysis of variance. When significant, means compared by Scott-Knott test or polynomial regression at 5% level of probability. We used the statistical program Sisvar for Windows, version 4.0. The accuracy of the experiment measured by selective accuracy (AS), calculated by $\sqrt{x+0,5}$ and the data presented only for the factors with significant effects. The results presented are the original averages.

Results and discussion

Experiment 1 - Agar Concentration

Root formation (%), callus formation (%) and number of roots were affected by agar concentration (Table 1). For all this variables, the Selective Accuracy (AS) was high to very high according to the classification of RESENDE & DUARTE (2007).

Callus formation (%) had a curve with negative quadratic behavior (Figure 1). Maximum callus formation was obtained with 5,62 g L^{-1} of agar. After that, callus formation decreases until reach 0% of callus formation in the explants at a concentration of 10 g L^{-1}.

Probably decrease callus formation is related to the lower nutrient diffusion in solid medium, as callus formation is related to the high availability of salts in the culture medium and the hormonal balance. It is possible that the optimal concentrations of salts in a solid medium, are higher than the optimal concentrations for growth in liquid medium (Caldas et al., 1998).

For root formation *in vitro* was observed a linear behavior, increasing root formation with increment of agar concentration (Figure 2A). Using 4 g L^{-1} there was no root formation, while using 10 g L^{-1} showed practically all formation of roots. Otherwise, number of roots, presenting a positive quadratic behavior (Figure 2B). With the use of 10 g L^{-1} gave the highest number of roots per explant (2.50 roots).

Table 1. Analysis of variance (mean square) for contamination (%), establishment (%) formation of roots (%), callus formation (%), number of roots and number of leaves in explants of yellow ipe after 30 days of *in vitro* culture in the different agar concentrations in ½ WPM culture medium.

FV	GL	Contamination (%)	Establishment (%)	Formation of roots (%)	Callus formation (%)	N° of roots	N° of leaves
Treatment	3	4916,6^{ns}	666,66^{ns}	15229,1*	5729,1*	11,4*	3,2^{ns}
Residue	36	2027,7	1083,33	743,0	1381,9	1,0	2,6
Mean (%)	-	32,50	85,00	48,75	33,75	1,47	3,92
AS	-	0,76	0,45	0,97	0,87	0,95	0,42

^{ns} non-significant by F test at P<0.05; * means significant by F test at P<0.05; AS = selective accuracy, ranges: ≤ 0.5 (Low); 0.5 <AS 0.7 (Moderate); 0.7 <AS 0.9 (High); > 0.9 (Very High).

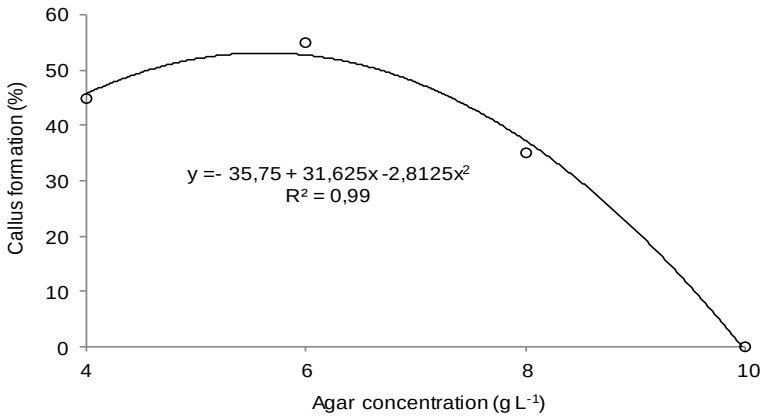

Figure 1. Percentage of callus formation in explants of yellow ipe after 30 days of *in vitro* culture in different agar concentrations in the ½ WPM culture medium.

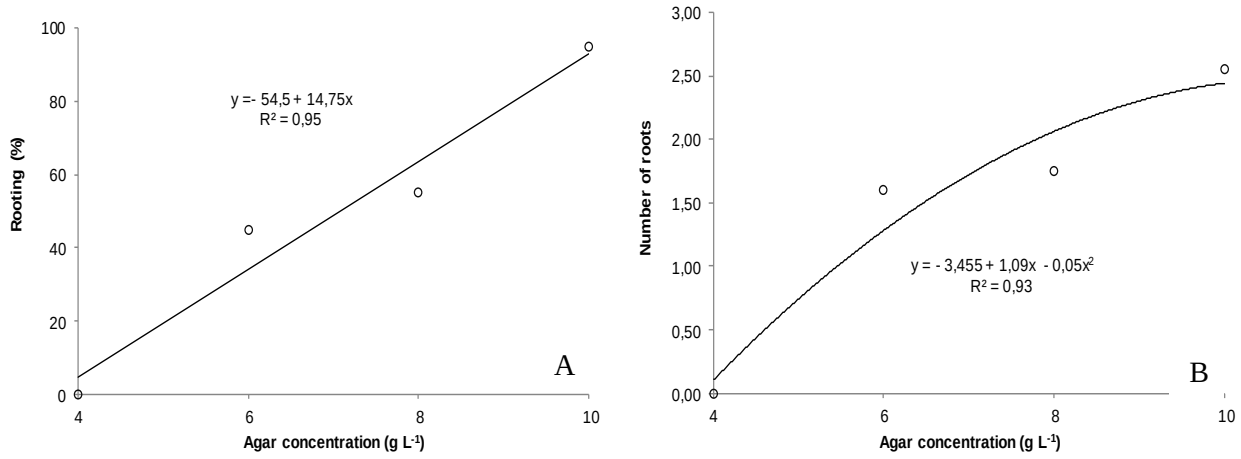

Figure 2. Rooting percentage (A) and number of roots (B) in nodal apical segments of yellow ipe after 30 days of culture *in vitro* in different agar concentrations in ½ WPM culture medium.

According to Grattapaglia & Machado (1998) solid medium with more than 6 g L^{-1} of agar concentrations can cause problems with rooting. However, this was not observed for the yellow ipe, which presented a higher rooting with higher agar concentrations.

A lower rooting may be related to increased anaerobic medium, which tissue of the bases of the shoots are exposed. This fact was also observed in *Kielmeyera coriaceae* where testing agar concentrations there was a higher number of roots using 6-9 g L^{-1} agar (Pinto et al., 1995).

Experiment 2 - Culture medium

Yellow ipe establishment, root formation, number of roots and number of leaves were affected by culture medium (Table 2). For all this variables, the selective accuracy (AS) was high to very high according to the classification of Resende & Duarte (2007).

Establishment of yellow ipe was lower in the treatment control (agar + water). However, among the MS and WPM culture medium and their dilutions no difference were observed. Establishment were greater than or equal to 90% for all treatments with culture medium. The nutrients in the culture medium provide the essential substances for the development of tissues and control, in large part, the *in vitro* development pattern (Torres et al., 1998) and is quite variable depending on the plant species and the source of the explant.

For the percentage of explants with root formation (Table 3), the WPM and their dilutions showed the best results (averages above 85%). The MS medium and their dilutions showed intermediate means of roots formation with values close to 70%. While in the control treatment, the lowest average root formation was observed. This can be indicated that to maximize root formation is necessary to add nutrients and vitamins to the culture medium. Besides, induction of root formation is favored in some species in culture medium with less salts (Junior & Pereira, 2012; Stachevski et al. 2013; Gomes & Paiva, 2014).

Table 2. Analysis of variances (mean square) for establishment (%), formation of roots (%), number of roots and number of leaves in explants of yellow ipe after 30 days of *in vitro* culture in different culture medium.

FV	GL	Establishment (%)	Formation of roots (%)	N° of roots	N° of leaves
Treatment	6	2.483,0*	3.316,4*	23,15*	13,03*
Residue	62	370,96	693,54	4,29	0,95
Mean (%)	-	89,85	76,81	4,52	4,44
AS	-	0,85	0,79	0,81	0,96

^{ns} non-significant by F test at P<0.05; * means significant by F test at P<0.05; AS = selective accuracy, ranges: ≤ 0.5 (Low); 0.5 <AS 0.7 (Moderate); 0.7 <AS 0.9 (High); > 0.9 (Very High).

Table 3. Establishment (%), formation of roots (%), number of roots and number of leaves per explant in nodal apical segments of yellow ipe after 30 days of culture *in vitro* in different culture medium.

Treatment	Establishment (%)	Formation of roots (%)	N° of roots	N° of leaves
¼ WPM	100,0 a*	100,0 a	5,66 a	4,75 b
½ WPM	95,0 a	95,0 a	6,90 a	4,50 b
WPM	95,0 a	85,0 a	5,00 a	6,33 a
MS	90,0 a	75,0 b	4,00 b	4,60 b
½ MS	95,0 a	70,0 b	4,40 b	4,50 b
¼ MS	100,0 a	70,0 b	3,70 b	4,20 b
Agar + water	55,5 b	45,0 c	2,10 c	2,22 c

* Means followed by the same letter in the column do not differ by the Scott-Knott test at 5% probability. The letter "a" refers to the best response in vitro.

In most cases, explants does not start rooting process in medium with high concentrations of salts, such as MS medium. High salts concentrations tend to inhibit rooting and the growth of roots, mainly due to the action of macronutrients in the culture medium (Grattapaglia & Machado, 1998). In this way, WPM medium, which was initially developed for woody species, had its concentration of most macronutrients reduced, and this could be favored yellow ipe root formation. In *Luehea divaricata* WPM medium also promoted higher root formation with 66.80% versus 30.0% in DM medium (Flores et al., 2011). In many other woody species has already been registered that culture medium as WPM and Creishoff and Doy (CD) increase the *in vitro* rooting percentage by low concentration of salts (Thorpe et al., 1991).

For number of roots per explant (Table 3), WPM and their dilutions also had the highest number of roots, among 5-7 roots per explant compared to the other treatments. The MS medium and their dilutions showed an intermediate number of roots, reaching an average close to four roots per explant. The control had the lowest number of roots per explant. These results confirm the previous observation related to rooting: the use of culture medium with lower salt concentrations or dilutions provide more and better rooting (Hu; Wang, 1983).

The Figure 3 shows differences in rooting of yellow ipe explants under different culture medium. WPM medium provided a higher number of roots (A) and better formation (B), while MS medium resulted in a lower number of root (C) and hindering the growth of these roots, getting aspect of burned and sliced roots (D).

Figure 3. Rooted plants of yellow ipe in ½ WPM culture medium (A, B) and in MS culture medium (C, D) after 30 days of *in vitro* culture.

The WPM medium was responsible for most leaves formation with an average of 6.33 leaves per explant. The MS medium and dilutions as well as the dilutions of WPM had an intermediate number of leaves. Like the other variables, the control had the lowest leaves formation (Table 3). This confirm that WPM is more suitable to early development *in vitro* of yellow ipe. The WPM has higher concentration of vitamins in relation to MS medium, especially thiamine. This vitamin promotes cell growth (Lopes et al., 1997) which can be responsible for the highest number of leaves.

Choosing the best culture medium, besides technical efficiency, must be considered the cost of each medium. The WPM is more economical when compared to MS, which makes it a potential medium for use mainly in native tree species.

Conclusion

Increasing agar concentrations in the culture medium improve root formation and number of roots in yellow ipe explants on the establishment *in vitro*. It also follows that the higher the concentration of agar is less callus formation.

WPM medium and its dilution in ½ and ¼ are more efficient than MS medium to promote establishment *in vitro* of yellow ipe.

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