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An efficient protocol for micropropagation of *Melaleuca alternifolia* Cheel

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Abstract *Melaleuca alternifolia* is cultivated for the production of an essential oil useful in the cosmetic and pharmaceutical industries. Despite the economic importance of this species, there is little knowledge about its *in vitro* propagation. The aim of this study was to establish an efficient protocol for micropropagation of *M. alternifolia.* With the goal of *in vitro* multiplication by axillary shoot proliferation, both solid and liquid MS and WPM media were tested with supplementation with BA at 0, 0.55, 1.11, 2.22, 3.33, and 4.44 μM. The best result for shoot multiplication was obtained when either 0.55 μM BA was added into solid MS medium or 1.11 μM BA was added into liquid MS medium, with 5.6 and 11.8 shoots per explant generated, respectively. On solid or liquid WPM medium supplemented with 0.55 μM BA, the proliferation rates were 5.5 and 4.7, respectively. Three auxins (NAA, IAA, and IBA) were tested at 0.53 and 2.64 μM during the rooting stage. Several sucrose concentrations (15, 30, and 45 gL^{-1}) were compared to a sucrose-free medium. Rooting performances on four culture media were then compared: MS, half-strength MS (MS/2), MS+activated charcoal (AC), and MS/2+AC. The results showed that auxin addition to culture medium is not necessary for *in vitro* rooting. Rooted microcuttings from different culture media were acclimatized in a greenhouse, and the survival percentage was evaluated. All shoots cultured in an auxin-free MS medium supplemented with sucrose (30 gL^{-1}) produced roots, and all plants survived during acclimatization. Activated charcoal added in rooting medium reduced rooting rates.

Keywords Growth regulator · *In vitro* rooting · Medicinal plant · Shoot multiplication · Tea tree

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Introduction

Melaleuca alternifolia Cheel (Myrtaceae) or "tea tree" is native to New South Wales, Australia, where it grows in water courses, swampflats, and springs (Homer *et al*. 2000) or near rivers (Riedl 1997). The main product of the tea tree is an essential oil with antimicrobial and anti-inflammatory properties (Kiong *et al*. 2007) used by the pharmaceutical industry and for cosmetics and toiletries. One of the disadvantages of essential oil production by plants is the compositional variability, particularly when plants are propagated sexually, given that secondary metabolite production is directly related to plant genotype. A method of vegetative propagation for elite individuals is therefore important in order to minimize variation. Clonal propagation through tissue culture offers great potential for the establishment of production fields from plants with high essential oil production (List *et al*. 1996). Although *Melaleuca* culture is economically important, there are few reports on its propagation and little information about its micropropagation in the literature. Up to now, List *et al*. (1996) were the only authors who described a protocol for bud proliferation from nodal segments.

Different genotypes of tea tree may have different nutritional and hormonal needs during *in vitro* multiplication. This study was carried out in order to optimize a micropropagation system that combines high multiplication and rooting rates and good chances of survival during acclimatization for the genotype currently in our lab.

Materials and Methods

Culture establishment. The mother plants were grown from cuttings in a greenhouse for 3 mo and then cut at 20 cm above the crown. All cuttings were from the same genotype. After 45 d, the young shoots were randomly chosen and used for *in vitro* establishment. They were disinfested with thiophanate-methyl (2 gL^{-1}) for 40 min followed by 70% (*v*/*v*) ethanol containing 15 drops/L Tween 20® for 30 s, 1% NaOCl (*v*/*v*) for 20 min, and three 10-min rinses in sterile, distilled water. The apexes were discarded and the shoots divided into 1 cm segments, each containing two nodes. The segments were cultured on MS (Murashige and Skoog 1962) culture medium solidified with 6 gL^{-1} agar (Vetec®).

Culture conditions. Multiplication and rooting experiments were carried out in glass flasks 6 cm in diameter and 9 cm in height, covered with polypropylene caps. All media had their pH adjusted to 5.8 and were autoclaved at 1 atm and 121°C for 20 min. The cultures were kept at 25 ± 2°C under white fluorescent light (20 μmol $m^{-2}s^{-1}$) with a 16-h photoperiod.

Multiplication. Media were tested at two densities, solid (6 gL^{-1} agar) and liquid, and with five different concentrations of BA. Nodal segments were isolated from 20-d cultures that had been grown in establishment medium, MS medium, as indicated in Culture establishment of "Materials and Methods". The freshly cut segments were then cultured on either MS or WPM (Lloyd and McCown 1980) media in separate assays. In both media, BA was supplemented at 0.55, 1.11, 2.22, 3.33, and 4.44 μM and compared with a BA-free medium. The results of two subcultures, carried out every 30 d, were compared. The number of new shoots per explant, the fresh and dry weights, and percentage of necrosis (dead explants) were recorded for every subculture. Experimental design was totally randomized following a tri-factorial arrangement (2×2×6) with five replicates per treatment and five explants per flask. Elongation media were not tested.

In vitro rooting. One-centimeter shoots from liquid MS medium supplemented with 1.11 μM BA were used to evaluate several different rooting media. In three different experiments, the basic media was MS solid culture medium supplemented with 30 gL^{-1} sucrose. In the first experiment, three types of auxin (NAA, IAA, and IBA) at two concentrations (0.53 and 2.64 μM) were compared to an auxin-free medium. The auxin was added to the culture media before autoclaving. In a second experiment, MS and half-strength MS salts (MS/2) medium, with or without 2 g L^{-1} AC, were compared. In the third experiment, three sucrose concentrations (15, 30, and 45 gL^{-1}) and a sucrose-free control were tested in MS solid culture medium. Experimental design was totally randomized following a bifactorial arrangement (3×3) with five replicates per treatment and five explants per flask. After 30 d on a rooting medium, the percentage of rooted plants, mean root number per explant, mean root length, shoot height, and aerial fresh weight were recorded.

The experiments were repeated three times, and the values used for statistical analyses were the means obtained from three experiments. For percentage results, the means were transformed by $\arcsin\sqrt{x/100}$ and those of numbers by $\sqrt{x+1}$, analyzed by ANOVA and means compared by Duncan's test using the SOC program (Embrapa 1990) (α=0.05).

Acclimatization. Rooted plants were transferred to a commercial substrate in polypropylene tubes (53 cm^3) and acclimatized in a greenhouse under intermittent mist for 30 d. They were then kept without mist and irrigated manually for 30 d. After 60 d, the percentages of survival were calculated.

Results and Discussion

In vitro establishment. The disinfestation process and culture conditions resulted in a survival rate of 80% for the nodal segments after 20 d.

In vitro multiplication. In WPM, an interaction was found between the factors consistency of culture medium (solid and liquid) and BA concentration (0.00, 0.55, 1.11, 2.22, 3.33, and 4.44 μM), but there were no significant differences between the mean shoot numbers of both subcultures (Table 1).

The WPM supplemented with 0.55 μM BA gave the highest number of new shoots per explant, on solid as well as in liquid WPM, with 5.5 and 4.7 shoots per explant, respectively (Table 1). Solid WPM was superior to liquid for the other BA concentrations tested. This result can be due to the low aeration of liquid medium, since the flasks were not shaken. As indicated by Murashige (1974), the explants that remain immersed in the liquid medium are not

oxygenated. Additionally, absorption of nutrients and growth regulators may be affected by this lack of aeration.

Shoots cultured in solid WPM medium were short (Fig. 1*A*) and had small chlorotic leaves with necrotic spots. Necrosis was high in WPM liquid medium (Table 1). When shoots were cultured on WPM medium supplemented with low concentrations of BA (0.55 and 1.11 µM), their fresh and dry weights were higher than those of control shoots, due to an increase in shoot proliferation (data not shown). Shoot fresh weight was greater in liquid than in solid medium, due to high water absorption in liquid medium, while dry weight was higher in solid medium.

In cultures on MS medium, shoot per explant numbers were generally more consistent at the varying BA concentrations and necrosis was generally lower. The multiplication rate decreased at BA concentrations greater than 2.22 µM. The highest multiplication rate (11.8) was found in liquid MS medium supplemented with 1.11 µM BA (Table 2). In contrast to the result obtained on WPM, shoots elongated on MS medium, rendering transfer to a specific elongation medium unnecessary (Fig. 1*A*, *B*).

Shoots obtained on MS medium were more vigorous than those developed on WPM, for all BA concentrations tested. This indicates a differing response of the shoots to the basic media composition. For *Acacia* (Vengadesan *et al*. 2002) and *Anacardium occidentale* (Mneney and Mantell 2002), the MS formula also gave the best results during multiplication. However, for other woody species, such as *Myrica esculenta*, a better multiplication response was observed on WPM (Bhatt and Dhar 2004).

WPM and MS formulas are quite different: WPM medium possesses only 45% of total ionic strength of MS medium, as MS contains 94.21 mM total salts and WPM 41.06 mM. Moreover, the nitrate and ammonia concentrations are different: MS has 39.4 mM NO_3^- and WPM 9.71 mM NO_3^-; MS has 20.61 mM NH_4^+ and WPM 4.94 mM NH_4^+. Therefore, the total N_2 concentration is low in WPM (14.71 mM) when compared to MS medium (60.01 mM).

List *et al*. (1996) tested several concentrations of BA (0.0045, 0.045, 0.45, and 4.5 µM) added to solid MS medium during the *in vitro* multiplication of *M. alternifolia* shoots. The best result was encountered on medium supplemented with 4.5 µM BA, which induced the formation of an average of 5.5 shoots per segment at the end of 9 wk of culture. In the present study, after 8 wk of culture in similar conditions, every explant produced an average of 3.3 shoots. However, the addition of 1.11 µM BA to liquid medium gave a better multiplication rate (11.8) than those reported by List *et al*. (1996) (4.9 and 5.5 at the level of 0.45 and 4.5 µM BA, respectively). In liquid medium, nutrient and BA diffusion and absorption are easier than on solid medium, as was also shown for pineapple micropropagation (Costa and Zaffari 2005).

In vitro rooting. First, three types of auxin at two different concentrations were compared with an auxin-free medium. Adventitious roots were visible after 8 d in auxin-containing medium and after 12 d in auxin-free medium. The results of rooting varied statistically according to auxin presence and concentrations. The rooting rate of shoots kept on an auxin-free medium and on 0.53 µM auxin was 100% and 96%, respectively, and lower (90.6%) in the presence of 2.64 µM auxin (data not shown). The highest auxin concentration induced both a high root number (Table 3) and a large fresh weight of the plants (data not shown).

In *Bambusa atra*, rooting was satisfactory on an auxin-free medium (Ramanayake *et al*. 2008), while in other species, the addition of an auxin to the last medium was necessary. For *Eucalyptus* species, such as *Eucalyptus botryoides*, *Eucalyptus camaldulensis*, *Eucalyptus deglupta*, and *Eucalyptus grandis* (Ito *et al*. 1996), root induction occurred with low concentrations or in the absence of auxin. List *et al*. (1996) tested two concen-

Table 1. Effect of BA on *in vitro* multiplication of *M. alternifolia* after 60 d on WPM (means of two subcultures)

BA concentration (µM)	Shoot number per explant		Necrosis (%)	
	Solid	Liquid	Solid	Liquid
0.00	0.0 eA	0.0 dA	0.0 cB	52.5 deA
0.55	5.5 aA	4.7 aB	5.5 cB	44.5 eA
1.11	4.5 bA	1.0 bcB	4.5 cB	70.5 bA
2.22	3.6 cA	1.2 bB	16.0 bB	60.5 cdA
3.33	3.4 cA	1.0 bcB	31.5 aB	64.5 bcA
4.44	2.7 dA	0.9 cB	40.0 aB	82.5 aA

Means within a *column* followed by the same *lower case letter* and means within a line followed by the same *capital letter* for each parameter are not different at $P<0.05$ by Duncan's test.

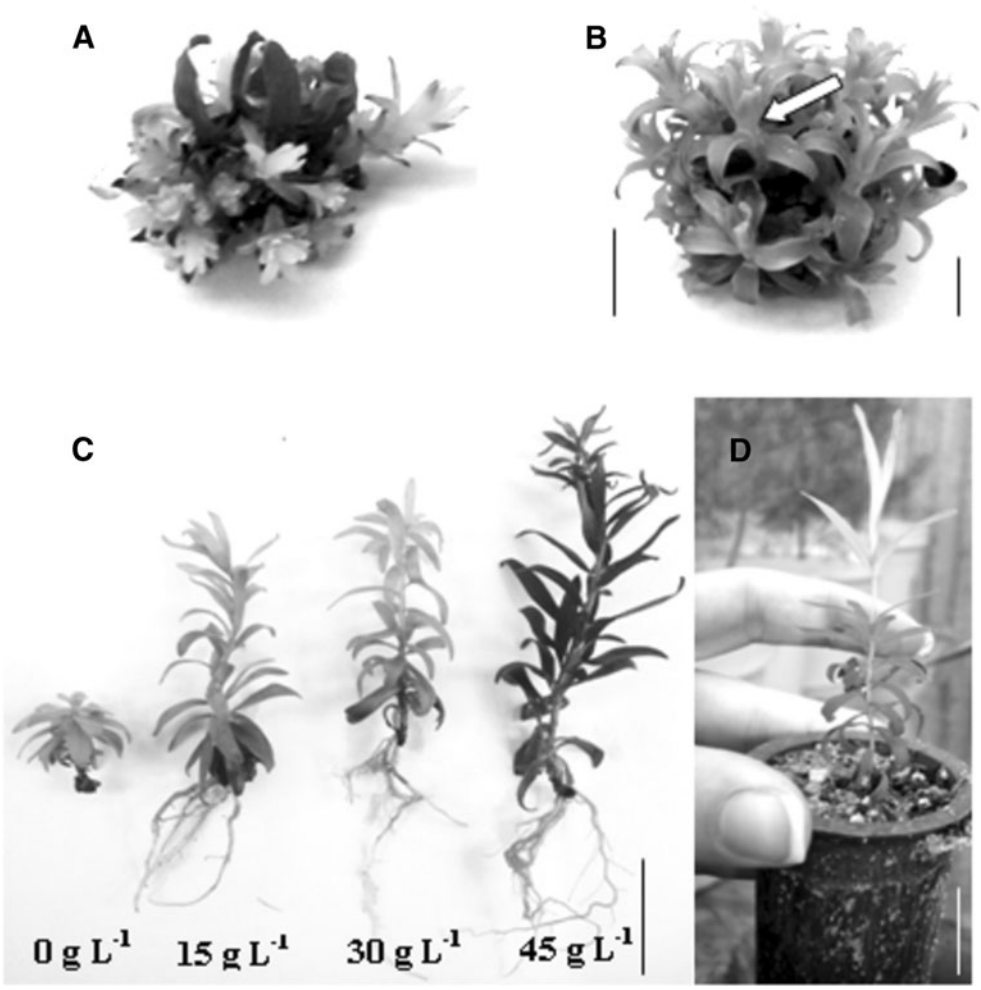

Figure 1. (*A*) *M. alternifolia* shoots cultured for 30 d in solid WPM supplemented with 0.55 μM BA. (*B*) Shoot cluster with elongated shoots after 30 d in liquid MS culture medium supplemented with 1.11 μM BA. (*C*) *M. alternifolia* micro-cuttings rooted in MS culture medium supplemented with different sucrose concentrations. (*D*) *M. alternifolia* plants after a 30-d acclimatization. *Bar* 1 cm.

trations of IAA (0.1 and 1 μM) for *M. alternifolia* rooting in MS solid medium. Both concentrations resulted in a rooting rate of 80% after 8 wk, lower than the values encountered in our study.

In the auxin-free medium, the root number was lower than in auxin-containing media (Table 3). A similar result was found in *Metrosideros excelsa* (Myrtaceae), for which rooting occurs in the absence of auxin (Iapichino and Airò 2008). In our case, the auxin induced big roots or calluses at the shoot base, which is not desirable. Calluses may interfere with root system functionality and hamper plant acclimatization (Compton *et al*. 2001).

Table 2. Effect of BA on *in vitro* multiplication of *M. alternifolia*, after 60 d on MS culture medium (means of two subcultures)

BA concentration (μM)	Shoot number per explant		Necrosis (%)	
	Solid	Liquid	Solid	Liquid
0.00	0.0 Ea	0.0 dA	0 bA	5.2 cA
0.55	5.6 aA	3.2 cB	0 bA	4.8 cA
1.11	5.4 aB	11.8 aA	0 bA	6.0 cA
2.22	4.7 bB	5.1 bA	4 bA	4.0 cA
3.33	4.0 cA	3.2 cB	4 bB	65 bA
4.44	3.3 dA	1.0 dB	12 Ab	100 aA

Means within a *column* followed by the same *lower case letter* and means within a line followed by the same *capital letter* for each parameter are not different at $P<0.05$ by Duncan's test.

Table 3. Effect of auxins on rooting of *M. alternifolia* microcuttings cultured on MS medium

Concentration (μM)	NAA	IAA	IBA
Root number			
0.00	2.3 cA	2.3 cA	2.3 cA
0.53	7.5 bA	4.9 bB	4.5 bC
2.64	8.0 aB	10.1 aA	10.2 aA
Root length (cm)			
0.00	1.7 bA	1.7 bA	1.7 bA
0.53	2.1 aB	2.6 aA	2.4 aA
2.64	1.3 cB	1.7 bA	1.6 bA
Aerial part height (cm)			
0.00	2.3 aB	2.3 bA	2.3 cA
0.53	3.1 aB	3.6 aA	3.3 bAB
2.64	2.7 bB	3.5 aA	3.7 aA

Means within a *column* followed by the same *lower case letter* and means within a line followed by the same *capital letter* are not different at $P<0.05$ by Duncan's test.

Root length was higher in the presence of 0.53 μM auxin, especially when IAA or IBA was used. Growth of the aerial parts was higher on a medium containing either 2.64 μM IAA or IBA (Table 3).

In a second experiment, four auxin-free media were compared: MS and MS/2 with and without AC. MS medium was superior to MS/2 (Table 4). The salt concentration of the culture medium often affects the adventitious rooting process *in vitro*. The high salt concentration of MS medium has been reported to inhibit *in vitro* rooting, even in the presence of auxin (McCown 1988), but did not occur in this study. The reduction of the salt concentration of MS medium decreased the rooting rate from 100% to 64%.

The addition of AC to MS and MS/2 media reduced the rooting percentage and number of adventitious roots per explant, while other variables were poorly affected (Table 4). Several authors showed that AC can adsorb auxins and nutrients present in a culture medium that are needed for rooting and normal development of the aerial parts (McCown 1988; Thomas 2008).

Table 4. Effect of MS salt concentration and activated charcoal on *Melaleuca alternifolia* rooting

Medium	Rooting (%)	Mean root number	Mean root length (cm)	Aerial part height (cm)	Total fresh weight (g)
MS/2	64 b	2.72 a	3.34 a	2.06 c	0.137 c
MS	100 a	3.00 a	2.20 c	3.27 a	0.189 a
MS/2+AC	28 c	1.36 b	3.32 a	2.60 b	0.148 c
MS+AC	36 c	1.18 b	2.78 b	3.42 a	0.165 b

Means within a *column* for each parameter followed by the same *lower case letter* are not different at $P<0.05$ by Duncan's test.

In a third experiment, the effect of sucrose on rooting was tested. With regard to the rooting rate, sucrose was most effective at 30.8 gL^{-1} (Fig. 2*a*). These results further demonstrated that carbohydrates affect adventitious root formation, thus confirming the results of other researchers. For Cydomalus (*Malus communis*×*Cydonia oblonga*), rooting was difficult in sucrose-free medium (De Paoli *et al*. 2002). Similar to our results, those of Lane (1978) showed that sucrose concentrations under 20 gL^{-1} and greater than 50 gL^{-1} negatively affected apple tree rooting. According to George (1996), *in vitro* root formation requires the energy and carbohydrates that are either provided by photosynthesis (in auxotrophic conditions) or by an exogenous sugar sink (in heterotrophic or mixotrophic conditions).

In *Asparagus racemosus*, 30 gL^{-1} sucrose was sufficient for root induction (Bopana and Saxena 2008), while for *Passiflora edulis*, the presence of 20 or 30 gL^{-1} sucrose resulted in the same rooting rate (Isutsa 2004). These results demonstrate a variation in the rhizogenic response of different species. However, the exact relation between auxins, carbohydrates, and rooting is complex and has not yet been entirely elucidated.

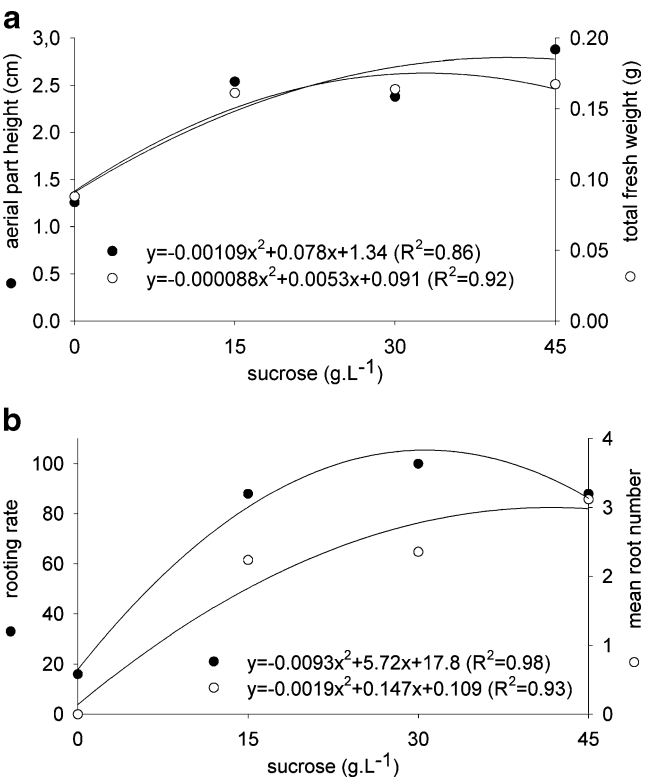

Figure 2. Effect of sucrose concentration on *M. alternifolia* rooting. (*a*) Rooting rate and mean root number; (*b*) aerial parts height and total fresh weight.

For tea tree, the explants cultured in the absence of sucrose showed the lowest rooting rate (ca. 20%) (Fig. 2*a*). This is probably caused by the lack of starch accumulation in the plant tissue, which is promoted by the presence of sucrose in culture medium (Li and Leung 2000).

The rooting rate, plant height, and fresh weight increased up to the concentration of 30 gL^{-1} sucrose (Figs. 2*a*, *b*), but tended to decrease above that concentration. During the rooting process, carbohydrates are a source of energy and carbon for the synthesis of compounds essential for organogenesis; moreover, sugars function as an osmotic agent (Verma and Dougall 1977).

During acclimatization, all microcuttings that rooted on MS medium supplemented with 30 gL^{-1} sucrose survived after 60 d, while the plants derived from other treatments had a lower survival rate (data not shown). Sucrose was therefore important for providing energy and carbon products during the acclimatization process and for new root initiation and elongation. The survival of plants rooted in the presence of auxin was low (data not shown) and the roots were easily broken during transfer to substrate. Furthermore, in these cases, roots may not be functional. Another factor that may have affected the survival rate was the callus formation at the cut base observed in several plants, caused by the long contact of the explant with auxin (30 d).

Microcuttings cultured on MS/2 medium had a survival rate of 80% after 60 d of acclimatization (data not shown). The same survival rate (80%) was obtained in *M. alternifolia*, using microcuttings cultured in full-strength MS medium with 0.1 or 1.0 μM IAA (List *et al*. 1996). Nevertheless, in our study, microcuttings cultured in full-strength MS in the absence of auxin reached a 100% survival rate during acclimatization (Fig. 1*D*).

Conclusion

We recommend MS liquid medium supplemented with 1.11 μM BA for *in vitro* multiplication of *M. alternifolia*, as this medium promoted the highest rate of shoot proliferation (11.8 shoot per stem after 60 d), higher than the seven plants per stem obtained by List *et al*. (1996) after 140 d. Moreover, no symptoms of hyperhydricity were observed. An elongation phase was found to be unnecessary in these multiplication conditions. Ample rooting can be achieved on a PGR-free MS culture medium with 30 gL^{-1} sucrose, followed by acclimatization with a high survival rate.

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References

Bhatt I. D.; Dhar U. Factors controlling micropropagation of Myrica esculenta buch.–Ham, ex D. Don: a high value wild edible of Kumaun Himalaya. Afric. *J Biotechnol* 3: 534–540; 2004.

Bopana N.; Saxena S. In vitro propagation of a high value medicinal plant: Asparagus racemosus Willd. *In Vitro Cell Dev Biol Plant* 44: 525–532; 2008.

Compton M. E.; Pierson B. L.; Staub J. E. Micropropagation for recovery of Cucumis hystrix. *Plant Cell Tiss Organ Cult* 64: 53–67; 2001.

Costa T. D.; Zaffari G. R. Micropropagation of Ananas bracteatus (Schultz) var.striatus Hort. *Rev Bras Hortic Ornam* 11: 109–113; 2005.

De Paoli G.; Subirà E.; Battistini A. In vitro rooting of Pyrodwarf and Cydomalus, two rootstocks for pear, under photoautotrophic conditions. *Acta Hortic* 596: 463–467; 2002.

EMBRAPA. Núcleo Tecnológico para Informática. SOC–Software Científico. Campinas, 1990.

George E. F. Plant propagation by tissue culture. Part 2: In Practice. 2nd ed. Exegetics, Edington, p 1361; 1996.

Homer L. E.; Leach D. N.; Lea D.; Lee L. S.; Henry R. J.; Baverstock P. R. Natural variation in the essential oil content of Melaleuca alternifolia Cheel (Myrtaceae). *Biochem Syst Ecol* 28: 367–382; 2000.

Iapichino G.; Airò M. Micropropagation of Metrosideros excelsa. *In Vitro Cell Dev Biol Plant* 44: 330–337; 2008.

Isutsa D. K. Rapid micropropagation of passion fruit (Passiflora edulis Sims.) varieties. *Sci Hortic* 99: 395–400; 2004.

Ito K.; Doi K.; Tatemichi Y.; Shibata M. Plant regeneration of Eucalyptus from rotating nodule cultures. *Plant Cell Rep* 16: 42–45; 1996.

Kiong A. L. P.; Huan H. H.; Hussein S. Callus induction from leaf explants of Melaleuca alternifolia. *Intern J Agric Res* 2: 227–237; 2007.

Lane W. D. Regeneration of apple plants from shoot meristem tips. *Plant Science Letters* 13:3 281–285, 1978.

Li M.; Leung D. W. M. Starch accumulation is associated with adventitious root formation in hypocotyl cuttings of Pinus radiata. *J Plant Growth Regul* 19: 423–428; 2000.

List S. E.; Brown P. H.; Low C. S.; Walsh K. B. A micropropagation protocol for Melaleuca alternifolia (tea tree). *Aust J Exp Agric* 36: 755–760; 1996.

Lloyd G.; McCown B. Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture. *International Plant Propagator's Society. Proceedings* 30: 421–427; 1980.

McCown B. H. Adventitious rooting of tissue cultured plants. In: Davis T. D.; Haissig B. E.; Sankhla N. (eds) Adventitious root formation in cuttings, vol. 2. Dioscorides, Portland, pp 289–302; 1988.

Mneney E. E.; Mantell S. H. Clonal propagation of cashew (Anacardium occidentale L.) by tissue culture. *J Hortic Sci Biotechnol* 6: 649–657; 2002.

Murashige T. Plant propagation through tissue cultures. *Annu Rev Plant Physiol* 25: 135–166; 1974.

Murashige T.; Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant* 15: 473–497; 1962.

Ramanayake S. M. S. D.; Maddegoda K. M. M. N.; Vitharana M. C.; Chaturani G. D. G. Root induction in three species of bamboo with different rooting abilities. *Sci Hortic* 118: 270–273; 2008.

Riedl, R. W. Practical methods for using tea tree oil. Agro-Food Industry/Hi Tech. Ballina, set/oct, p. 34–36, 1997.

Thomas T. D. The role of activated charcoal in plant tissue culture. *Biotechnol Adv* 26: 618–631; 2008.

Vengadesan G.; Ganapathi A.; Amutha S.; Selvaraj N. In vitro propagation of Acacia species—a review. *Plant Sci* 163: 663–671; 2002.

Verma D. C.; Dougall D. K. Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. *Plant Physiol* 59: 81–85; 1977.