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Micropropagation of *Handroanthus heptaphyllus* (Vellosso Mattos)

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ABSTRACT

Background: The *Handroanthus heptaphyllus* (Vell. Mattos) is an arboreal species with high economic value, particularly for providing excellent quality of wood and medicinal properties of its bark. The usual method of propagation of this species is via seminal, however their seeds have short viability, which hinders the production of seedlings throughout the year. Objective: The aim of this study was to investigate the use of micropropagation for the production of *H. heptaphyllus* plantlets. Methodology: Aseptic seedlings were transferred to the MS or WPM culture media and evaluated for survival percentage, number of internodes and length of shoots and primary root. For *in vitro* multiplication, nodal segments from aseptic seedlings were cultivated in a WPM medium with 0, 6.7 and 13.3 µM of 6-benzylaminopurine (BAP) or 0, 4.6 and 9.3 µM of kinetin (KIN), and evaluated for survival percentage and rooting, the number of shoots and internodes and the length of shoots. For *in vitro* rooting, shoots of nodal segments and microstumps were grown in WPM medium with 0, 4.9, 9.8, 19.6 and 29.4 µM of indolebutyric acid (IBA), and for *ex vitro* rooting, micro-cuttings with one pair of leaves reduced to 50% of their area were treated or not with 4920 µM of IBA and grown in different substrate compositions (v/v): commercial substrate and vermiculite (1:1); commercial substrate and vermiculite (1:2); and commercial substrate, vermiculite and sand (1:1:1). Results: WPM was found to be the most adequate culture medium for obtaining aseptic *H. heptaphyllus* seedlings, and it was possible for the nodal segments to be grown in of cytokinin -free medium. In the *in vitro* rooting, nodal segments did not present roots in any treatments. On the other hand, the shoots of microstumps cultivated *in vitro* presented 2.4% of rooting, but when kept in *ex vitro* conditions rooting reached 40%. Conclusion: Growth of aseptic seedlings of *H. heptaphyllus in vitro* was found to be more adequate in WPM culture medium and these plants can be used as microstumps to produce vegetative propagules. Shoots of microstumps can be *ex vitro* rooted after treatment with IBA and grown in commercial substrate and vermiculite (1:2 v/v). The *ex vitro* rooting is more efficient in the formation of roots in microstumps shoots when compared to *in vitro* rooting, making it possible the micropropagation technique for the production of plantlets of *Handroanthus heptaphyllus*.

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INTRODUCTION

Brazil possesses the world's second largest area of forest coverage, accommodating around 4.6 million km^2 of natural and planted forests, which represents 54.4% of its territory. The natural Brazilian forests are significant because they hold one of the greatest diversities of arboreal species of economic and ecological relevance (SFB, 2013), including the noteworthy species *Handroanthus heptaphyllus* (Velloso Mattos), which belongs to the Bignoniaceae family (Lorenzi, 2008).

H. heptaphyllus occurs naturally in Brazil, Argentina, Bolivia and Paraguay (Lorenzi, 2008; Carvalho, 2003). It is an arboreal species that provides wood of excellent quality and high economic value, due to its high density, durability, resistance and low volumetric retractability (Paula and Alves, 2007). In addition, extractives from leaves and bark present medicinal properties (Ettori *et al*., 1996), including hypoglycemic, depurative and bactericidal activities (Neto and Morais, 2003). Thus, interest in *H. heptaphyllus* from the wood industry and for therapeutic purposes has spurred a chaotic exploration of this species and consequently the reduction of number of individuals who are still found in naturally occurring areas (Ettori *et al*., 1996).

The development of efficient strategies for propagation of native species is fundamental for production of seedlings with high genetic and phytosanitary quality, both for commercial purposes and forest replacement. However, the production of *H. Heptaphyllus* seedlings is hindered due for short viability of the seeds (Cabral *et al*., 2003). The vegetative propagation might be an alternative to maximize seedling production for those species that present seedling production limited by germination difficulties (Hartmann *et al*., 2011). Among the vegetative propagation techniques, can mention the micropropagation, which is composed of distinct stage as the selection of the explant, the multiplication of the vegetative propagules and *in vitro* or *ex vitro* rooting (Xavier *et al*., 2013).

In vitro multiplication helps maximize the production of shoots, while i*n vitro* rooting is characterized by induction of adventitious roots in elongated shoots, generally kept in culture medium supplemented with auxins, in order to obtain complete plantlets that will be subsequently acclimatized (Oliveira *et al*., 2013). However, McClelland *et al*. (1990) reported plants produced by *in vitro* rooting to present low survival during acclimatization, when the transition from heterotrophism to autotrophism occurs, because the adventitious root system produced *in vitro* generally presents little branching and is inefficient in the absorption of water and nutrients (Hoffmann *et al*., 2001). On the other hand, *ex vitro* rooting of explants can minimize these problems, because at that stage the explants are treated, or not, with auxin and planted in the substrate for root formation concomitant to acclimatization, reducing mortality and production costs of micropropagated plantlets (Augusto *et al*., 2006).

The micropropagation is an important technique for multiplication and/or rooting of a number of arboreal species of economic interest, including *Apuleia leiocarpa* (Vogel J. F. Macbride) (Lencina *et al*., 2016), *Caryocar brasiliense* (Cambess) (Santos *et al*., 2006) and *Aspidosperma polyneuron* (Müller Argoviensis) (Ribas *et al*., 2005), as well as medicinal species such as *Stryphnodendron barbatiman* (Mart.) (Nicioli *et al*., 2008), *Melissa officinalis* (L.) (Reis *et al*., 2008) and *Malva sylvestris* (L.) (Filter *et al*., 2014). However, studies on micropropagation of arboreal species native, including those with great medicinal potential as the case of *H. heptaphyllus*, are still lacking. The rooting step of those species presents one of the greatest challenge to defining micropropagation protocols, due to the influence of diverse endogenous and exogenous factors that have not been elucidated for this and other woody species (Souza and Pereira, 2007) and due to the high genetic diversity of these species, resulting in genotype-dependent responses (Sobrosa and Corder, 2003).

The objective of this work was to evaluate the *in vitro* growth and multiplication, *in vitro* and *ex vitro* rooting of *H. heptaphyllus* explants for the production of micropropagated plantlets.

MATERIALS AND METHODS

This work was carried out between January 2012 and December 2013 in the Department of Plant Science of the Federal University of Santa Maria, in the state of Rio Grande do Sul, Brazil. All cultures were maintained in a growth room at a temperature of 25 ± 2ºC on a 16 h photoperiod, under 14.3 µE m^{-2} S^{-1} of light intensity supplied by fluorescent lamps.

To obtain aseptic *H. heptaphyllus* seedling, seeds were immersed in distilled water for 30 minutes, followed by a solution of 70% alcohol during 7 minutes. The seeds were then immersed in 2% sodium hypochlorite during 30 minutes. Between each disinfestation stage, the seeds were rinsed three times in distilled and autoclaved water. All processes were performed in a laminar flow chamber under aseptic conditions. After disinfestation, the seeds were cultivated in glass culture tubes (10 mL) containing 5 mL of culture medium with 30 g L^{-1} sucrose and 7 g L^{-1} agar. After 15 days in culture, the aseptic seedlings were transferred to Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) or Wood Plant medium (WPM) (Lloyd and Mccown, 1980) in order to determine the most adequate nutritive medium for *in vitro* growth of the species. Both media were supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar and placed in glass flasks (150 mL) containing

approximately 25 mL of culture medium. The pH of the media was adjusted to 5.8 with subsequent autoclaving for 20 min. at 121 ºC and 1 atm of pressure. The experiment was a completely random design with 14 repetitions of 4 seedlings. Survival percentage, number of internodes and leaves and length of shoots and primary roots were evaluated at 45 and 60 days.

For *in vitro* multiplication, nodal segments (1.0 cm in length) from aseptic seedlings (60 days of age) were transferred to glass culture tubes (60 mL), containing approximately 15 mL WPM medium with 30 g L^{-1} sucrose, 7g L^{-1} agar, 1.5 g L^{-1} activated charcoal and supplemented with 0; 6.7 and 13.3 µM of 6-benzylaminopurine (BAP) or 0; 4.6 and 9.3 µM of kinetin (KIN). The experiment was a completely random design with 5 repetitions of 4 explants. After 45 days, survival and rooting percentages, number of shoots and internodes and shoot lengths were evaluated.

For *in vitro* rooting, nodal segments and microstumps obtained from 90-day-old aseptic *H. heptaphyllus* seedlings were utilized. To produce the microstumps (Figure 1A), the aseptic seedlings underwent drastic pruning in the region above the first pair of leaves. The nodal segments and microstumps were kept in WPM medium with 30 g L^{-1} sucrose, 7 g L^{-1} agar and 1.5 g L^{-1} activated charcoal for 60 days for shoot production. The shoots of nodal segments and microstumps were grown in WPM medium containing 30 g L^{-1} sucrose, 7 g L^{-1} agar, 1.5 g L^{-1} activated charcoal and supplemented with 0; 4.9; 9.8; 19.6 and 29.4 µM indolebutyric acid (IBA). The factorial (2 x 5) experiment utilized a completely random design with 5 repetitions of 5 shoots. The percentage of survival, rooting and callus formation and total number and length of roots were evaluated after 90 days of growth.

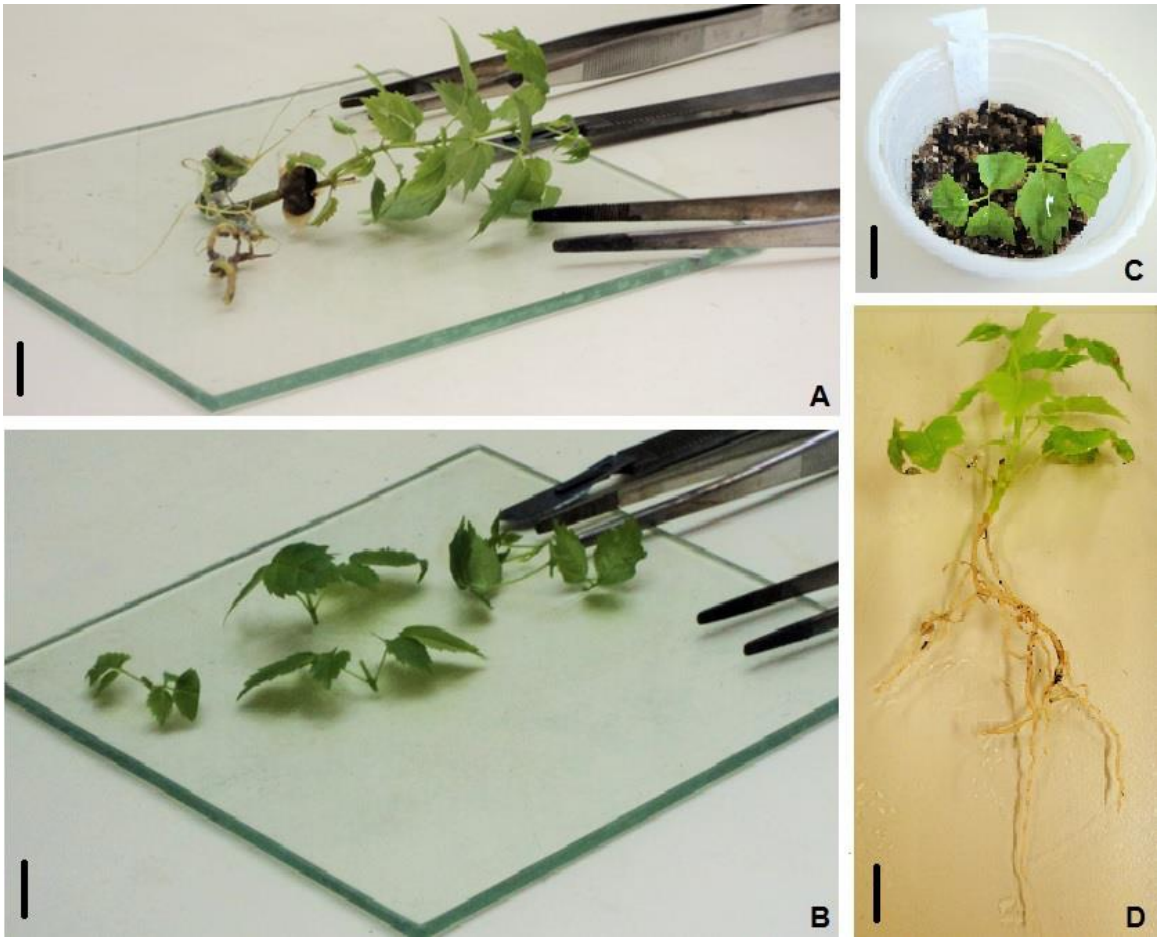

Fig. 1: Microstumps of *Handroanthus heptaphyllus* after 60 days of growth in WPM culture medium supplemented with 1.5 g L^{-1} activated charcoal for shooting (A). Shoots from microstumps with one pair of leaves (B) reduced to 50% of their area, treated with 4.920 µM IBA and grown in commercial substrate and vermiculite (1:2 v/v) (C) for the production of plantlets by *ex vitro* rooting (D). Santa Maria, RS, Brazil, 2015. Bar = 1 cm of length

The recipients containing the shoots were placed in polyethylene trays covered by transparent PVC film and were maintained in a growth room for 60 days. Substrate humidity was maintained by three weekly irrigations with distilled water. After 45 days, the plants were transferred to larger recipients (200 cm^3) containing 100 g of

the same compositions of substrate for each treatment. The factorial (2 x 3) experiment utilized a completely random design with 5 repetitions of 5 shoots. At 30, 45 and 60 days of growth, the rooting percentage, number and total length of roots were evaluated.

To account for normality, percentage data were transformed to arc-sine ($\sqrt{x}/100$) and submitted to analysis of variance. The treatment means with significant differences were compared by Tukey test, at a 5% probability of error.

RESULTS AND DISCUSSION

Upon evaluating the effect of the culture media on *in vitro* growth of *H. heptaphyllus* seedlings, no significant differences were found among treatments after 45 days of cultivation. Plants grown either in MS or WPM medium presented vigorous growth, with no symptoms of nutritional deficiency, and a 100% rate of survival. However, after 60 days of growth, a significant difference was observed between the WPM and MS media, where plants grown in WPM presented significantly improved responses for number of internodes and leaves, length of shoots and the primary root (Table 1). A similar result was obtained in *Tabebuia donnell-smithii* (Rose (Bignoniaceae)), where shooting was 40% lower in MS medium when compared to WPM medium (Gonzalez-Rodriguez *et al*., 2010). WPM also showed superior results when compared to MS for *in vitro* growth of other arboreal species, such as *Didymopanax morototoni* (Aublet) Decaisne et Planchon) (Mantovani *et al*., 1999), *Cordia Trichotoma* (Vellozo) Arrabida ex Steud) (Fick *et al*., 2007), *Olea europaea* (Linneo) (Donini *et al*., 2008) and *Luehea divaricata* (Martius et Zuccarini) (Flôres *et al*., 2011), corroborating with our results obtained for *H. heptaphyllus*.

According to Larraburu *et al*. (2012), the responses observed in different species may be influenced by the different compositions of micronutrients in the two culture media. WPM medium contains copper sulfate, which is absent in MS medium and studies of tissue culture have shown the presence of copper sulfate to improve organogenesis (Joshi and Kothari, 2007; Nassar, 2004). In addition, WPM contains higher contents of potassium and sulfate ions (Pasqual, 2001) and MS contains a high content of nitrate, which could induce metabolic stress, and this could explain the lower growth *in vitro* of *H. heptaphyllus* grown in this medium. Moreover, considering the economic viability of micropropagation techniques, the use of WPM is more advantageous when compared to MS, because it contains only 25% of the concentration of nitrate ions and ammonium used in MS (Melo *et al*., 1999).

Table 1: Survival percentage, number of internodes and leaves, length of shoots and primary root of *Handroanthus heptaphyllus* seedlings in WPM and MS culture medium at 45 and 60 days of growth

Culture medium	Survival (%)	Number of internodes	Number of leaves	Length of shoots (cm)	Length of primary root (cm)
		----------Evaluation at 45 days of growth ----------			
WPM	100 a*	2.8 a	5.2 a	2.7 a	7.6 a
MS	100 a	2.8 a	5.3 a	2.6 a	6.4 a
Mean	100	2.8	5.25	2.65	7.0
CV (%)	-	8.38	8.78	7.09	13.49
		----------Evaluation at 60 days of growth ----------			
WPM	98.2 a*	3.6 a	8.1 a	3.4 a	8.8 a
MS	89.3 a	2.9 b	5.6 b	2.7 b	6.1 b
Mean	93.75	3.25	6.85	3.05	7.45
CV (%)	15.77	14.09	14.98	13.54	14.41

*Values followed by the same letter do not differ by the Tukey test at 5% probability of error.

In the *in vitro* multiplication, there was no significant difference observed between the evaluated treatments after 45 days of growth. A high explant survival percentage was observed (83%), suggesting that the BAP and KIN concentrations tested did not provoke phytotoxic effects in the tissues. It was also found that shooting occurred even in WPM medium without the presence of BAP or KIN (Table 2), indicating that endogenous concentrations of cytokinin were sufficient for shoot regeneration in the explants. A similar result was observed for *Luehea divaricata* (Martius et Zuccarini), where shooting in explants was efficient in culture medium free of cytokinin (Flôres *et al*., 2011). Considering that the *in vitro* production of plantlets of arboreal species has as of yet not been economically viable (Xavier and Wendling, 1998), the possibility of producing plantlets in media free of cytokinins is desirable in order to reduce micropropagation costs. However, the response of the species to treatment with phytohormones are not universal, because certain species in the presence of cytokinins do not form new shoots, as is the case of explants of *Jatropha curcas*. This species did not induce shoot formation when grown in culture medium supplemented with BAP or KIN (Soong *et al*., 2016).

It is also important to highlight the formation of roots in explants (2.10 %) observed during multiplication *in vitro* (Table 2), which allowed the production of plantlets of *H. heptaphyllus.* When concentrations of auxin are higher than those of cytokinin, the morphogenetic response is associated to root formation, while cytokinin concentrations higher than those of auxin result in the induction of the shoots (Xavier *et al*., 2013). Since the *H. heptaphyllus* originated from seedlings, it is possible that some genotypes have a greater potential for root

formation in comparison to others, suggesting that in these individuals there was a high auxin/cytokinin ratio in the vegetal tissues, besides a natural competence toward rooting.

Table 2: Percentage of survival and rooting, number of shoots and internodes and length of shoots in explants of *Handroanthus heptaphyllus* grown in WPM medium supplemented by different concentrations of benzylaminopurine (BAP) or kinetin (KIN), at 45 days of growth.

Treatments	Survival (%)	Rooting (%)	Number of shoots	Number of internodes	Length of shoots (cm)
BAP (µM)					
0	83.0 a*	0.0 a	1.0 a	2.5 a	2.1 a
6.7	72.8 a	0.0 a	1.0 a	2.0 a	1.7 a
13.3	90.0 a	6.3 a	1.0 a	1.9 a	2.1 a
KIN (µM)					
0	90.0 a	0.0 a	1.1 a	2.8 a	1.9 a
4.6	83.0 a	6.3 a	1.1 a	2.6 a	1.9 a
9.3	79.1 a	0.0 a	1.1 a	2.4 a	2.2 a
Mean	83.00	2.10	1.05	2.36	1.98
CV (%)	24.8	225.71	2.29	17.08	11.73

*Values followed by the same letter do not differ by the Tukey test at 5% probability of error.

In the *in vitro* rooting, there was not a significant interaction among the evaluated parameters, nor did shoot type or IBA concentration affect the percentages of survival, rooting and callus formation and total length of roots, after 90 days of growth. A mean survival of 28.8% and a mean rooting in the shoots of 1.2% were observed regardless of the type of shoot tested (Table 3). The high rate of callus formation (26.4%) (Table 3) observed at the base of the explants suggests that the type and/or concentration of auxin in the present study was not efficient to promote root differentiation. These results contradict those reported by Hartmann *et al*. (2011) and Watt *et al*. (2003), who claimed that both shoot origin (nodal segments and microstumps) are originated from pre-formed meristematic organs (axillary bud), which generally present satisfactory rooting potential, due to the high degree of juvenility.

Table 3: Percentage of survival, rooting and callus formation and number and total length of roots obtained from microstumps or nodal segments of *Handroanthus heptaphyllus* at 90 days of growth in WPM medium.

Shoot origin	Survival (%)	Rooting (%)	Callus (%)	Number of roots	Total length of roots (cm)
Microstumps	28.8 a*	2.4 a	28.0 a	1.5 a	6.4 a
Nodal segment	28.8 a	0.0 a	24.8 a	0.0 b	0.0 a
Mean	28.8	1.2	26.4	0.75	3.2
CV	123.9	253.4	127.3	28.04	129.04

*Values followed by the same letter do not differ by the Tukey test at 5% probability of error.

In terms of number of roots, the shoots obtained from microstumps presented significantly superior results when compared to shoots from nodal segments after 90 days of growth (Table 3). The content of water and of nutritional reserves of the plants used to obtain the vegetative propagules can affect the process of root formation in explants (Hartmann *et al*., 2011), which may explain the superior rooting responses found for *H. heptaphyllus* shoots generated from microstumps, since these possess a root system, and thus continually absorb nutrients and water from the culture medium.

In addition, it is important to consider that the presence of natural or synthetic auxins in the vegetal tissues may not always induce adventitious root primordia *in vitro* explants. Several vegetal species, especially woody ones, present a low rooting capacity even in culture media supplemented with auxins, indicating that other factors, besides the use of phytohormones, influence tissue differentiation during this process (Souza and Junghans, 2006). Inefficient rooting *in vitro* was also observed in shoots of *Lavandula angustifolia* (Miller) treated with 0.5, 1.0 and 2.0 µM of IBA (Machado *et al*., 2013).

For *ex vitro* rooting of *H. Heptaphyllus*, there was not a significant interaction between substrate and IBA for the variables of survival and callus formation percentage at 30, 45 and 60 days of growth. The greatest survival responses were observed in shoots grown in commercial substrate and vermiculite (1:2 v/v) followed by commercial substrate, vermiculite and sand (1:1:1 v/v) (Supplemental Table 1). Based on physical analysis, these substrate compositions also presented adequate aeration space (AS) (Supplemental Table 2), as the optimal range is between 20 and 40% (De Boodt and Verdonck, 1972). For production of *Sesbania virgata* (Cav. Pers) seedlings, aeration space of up to 25% is considered optimal (Delarmelina *et al*., 2014).

Supplemental Table 1: Percentage of survival and callus formation in *ex vitro* rooting of *Handroanthus heptaphyllus* shoots grown for 30, 45 and 60 days in different compositions of substrates.

Composition of substrate	Survival (%)	Callus formation (%)
	---------- 30 days of growth ----------	
CS & V (1:1 v/v)**	42.0 b*	4.0 a
CS & V (1:2 v/v)	84.0 a	18.0 a
CS, V & S (1:1:1 v/v)	78.0 a	12.0 a
Mean	68.0	11.3
CV (%)	36.0	115.3
	---------- 45 days of growth ----------	
CS & V (1:1 v/v)	16.0 b	0.0 b
CS & V (1:2 v/v)	60.0 a	18.0 a
CS, V & S (1:1:1 v/v)	30.0 ab	12.0 ab
Mean	35.4	10.0
CV (%)	71.6	101.8
	---------- 60 days of growth ----------	
CS & V (1:1 v/v)	0.0 b	0.0 b
CS & V (1:2 v/v)	36.0 a	14.0 a
CS, V & S (1:1:1 v/v)	16.0 ab	8.0 ab
Mean	17.3	7.3
CV (%)	87.3	95.7

*Values followed by the same letter do not differ by the Tukey test at 5% probability of error. **CS- Commercial substrate made of pine bark; V- Vermiculite of medium granular size; S- Sand of coarse granular size.

However, there was a significant interaction between substrate and IBA for rooting percentage, number and total length of roots. The best rooting responses (40%) were obtained in shoots treated with 4.920 µM IBA and grown in commercial substrate and vermiculite (1:2 v/v), regardless of the evaluation period. However, an increase in root number and length was observed throughout the growth period. At 60 days, the best results for these variables were observed in shoots treated with 4.920 µM IBA, with no significant difference observed between the compositions of commercial substrate, vermiculite (1:2 v/v) and commercial substrate,vermiculite, sand (1:1:1 v/v) (Table 4). Similar responses have been verified in shoots of *Tectona grandis* (Linn), which presented high *ex vitro* rooting when treated with 4.920 µM of IBA and grew in commercial substrate or vermiculite (Fermino Júnior *et al*., 2011).

Table 4: Rooting percentage, number and total length of roots of *Handroanthus heptaphyllus*, treated or not with 4.920 µM of IBA and grown in different substrate compositions for 30, 45 and 60 days.

Substrate	Rooting (%)		Number of roots		Length of roots (cm)	
	0 µM IBA	4.920 µM IBA	0 µM IBA	4.920 µM IBA	0 µM IBA	4.920 µM IBA
	---------- 30 days of growth ----------					
CS & V (1:1 v/v)**	0.0 Aa*	0.0 Ba	0.0 Aa	0.0 Ba	0.0 Aa	0.0 Ba
CS & V (1:2 v/v)	0.0 Ab	40.0 Aa	0.0 Ab	1.2 Aa	0.0 Ab	3.0 Aa
CS, V & S (1:1:1 v/v)	0.0 Ab	12.0 Ba	0.0 Ab	1.1 Aa	0.0 Ab	3.1 Aa
Mean	0	17.33	0	0.77	0	2.04
CV (%)	98.6	98.6	13.4	13.4	25.1	25.1
	---------- 45 days of growth ----------					
CS & V (1:1 v/v)	0.0 Aa	0.0 Ba	0.0 Ba	0.0 Ca	0.0 Ba	0.0 Ba
CS & V (1:2 v/v)	8.0 Ab	40.0 Aa	1.0 Ab	1.7 Aa	1.3 Ab	4.1 Aa
CS, V & S (1:1:1 v/v)	0.0 Ab	16.0 Ba	0.0 Bb	1.2 Ba	0.0 Bb	3.6 Aa
Mean	2.66	18.66	0.33	0.97	0.43	2.57
CV (%)	77.2	77.2	12.7	12.7	25.8	25.8
	----------60 days of growth ----------					
CS & V (1:1 v/v)	0.0 Aa	0.0 Ca	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba
CS & V (1:2 v/v)	8.0 Ab	40.0 Aa	1.0 Ab	1.7 Aa	2.7 Ab	5.6 Aa
CS, V & S (1:1:1 v/v)	0.0 Ab	20.0 Ba	0.0 Bb	1.5 Aa	0.0 Bb	3.8 Aa
Mean	2.66	20	0.33	1.07	0.9	3.13
CV (%)	70.1	70.1	14.1	14.1	32.6	32.6

*Values followed by the same letter, capital letter vertically and lowercase horizontally, do not differ by the Tukey test at 5% probability of error. **CS- Commercial substrate made of pine bark; V- Vermiculite of medium granular size; S- Sand of coarse granular size.

Fermino Júnior *et al*. (2011) and Schuck *et al*. (2012) pointed out that substrate is one of the most important factors in successful *ex vitro* rooting, together with acclimatization of micropropagated plantlets, and that it should present good water and air retention. In this study with *H. heptaphyllus*, shoots grown in commercial substrate and vermiculite (1:1 v/v) did not present root formation without the use of IBA (Table 4), confirming that substrate is indeed important for successful *ex vitro* rooting of this species. This is due to the fact that the composition of different substrates may facilitate or impair root growth, depending on its physical and chemical properties (Skrebsky *et al*., 2006). Physical analysis of the compositions of substrate used in this study revealed that commercial substrate and vermiculite (1:1 v/v) presented greater water retention associated to the smaller aeration space (Supplemental Table 2). The excess water in the substrate during the rooting phase can make the

emission of adventicious roots more difficult because of the lack of tissue oxygenation (Arruda *et al*., 2007), which may explain the absence of roots in shoots of *H. heptaphyllus* grown in this substrate.

Supplemental Table 2: Aeration space and capacity of water retention at 10, 50 and 100 cm in a water column with the substrate compositions used for *ex vitro* rooting of shoots of *Handroanthus heptaphyllus.*

Substrate	Aeration space (%)	Capacity of water retention (%)		
		10 cm (%)	50 cm (%)	100 cm (%)
CS & V (1:1 v/v)*	19.9	57.2	46.3	44.3
CS & V (1:2 v/v)	20.0	54.6	45.5	44.0
CS, V & S (1:1:1 v/v)	25.3	38.7	31.1	30.0

*CS- Commercial substrate made of pine bark; V- Vermiculite of medium granular size; S- Sand of coarse granular size.

The faster rooting (30 days), associated to a higher percentage of rooting (40%) and survival (84.0%) in the plants cultivated in commercial substrate and vermiculite (1:2 v/v) (Table 4 and Supplementary Table 1) may be due to a water/air ratio that is favorable to the induction and growth of *H. heptaphyllus* root primordia. The larger quantity of vermiculite in this composition (67% total volume) may have provided greater aeration, a factor that is essential for supplying tissues with the energy necessary for emission and rapid growth of root primordia.

In addition, *ex vitro* rooting of *H. heptaphyllus* was found to be significantly stimulated when shoots were treated with 4.920 µM IBA (Table 4), demonstrating that application of exogenous auxin is an important factor in the obtainment of plantlets of *H. heptaphyllus* (Figure 1D). Treatment with phytohormones is considered an efficient method for obtaining adventitious roots, as it supports speed of emission and the number and quality of roots (Hartmann *et al*., 2011). *Ex vitro* rooting, where roots are induced directly in the substrate, is an important methodology in the development of viable micropropagation protocols, since it allows a reduction of production costs of up to 50% and greater survival percentages when compared to *in vitro* rooting (Pedrotti and Voltolini, 2001). Moreover, plants produced by *ex vitro* rooting generally form stronger roots with more normal morphology than those produced *in vitro*, because they possess less swollen cortical cells and a functional vascular system (Hartmann *et al*., 2011), allowing water and nutrient needs of the plants to be met.

The *H. heptaphyllus* is among the medicinal plants with recognized pharmacological value, which makes a constant search for plants of this species by local people and the pharmaceutical industries. In this context, the global demand of herbal medicines on an industrial scale (Brasil, 2009), makes the vegetative propagation, especially micropropagation of medicinal plants, an alternative to be adopted. It is important, because it allows the commercial production of quality homogeneous plantlets and assists with the selection and the breeding new genotypes with potential use by the pharmaceutical industry (Rao and Ravishankar, 2002). The results of this study showed that the production of *H. heptaphyllus* plantlets through micropropagation technique is feasible. The WPM culture medium free of cytokinins (BAP or KIN) showed the best growth and *in vitro* multiplication of this species. Acclimatization and *ex vitro* rooting of *H. heptaphyllus* can be performed simultaneously, which required the treatment of explants with 4.920 µM IBA and growing in commercial substrate and vermiculite (1: 2 v / v), allowing the reduction of the period of plantlet formation and final production costs of selected genotypes.

The micropropagation protocol developed in this study may be used as a biotechnological tool for *in vitro* production of secondary metabolites, thus ensuring an alternative way for sustainable exploitation of *H. heptaphyllus*. In addition, the *ex vitro* rooted plantlets can be utilized in micro-clonal hedge, which would provide mass production of economically viable plantlets, satisfying the demand of the pharmaceutical industry. However further studies are needed in order to evaluate the survival, growth and production of micro-cuttings of these micropropagated plantlets in micro-clonal hedge.

Conclusions:

Growth of aseptic seedlings of *H. heptaphyllus in vitro* was found to be more adequate in WPM culture medium.

The nodal segments can be multiplied *in vitro* in WPM cytokinin-free culture medium, however they did not produce any roots *in vitro*.

Microstumps can be maintained *in vitro* for supplying vegetative propagules.

Shoots of microstumps can be used in the *ex vitro* rooting when treated with IBA and grown in commercial substrate and vermiculite (1: 2 v / v).

The *ex vitro* rooting is more efficient in the formation of roots in shoots of microstumps when compared to *in vitro* rooting, making possible the micropropagation technique for the production of plantlets of *Handroanthus heptaphyllus*.

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