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ORIGINAL ARTICLE

Micropropagation of *Melaleuca alternifolia* by shoot proliferation from apical segments

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Abstract

Key message **The addition of cytokinin drastically increases shoot proliferation in *Melaleuca*. Individual in vitro shoots previously treated with BA showed better rooting development than BA-free culture medium. Reduction of the osmotic potential in the culture medium decreased dehydration of micropropagated plantlets.**

Abstract *Melaleuca alternifolia*, known as tea tree, is an Australian medicinal plant widely used in the cosmetic and pharmaceutical industries due to its antibacterial and antifungal properties. Propagation of *Melaleuca* is limited due to low rates of seed germination and multiplication and the poor rooting of stem cuttings. Thus, micropropagation can be an alternative to the propagation of this woody medicinal species. In this study, different concentrations of 6-benzyladenine (BA) (0, 0.55, 1.11 and 2.22 µM) were tested during the in vitro multiplication phase. It was observed that even the lowest concentration of BA (0.55 µM) could drastically increase the multiplication rate in *Melaleuca*, compared to the BA-free treatment, due to multiple shoot proliferation. At the rooting stage, in the culture medium without phyto-regulators, individual shoots previously treated with BA had a higher rooting percentage (91–97%) and considerable height growth compared to those of the control treatment (without BA) (66%). However, none of the in vitro plantlets survived to acclimatization stage due to excessive and rapid dehydration of the plantlets under ex vitro conditions, making it the most challenging phase for the micropropagation of *Melaleuca*. Therefore, a second experimental setup was designed, which included treatments with sucrose, sucrose + sorbitol and sucrose + mannitol in the culture medium at − 0.2170, − 0.3255 and − 0.4340 MPa, respectively, to determine the effects of these osmotic agents on the development of *Melaleuca* in rooting and acclimatization stages. Sorbitol with sucrose at $\Psi\pi = -0.4340$ decreased stomatal density in leaves and reduced dehydration of plantlets under ex vitro conditions, but was not enough to provide successful plantlets acclimatization.

Keywords *Melaleuca alternifolia* · Woody medicinal plant · Multiplication · Acclimatization · Water stress · Osmotic agents

Introduction

Melaleuca is a genus belonging to the Myrtaceae family comprising more than 240 species (The Plant List 2020). *Melaleuca alternifolia*, known as tea tree worldwide, is a species native to New South Wales, Australia (Butcher et al. 1994). It is a small evergreen tree (List et al. 1995) with smooth leaves containing oil glands (Holliday 2004; Biasi and Deschamps 2009). The main product of this plant with medicinal properties is the essential oil, known as tea tree oil (TTO) (Kiong et al. 2007), which is widely used in the formulations of many products, such as soaps, shampoos, gels, moisturizers and cleaning products (Yadav et al. 2016), due to its antibacterial (Cox et al. 2000), antifungal (Hammer et al. 2006), anti-inflammatory (Hart et al. 2000),

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antiviral (Carson et al. 2008) and antiparasitic properties (Baldissera et al. 2014) and commonly used as an antiseptic agent for acne treatment (Hammer et al. 2006). Australia is the world's largest producer of TTO, with the production of around 900,000 kg of pure oil in 2016/17, totaling $35.32 million US (AgriFutures 2020).

Sexual propagation of *Melaleuca* by seeds precludes the complete inheritance of the desired characteristics of the mother plant (Chen et al. 2016), such as high oil contents and oil composition in leaves, resulting in non-uniform progenies, affecting composition and productivity of TTO and difficulty the clonal propagation of new genetic materials with potential for the commercial production (Raut and Karuppayil 2014; Uchoi et al. 2018). Clonal propagation using cuttings is rare in *Melaleuca* establishment because it is not economically viable on a large scale when compared to rapid seedling production systems (Uchoi et al. 2018), in addition to low multiplication and rooting rate of cuttings (Guo 2007).

In comparison to the conventional seedling production of tea tree, a promising plantlet production system for *Melaleuca* species would be micropropagation, which is an efficient technique for rapid and large-scale multiplication and production of clonal micropropagated medicinal plants derived from explants obtained from high-quality and superior genotypes (Cardoso et al. 2019).

In micropropagation, cytokinins have been widely used to increase induction of multiple shoots in micropropagated plantlets, aiming at large-scale micropropagation and among these, benzyladenine (BA) is the most used one for several plant species, including *Melaleuca*. List et al. (1996) examined the effects of different concentrations of BA in solid Murashige and Skoog (1962) culture media on the in vitro multiplication of *Melaleuca* shoots and found the highest number of shoots per segment with 4.5 µM of BA. Oliveira et al. (2010) noticed a greater shoot multiplication rate in liquid MS medium with 1.11 µM of BA than in solid medium with 0.55 µM of BA. Jala and Chanchula (2014) observed that the number of leaves and roots per plant reached the highest values in MS medium supplemented with 2.22 µM of BA combined with 10.74 µM of naphthaleneacetic acid (NAA). Chen et al. (2016) reported a good multiplication rate using the MS culture medium containing 1.33 µM of BA and 0.81 µM of NAA.

Plantlet acclimatization represents another challenging phase in micropropagation of tree species. Since the in vitro and ex vitro conditions are completely different, there is an increase in plant transpiration rates, resulting in loss of water (Lima-Brito et al. 2016) during the acclimatization process, which may cause a reduction in vigor and survival of plants when transferred from the in vitro to ex vitro conditions (Cha-um et al. 2010). High mortality is observed after the ex vitro transfer of micropropagated plantlets because, in general, these plantlets have non-functional stomata and poorly developed root system and cuticle (Indravathi and Babu 2019); thus, this phase is considered critical in micropropagation due to plant losses (Bag et al. 2019). When plantlets are subjected to a pre-acclimatization stage, there is a reduction in the damages observed in the plantlets after transfer to ex vitro conditions, which represents successful acclimatization (Chandra et al. 2010; Cardoso et al. 2013).

Pre-acclimatization can be conducted using different strategies, including the use of chemical substances in the culture medium to assist in the pre-hardening process of the plantlets. It is important to emphasize that the water stress conditions cause an increase in the biosynthesis of organic osmolytes (Masouleh et al. 2019; Per et al. 2017). Sorbitol is one of the most commonly found polyols in plants and a photosynthesis product found in mature leaves, besides sucrose (Jain et al. 2010).

Due to its low molecular weight, sorbitol can be easily dissolved in the culture medium (Dubois and Inzé 2020), acts as an osmotic regulator (Muñoz et al. 2019) and induces water stress responses in plants by decreasing osmotic potential ($\Psi\pi$) of the culture medium (Marssaro et al. 2017; Razavizadeh and Adabavazeh 2017). Thus, addition of sorbitol and mannitol to culture medium simulates water stress conditions in vitro, thereby leading to plant responses such as stomatal closure, reduction in water transpiration and consequently, optimization of the acclimatization stage (Ellouzi et al. 2014).

Based on the difficulties encountered previously in conventional propagation of *Melaleuca* and the low number of papers reporting micropropagation and acclimatization of this important medicinal and industrial crop, the main aim of this study was to establish an efficient protocol for clonal micropropagation of *Melaleuca* using shoot tips as explants, with the objective of increasing the efficiency of shoot proliferation, rooting and, specially, the acclimatization of plantlets under greenhouse conditions. Also, this study reported the influence of osmotic agents on rooting and acclimatization of this species.

Materials and methods

Donor plants and in vitro establishment of *Melaleuca*

Young sprouts were collected from a single source, that is, a mature *Melaleuca* tree (12 years old) in a commercial plantation, and were subsequently rooted in the pots containing a pine bark-based substrate. One-year-old greenhouse stem cutting-derived plants were used as donor plants for the in vitro experiments. Apical shoots approximately 2 cm in length were used for the establishment of the in vitro

culture. Surface disinfestation was performed by immersion of apical shoots in 70% ethanol for 30 s, followed by sodium hypochlorite solution (1.0–1.25% active chlorine) for 15 min and three consecutive washes with previously autoclaved deionized water. The length of apical shoots was reduced to 2–3 mm while retaining the shoot tip and they were placed vertically inside 240–250 mL glass flasks containing 35 mL of the semi-solid woody plant medium (WPM) (Lloyd and McCown 1980) with 4.44 µM of BA and 0.54 µM of NAA. The shoots were subcultured in the same culture medium every 14 days, three times; the browned tissues were removed and only green tissues were subcultured. Developed in vitro apical shoots were subcultured every 30 days on the WPM medium without phyto-regulators until a sufficient number of shoots for further experiments.

The effect of BA on shoot and root development

An experiment was carried out to determine the effect of cytokinin BA on the in vitro multiplication of *Melaleuca* and also on the quality and development of shoots during the next rooting phase. Nodal segments and shoot tips approximately 1.0 cm in length (microcutting) from the previous establishment phase were excised, subcultured and used as explants for this experiment. Different concentrations of BA (0, 0.55, 1.11 and 2.22 µM) were used to evaluate shoot multiplication in MS culture medium with half of the concentration of macronutrients (MS½) and with 20.0 g L^{-1} of sucrose. The pH was adjusted to 5.8 before the addition of 6.4 g L^{-1} agar as the gelling agent. Glass flasks (240–250 mL) covered with transparent polypropylene caps and containing 35 mL of semi-solid culture medium were autoclaved at 120 ºC and 1 kgf cm^{-2} for 25 min.

The experiment was conducted in a completely randomized design with seven flasks per treatment and each flask containing four microcuttings (each ≅ 1 cm in length) (four replicates), which were kept for 63 days at 26 ± 1 ºC and a 16-h photoperiod under the white and red (1:1) light-emitting diode (LED) lamps with an irradiance of 30–35 µmol m^{-2} s^{-1}.

After 63 days of culture, except for the four replicates from each treatment that were used to measure fresh and dry weight, microcuttings from each treatment were subcultured on the rooting medium consisting of MS½ medium supplemented with 20.0 g L^{-1} sucrose and 1.0 g L^{-1} activated charcoal without phyto-regulators. The pH was adjusted to 5.8 before the addition of 6.4 g L^{-1} agar as a gelling agent. The medium was autoclaved 120 ºC and 1 kgf cm^{-2} for 25 min. Shoots were cultured for 35 days under the same conditions as for the previous phase.

During multiplication phase, shoot length and numbers of shoots per nodal segments, as well as the final multiplication rate, were evaluated weekly. The number of multiple shoots produced per nodal segment was used as the variable "shoot proliferation", while the number of new 1-cm long microcuttings obtained from each explant was used as variable "multiplication rate". Fresh and dry weights were also measured 63 days after culture using the precision analytical balance Mettler ML201 (Mettler, Switzerland). To measure dry weight, clumps of shoots were dried in a drying oven at 65 ºC for 24 h. Similarly, at the completion of rooting phase (35 days), shoot length, number of roots per plantlet, rooting rate and fresh weight were evaluated.

The data obtained were subjected to Analysis of Variance (ANOVA), followed by the Tukey multiple comparison test at a 1% significance level using AgroEstat online software (Barbosa and Maldonado Jr 2015).

Acclimatization of micropropagated *Melaleuca* plantlets

The rooted plantlets (2.37 ± 0.65 cm in length) were removed from culture medium, washed gently under running tap water to remove culture medium from the roots and then immersed in a pre-treatment solution containing 800 mg L^{-1} fertilizer Plant-Prod 20–20–20 + micro (Plant Products, Leamington, Canada), 200 mg L^{-1} calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$), 150 mg L^{-1} magnesium sulfate ($MgSO_4{\cdot}7H_2O$), 0.22 µM BA and 1 mL L^{-1} Dioxiplus (Dioxide, Indaiatuba, Brazil) for 30 min before acclimatization to the ex vitro conditions.

The plantlets were acclimatized in the substrates Carolina Soil® (Carolina Soil, Pardinho, Brazil) and vermiculite (4:1), arranged in plastic trays with 128 cells, which were maintained in a greenhouse at the temperature range of 28.2 ± 6.8 ºC and relative air humidity of 71.3 ± 21.2% [measured by thermo-hygrometer (Incoterm®, Porto Alegre, Brazil)], controlled by the pad-fan cooling system and 50% Aluminet® shading. The plantlets were irrigated with tap water daily using micro-sprinklers and fertirrigated once a week with solution containing 1000 mg L^{-1} Plant-Prod 20–20–20 + micro, 300 mg L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$ and 250 mg L^{-1} $MgSO_4{\cdot}7H_2O$. Pad-fan cooling systems help in the maintenance of temperature in greenhouse. These greenhouse and environmental conditions are the standard conditions used for the successful acclimatization of other micropropagated plantlet species, such as banana, pineapple, orchids and others (> 90% survival). However, *Melaleuca* showed a recalcitrance response to acclimatization in these standard conditions. After rapid dehydration and no survival of *Melaleuca* plantlets under these conditions, we proposed a new experiment with osmotic agents for improving the acclimatization stage of *Melaleuca*, which is described below.

Effect of osmotic agents on the rooting and acclimatization of *Melaleuca*

The purpose of this experiment was to evaluate the effect of osmotic agents and reduced osmotic potential in the culture medium on the in vitro rooting of micropropagated shoots and acclimatization of plantlets of *Melaleuca.* Among the few studies conducted with *Melaleuca*, a study by Oliveira et al. (2010) reported difficulties in acclimatization of micropropagated plantlets, mainly related to low survival rate, similar to the findings of the previous experiment conducted using conventional acclimatization protocol for *Melaleuca*. These findings indicate that acclimatization was the most significant limiting factor for micropropagation of this species.

Microcuttings derived from rooting medium were subcultured to fresh rooting medium plus different concentrations of osmotic agents and a pH adjusted for 5.8 before the addition of 6.4 g L^{-1} agar. Sucrose, sorbitol and mannitol were used as osmotic agents. The culture media with osmotic potential values of − 0.2170, − 0.3255 and − 0.4340 MPa, equivalent to concentrations of 30.0, 45.0 and 60.0 g L^{-1} of sucrose, respectively, were used. The nine treatments consisted of 100% sucrose, 50% sucrose + 50% sorbitol and 50% sucrose + 50% mannitol in the culture medium for each osmotic potential (Table 1). The use of 50% mannitol or sorbitol instead of the complete replacement of sucrose prevented the complete absence of sucrose in the culture medium, which would otherwise be detrimental as sucrose is the main source of energy under in vitro conditions (photomixotrophy). Each treatment consisted of five flasks, each with four microcuttings (four experimental replicates), cultivated in osmotic treatments for 58 days. The experiment was performed as a completely randomized factorial design with three values of osmotic potential and three concentrations of carbohydrates.

Table 1 Osmotic potentials and the sucrose, sorbitol and mannitol concentrations used for each treatment composition

Osmotic potential (MPa)	Sucrose (g L^{-1})	Sorbitol (g L^{-1})	Mannitol (g L^{-1})
	30.0	–	–
− 0.2170	15.0	15.0	–
	15.0	–	7.99
	45.0	–	–
− 0.3255	22.5	22.5	–
	22.5	–	11.98
	60.0	–	–
− 0.4340	30.0	30.0	–
	30.0	–	15.97

The weekly measurements of shoot height, number of roots per plantlet and rooting rate were performed. After the removal of plantlets from the culture medium, fresh weight was measured using a precision analytical balance Mettler ML201 (Mettler, Switzerland).

The dehydration of plantlets was evaluated through the quantification of fresh weight loss after removal of plants from the in vitro culture flasks. Plantlet fresh weight was measured soon after removed from in vitro conditions, every 15 min and until 90 min, totalizing seven evaluations. During this period, plantlets were kept on trays with laboratory filter paper moistened with distilled water at a 26.3 ± 0.51 °C and relative humidity of 70.3 ± 3.1% (measured by thermo-hygrometer Incoterm®) to simulate the acclimatization under greenhouse conditions. Four plantlets from each treatment (four replicates) were used for this evaluation.

In addition, stomatal density on the abaxial leaf surface of *Melaleuca* was measured using the optical microscope Nikon Eclipse 201 with 400 × magnification, coupled with the high-resolution (5 Mp) camera Opticam. Leaves of the fourth–sixth nodes were collected after the removal of the plants from the in vitro culture flasks, followed by immediate fixing in Carnoy 3 (ethanol):1 (glacial acetic acid) solution (*v/v*) for 48 h and then storing the samples in 70% alcohol solution at 8 °C until evaluation. For stomata visualization, leaves were immersed in 70% alcohol for one minute, followed by 5 M potassium hydroxide solution at 45 °C for 20–30 s. For counting, four fields of view (0.06 mm^2) were analyzed per sample.

All data obtained from the experiments were analyzed as indicated in item 2.2, except for the correlation analysis between the fresh weight of plantlets and the duration of exposure to ex vitro conditions, which was realized using Microsoft Excel (Windows) software (version 1911).

Acclimatization was performed using ten rooted plantlets from each treatment that were acclimatized, as described in item 2.3. However, due to the difficulties observed previous in the acclimatization process, plantlets were incubated in a moist chamber with higher relative air humidity, constructed using a transparent 600-mL capacity square plastic container covered with a plastic lid that generated constant higher relative humidity (85 ± 2%) than greenhouse conditions, using the same substrate but replacing the plastic trays with 128 cells used previously in "Acclimatization of micropropagated *Melaleuca* plantlets" section (Fig. 1).

Nine moist chambers were constructed for each of the nine treatments with ten rooted plantlets per chamber. These moist chambers were maintained under greenhouse conditions similar to those described in "Acclimatization of micropropagated *Melaleuca* plantlets" section.

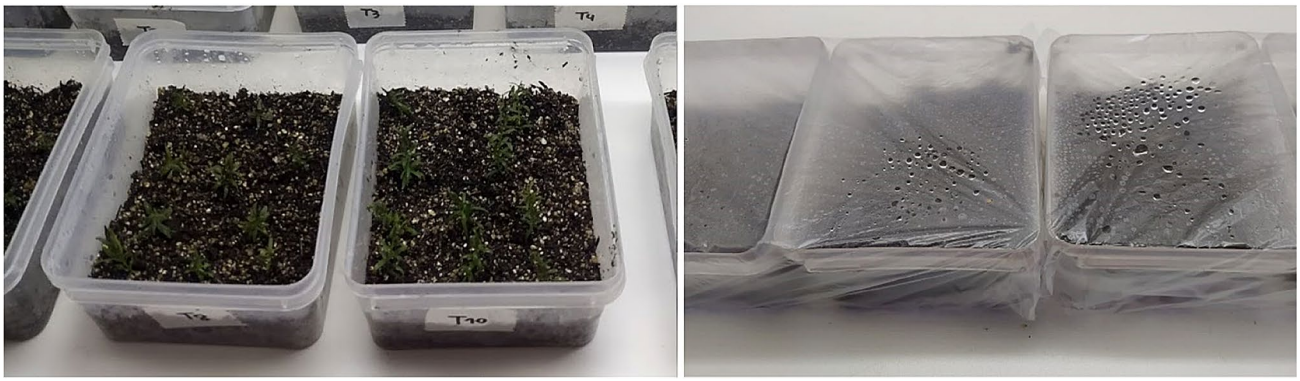

Fig. 1 Moist chamber with high relative humidity (85 ± 2%) created with plastic containers for acclimatization of *Melaleuca alternifolia*

Results

In vitro establishment of *Melaleuca*

Around 70% of the apical shoots were developed in vitro without microbial contamination (data not provided). Thus, the surface disinfestation protocol used, along with the use of explants from greenhouse-grown donor plants, was efficient for the in vitro establishment of *Melaleuca*.

After 30 days on in vitro culture, microcuttings were obtained by allowing the shoots to elongate on culture medium without cytokinin, allowing the production of more nodal segments for the experiments.

Effect of 6-benzyladenine (BA) on the in vitro shoot proliferation and development of *Melaleuca*

The cytokinin 6-benzyladenine (BA) had a significant effect on the in vitro development of *Melaleuca*. Even in the culture medium containing BA at the lowest concentration (0.55 µM), shoot proliferation increased to 6.54 shoot/explant, compared to the value of 1.32 shoots/explant in the BA-free medium. At the BA concentrations of 1.11 µM and 2.22 µM, shoot proliferation increased to 7.93 and 7.28, respectively. The corresponding multiplication rates were 7.5, 8.8 and 8.8 microcuttings/explant at 0.55, 1.11 and 2.22 µM of BA in the culture media, while it was 4.4 microcuttings/explant in the BA-free medium (Table 2; Fig. 2a).

The addition of BA to the culture medium, regardless of concentration, also increased 100% of dry matter in the clumps of shoots treated with 1.11 µM BA, compared to the control treatment (BA-free medium). However, in the presence of the highest concentration of BA, the shoot height was drastically reduced, from 3.55 (control) to 0.40 cm (2.22 µM). Interestingly, treatment resulting in a lower increase in height (BA at 2.22 µM) presented the highest fresh and dry weights, which could be explained by the highest shoot proliferation and stem development (Fig. 2a; Table 2).

The subculturing of shoots on rooting medium, in the absence of cytokinin, resulted in an interesting and significant residual effect of BA during the rooting and elongation phases. Despite the reduced size of the shoots in

Table 2 Effect of 6-benzyladenine (BA) on shoot multiplication of *Melaleuca alternifolia* evaluated after 63 days in MS½ medium

BA concentration (µM)	Height increase (cm)	Shoot proliferation rate[1]	Multiplication rate[2]	Mean fresh weight[3] (g)	Mean dry weight[3] (g)
0	3.55 a*	1.32 b	4.375 b	0.081 b	0.012 b
0.55	1.24 b	6.54 a	7.50 a	0.130 ab	0.022 a
1.11	0.90 b	7.93 a	8.75 a	0.157 a	0.024 a
2.22	0.40 c	7.82 a	8.75 a	0.177 a	0.022 a
p value	< 0.0001	< 0.0001	< 0.0001	0.0151	0.0040
CV (%)	65.95	12.94	15.20	10.67	5.69

*Means with different letters within a column are significantly different according to ANOVA and Tukey's test at 1% probability

[1]Mean number of shoots per explant

[2]Mean number of microcuttings per explant

[3]Mean fresh and dry weight of clumps of shoots

Fig. 2 Micropropagation of *Melaleuca alternifolia.* **a** *Melaleuca* shoots cultured in MS½ multiplication media containing concentrations of BA (µM) and BA effects on shoot proliferation, elongation and root formation. **b** *Melaleuca* shoots derived from MS½ multiplication culture media containing different concentrations of BA (µM) cultured in MS½ rooting medium, without plant growth regulators containing 1.0 g L^{-1} of activated charcoal

Table 3 Effect of 6-benzyladenine (BA) used in multiplication phase on the next rooting phase of *Melaleuca alternifolia* cultured in MS½ without plant growth regulators and addition of 1 g L^{-1} activated charcoal

BA concentration during multiplication phase (µM)	Rooting phase		
	Height increase (cm)	Number of roots per plant	Rooting rate (%)
0	1.38 b*	1.09 b	65.71
0.55	2.37 a	2.29 a	91.43
1.11	2.59 a	2.09 a	91.43
2.22	2.34 a	2.89 a	97.14
p value	0.0044	0.0009	
CV (%)	51.01	69.03	

*Means with different letters within a column are significantly different according to ANOVA and Tukey's test at 1% probability

BA-containing medium, their subculturing on rooting medium without BA resulted in well-developed shoots, with increased shoot height, rooting rate and the number of roots per plantlet compared to those in BA-free medium (Table 3; Fig. 2b).

Acclimatization of micropropagated *Melaleuca* plantlets

Although the best results were obtained from the in vitro micropropagation phase, the acclimatization of micropropagated plantlets has proved to be the most challenging phase for this technique, with all plantlets appearing dehydrated and brown-black, indicating the death of tissues during the first 48 h of ex vitro culture, which was caused mainly by the fast and excessive dehydration of tissues.

Effect of osmotic agents and osmotic potential on rooting and acclimatization stage

The effect of osmotic agents and osmotic potential during rooting phase was evaluated to solve the problem of acclimatization.

The effect of partial replacement of sucrose with sorbitol and mannitol on the in vitro development of *Melaleuca* shoots was important and resulted in a significant decrease in shoot height, plantlets fresh weight and root length. However, the reduction in osmotic potential was found to affect leaf stomatal density, although the final rooting percentage remained unaffected (Tables 4, 5 and 6; Fig. 3). Partial replacement of sucrose with sorbitol or mannitol was the most effective factor related to the reduced dehydration of micropropagated plantlets under ex vitro conditions. The treatments consisting of 30.0 g L^{-1} sucrose + 30.0 g L^{-1} sorbitol and 30.0 g L^{-1} sucrose + 15.97 g L^{-1} mannitol showed the lowest reduction in dehydration rates (%) under ex vitro conditions, preventing excessive dehydration of micropropagated plantlets of *Melaleuca* (Fig. 4b, c).

Stomatal density was affected by both osmotic agents and osmotic potential, as well as the interaction between these two factors (Table 6). The increase in sorbitol concentration in the culture medium led to a gradual reduction in stomatal density in the leaves of *Melaleuca*, while mannitol reduced it only in treatment with the lowest osmotic potential (− 0.4340 MPa).

Even with the positive effects of sorbitol and mannitol, which reduced plantlet dehydration under ex vitro

Table 4 Growth and rooting of *Melaleuca alternifolia* during the in vitro culture under different osmotic agents (sucrose, sorbitol and mannitol) on semi-solid MS½ rooting medium

Osmotic agent concentration (g L^{-1})	Ψπ (MPa)	Height increase (cm)	Mean fresh weight per plantlet (g)	Number of roots per plant	Root length (cm)
30.0 g L^{-1} sucrose 15.0 g L^{-1} sucrose + 15.0 g L^{-1} sorbitol 15.0 g L^{-1} sucrose + 7.99 g L^{-1} mannitol	− 0.2170	1.42 a	0.0685 a	1.61 a	2.86 ab
45.0 g L^{-1} sucrose 22.5 g L^{-1} sucrose + 22.5 g L^{-1} sorbitol 22.5 g L^{-1} sucrose + 11.98 g L^{-1} mannitol	− 0.3255	1.52 a	0.0645 a	1.77 a	3.54 a
60.0 g L^{-1} sucrose 30.0 g L^{-1} sucrose + 30.0 g L^{-1} sorbitol 30.0 g L^{-1} sucrose + 15.97 g L^{-1} mannitol	− 0.4340	1.53 a	0.1023 a	1.78 a	2.65 b
	DMS (5%)	0.5397	5.0535	0.2113	

*Means with different letters within a column are significantly different according to ANOVA and Tukey's test at 1% probability

Table 5 Growth and rooting of *Melaleuca alternifolia* during the in vitro culture on semi-solid MS½ rooting culture medium containing sucrose or the one with partial replacement of sucrose with sorbitol and mannitol

Carbohydrate (g L^{-1})	Height increase (cm)	Mean fresh weight per plantlet (g)	Number of roots per plantlet	Root length (cm)
100% sucrose (30, 45, 60)	2.37 a	0.1204 a	1.84 a	4.09 a
50% sucrose + 50% sorbitol (15 + 15, 22.5 + 22.5, 30 + 30)	1.06 b	0.0583 b	1.63 a	2.53 b
50% sucrose + 50% mannitol (15 + 7.99, 22.5 + 11.98, 30 + 15.97)	1.04 b	0.0567 b	1.70 a	2.43 b
DMS (5%)	0.5397	5.0535	0.2113	

*Means with different letters within a column are significantly different according to ANOVA and Tukey`s test at 1% probability

conditions, successful acclimatization could not be achieved and all plantlets died during the first 48 h of ex vitro culture in a growth chamber with high relative humidity (85 ± 2%). Similar observations were obtained in the previous experiment using conventional acclimatization under a greenhouse.

Discussion

The propagation methods for *Melaleuca,* such as stem cutting and seed germination, present several difficulties, such as low rooting rates, slow seed germination and genetic variability in seedlings, characteristics that are undesirable for its essential oil application in pharmaceutical and cosmetics industries. Thus, these conventional propagation methods fail to meet the high demand for *Melaleuca* plantlets related to oil productivity and composition (Chen et al. 2016; Doran et al. 2006; Huynh et al. 2016; Shepherd et al. 2013). Micropropagation of the medicinal plant species is currently the fastest and most efficient technique to produce, on a large scale, clonal and disease-free plantlets from the previously selected genotypes (Gosal and Wani 2018; Tripathi et al. 2019; Reshi et al. 2017). Moreover, micropropagation enables the production of plant-derived medicinal compounds (PMDCs), which are of interest to the target industry (Cardoso et al. 2019; Espinosa-Leal et al. 2018; Mukta et al. 2017). However, to increase economic viability, some factors as multiplication, rooting and successful acclimatization rate of the in vitro plantlets are required for the improved efficiency of the technique (Brunda et al. 2017; Cardoso et al. 2018; Uchoi et al. 2018).

The addition of BA affected the in vitro development of *Melaleuca* shoots

The cytokinin BA had significant effects on the in vitro development of *Melaleuca* shoots. In general, addition of BA to the culture medium, even in low concentration (0.55 µM), resulted in considerable increases in the shoot proliferation, from 1.32 (BA free) to 6.54 shoot/explants (with BA), through induction and development of multiple axillary/adventitious shoots (Table 2). Similar results were obtained with *Melaleuca alternifolia*, with the best shoot proliferation (5.6 shoots/explants) as a result of the addition of 1.11 µM BA to the MS medium (Oliveira et al. 2010). The BA has also been proved to be efficient

Table 6 Stomatal density and rooting rate of *Melaleuca alternifolia* in treatments with different osmotic potentials and osmotic agents

Ψπ (MPa)	Osmotic agents	Rooting rate (%)	Number of stomata mm^{-2}
	Sucrose	40.0	163 Cb
− 0.2170	Sucrose + sorbitol	30.0	313 Aa
	Sucrose + mannitol	25.0	271 Aa
	Sucrose	69.0	292 Aa
− 0.3255	Sucrose + sorbitol	30.0	196 Bb
	Sucrose + mannitol	40.0	271 Aa
	Sucrose	65.0	229 Ba
− 0.4340	Sucrose + sorbitol	20.0	121 Cc
	Sucrose + mannitol	65.0	167 Bb
Osmotic agent			31.08**
Osmotic potential			5.04*
Interaction			25.67**
CV (%)			2.56

Each mean represents four replicates (leaves). Means within a lowercase (osmotic agents) or lines (osmotic potential) followed by the same letter are not significantly different according to Tukey's test at 5% (*) and 1% (**) levels of probability

for shoot multiplication in other Myrtaceae species, such as *Campomanesia xanthocarpa* (Machado et al. 2020) and *Syzygium francissi* (Shatnawi et al. 2004). Moreover, the microcutting-based multiplication of *Melaleuca* (4.4 microcuttings/explant) without the addition of BA was achieved in this study, which was achieved by shoot elongation, instead of axillary/adventitious shoot proliferation, followed by the generation of 1-cm-longer new nodal segmentation, referred to as microcuttings. This technique provides an alternative for multiplication of woody plants, especially those that do not respond to cytokinin in the culture medium or when the use of BA results in undesirable effects, such as hyperhydricity in the in vitro development of shoots or plantlets (Cardoso and Teixeira da Silva 2013; Duarte et al. 2019).

In this study, an increase of up to 3.55 cm in shoot height of *Melaleuca* was observed with the use of MS½ medium without the addition of BA, which resulted in the multiplication rate of 4.4 despite low axillary/adventitious shoot proliferation (1.32). Oliveira et al. (2010) found that no shoot proliferation of *Melaleuca* occurred in both MS and WPM culture media without BA.

Jala and Chanchula (2014) observed practically no effects of BA (until the concentration of 2.22 µM) on shoot proliferation of *Melaleuca* (3.1 to 3.3 shoots/explant) and did not report reduction of shoot height as a result of the addition of this cytokinin to the MS culture medium. In contrast, results obtained from the present study showed a strong negative effect of 2.22 µM of BA on the shoot height, with the reduction from 3.55 to 0.40 cm, in response to the larger number of shoots from 1.32 to 7.82. Oliveira et al. (2010) also reported reduction in shoot length in *Melaleuca* in response to the addition

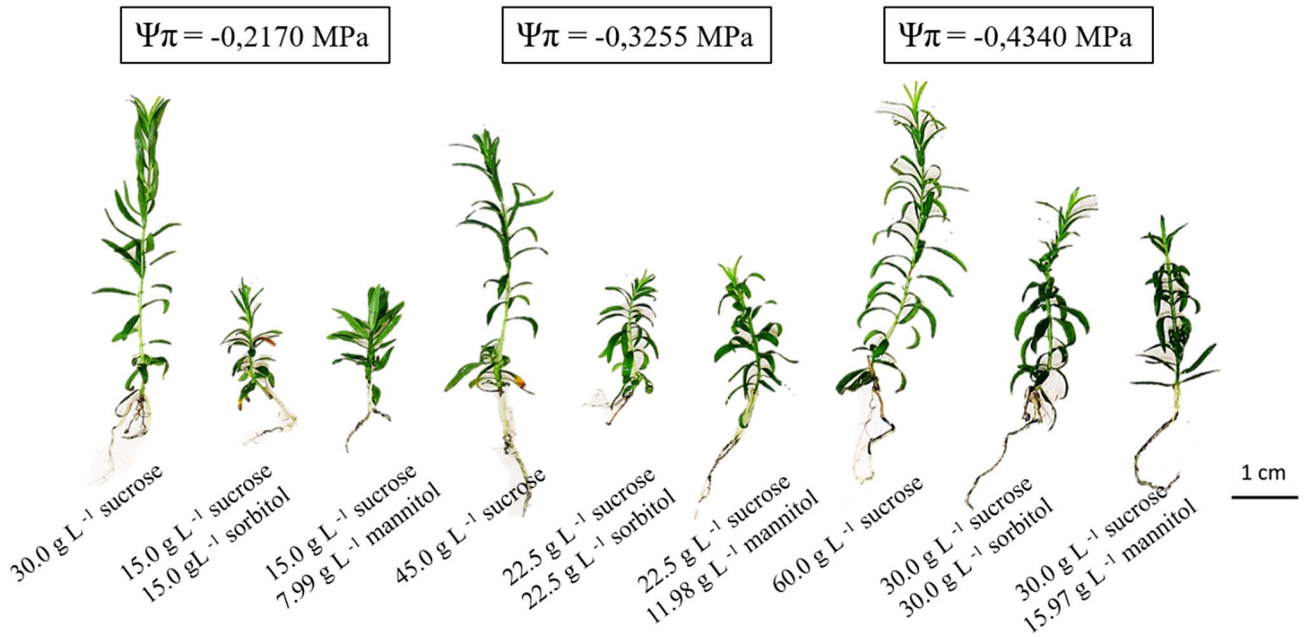

Fig. 3 Effects of different osmotic agents (sucrose, sorbitol and mannitol) and osmotic potential on in vitro development of *Melaleuca alternifolia* shoots/plantlets

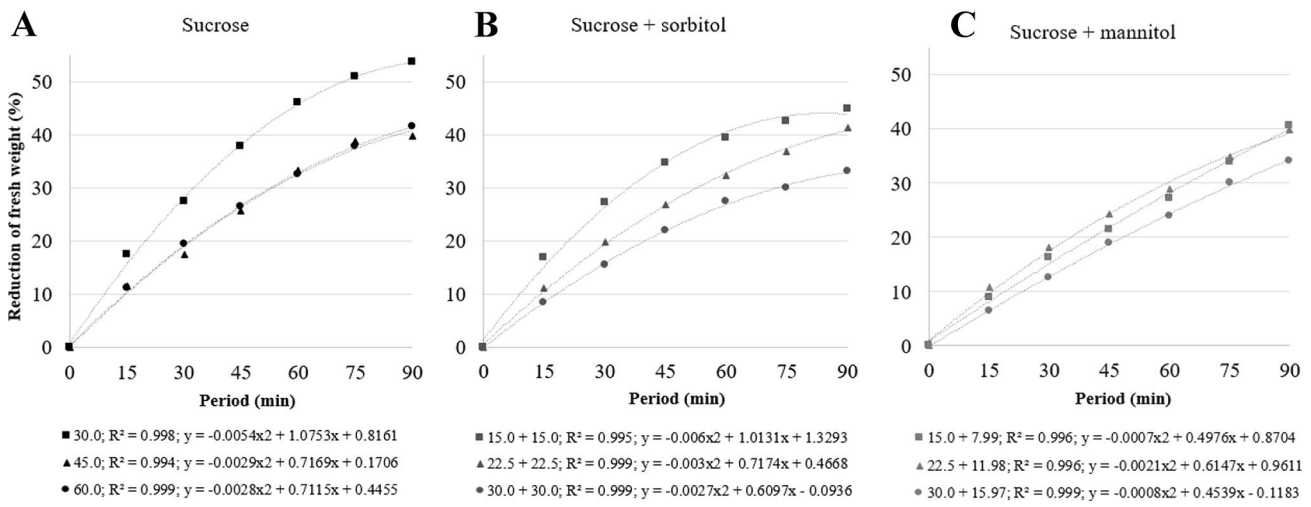

Fig. 4 Reduction in the fresh weight (dehydration) of *Melaleuca alternifolia*. **a** Effect of sucrose (30.0, 45.0 and 60.0 g L^{-1}) addition to the rooting MS½ medium; **b** Effect of sucrose and sorbitol combinations (15.0 + 15.0, 22.5 + 22.5 and 30.0 + 30.0 g L^{-1}) added to the rooting MS½ medium; **c** Effect of sucrose and mannitol combinations (15.0 + 7.99, 22.5 + 11.98 and 30.0 + 15.97 g L^{-1}) on the plantlets cultured under similar conditions of acclimatization

of BA to the culture medium. This reduction of shoot length in response to BA treatments was also observed in other Myrtaceae species, such as *Myrtus communis* (Cioć et al. 2018) and five plant populations produced from the crosses between *Corymbia torelliana* and *C. citriodora* (Hung and Trueman 2012).

Another undesirable effect associated with the addition of BA in the culture medium is the inhibitory effect on rooting. This inhibitory effect of BA on root formation was observed with *Melaleuca* in the presence of BA in the present study (Fig. 2a), similar to the results reported previously for *Myrtus communis* L. in the MS medium containing 0.89 µM BA (Canhoto et al. 1999). The cytokinin BA has been reported as an inhibitor of the in vitro rooting induction of shoots under in vitro conditions for different woody species (Bennet et al. 1994; Gentile et al. 2017).

However, interestingly, in the rooting phase, the *Melaleuca* plants derived from BA treatments had better rates of rooted shoots (91.4–97.1%) and the number of roots/shoots (2.1–2.9) compared to plants from the BA-free medium (65.7% and 1.1 roots/explants) after subculturing on MS½ medium containing 1.0 g L^{-1} activated charcoal (Fig. 2; Table 3). Such results were scarcely reported in the literature and involved the complex mechanism of interaction between the synthesis process of auxins and cytokinins (Jones and Ljung 2011).

In this study, the values of rooting percentage in shoots exposed to BA (> 90%) were higher than those reported by Oliveira et al. (2010), who reported achieving a rooting rate of 64% in *Melaleuca* with the use of MS½ without the activated charcoal and a rate of 100% using auxin-free MS medium, resulting in the conclusion that the addition of auxin to the culture medium was not needed for in vitro rooting of this species. However, Chen et al. (2016) reported that 100% of the *Melaleuca* shoots were rooted and the addition of 0.49–1.23 µM of Indolebutyric acid (IBA) to the MS½ for rooting resulted in 2.9–3.3 roots per plant.

Plantlets of *Melaleuca* grown on medium containing 1.11–2.22 µM of BA resulted in the highest multiplication rate in multiplication phase and in over 90% of shoots producing adventitious roots in rooting phase. Therefore, multiplication in culture medium containing 1.11–2.22 µM of BA along with the subculturing of shoots on the rooting medium with activated charcoal proved to be the best alternative to improve the efficiency of micropropagation of *Melaleuca.*

Acclimatization proved to be the most challenging phase of the micropropagation of *Melaleuca*

The conventional micropropagation protocol was not efficient for acclimatization of *Melaleuca* in the greenhouse under controlled temperature using the pad-fan cooling system, which has been conventionally used with successful results for the micropropagation of other species, such as banana, pineapple and orchids, using the mean temperature of 28.2 ± 6.8 ºC and relative air humidity of 71.3 ± 21.2%.

Rapid leaf dehydration was observed within the first 60 min after the removal of plantlets from the in vitro culture, followed by the subsequent darkening of both leaves and stem within the first 24 h of culturing in the substrate, regardless of the treatment. Moreover, the growth chamber with higher relative humidity (> 90%) did not provide any improvements and failed to solve the problems observed previously.

The low survival rate of plantlets during acclimatization of *Melaleuca*, related to excessive dehydration, can be the result of non-functional stomata and poorly developed leaf cuticle (Sáez et al. 2012). Acclimatization has been demonstrated to be the most challenging phase of micropropagation of woody plants (Tisarum et al. 2018a) like *Melaleuca*. The main limitation of this phase was the low survival rate, similar to that observed with the other woody species. In *Parasponia andersonii*, with 100% rooting (in vitro) of plantlets, the survival rate of only 20% was reported during acclimatization (Knyazev et al. 2018); similarly, in *Betula lenta* L., survival rate was 37% (Rathwell et al. 2016) and in *Prunus mume*, only 20–30% of the in vitro-rooted plantlets survived to acclimatization (Harada and Murai 1996).

Montalvo et al. (2010) also reported difficulties faced by two species belonging to *Melaleuca* family (Myrtaceae), including *Eugenia squarrosa* and *Eugenia subdisticha*, during acclimatization in a substrate based on organic matter and zeolite (4:1) due to the high sensitivity of plantlets to changes in relative humidity that caused the loss of water in the first few days.

Interestingly, Oliveira et al. (2010) reported that in vitro-derived microcuttings of *Melaleuca alternifolia*, previously cultured on MS½ medium, had a survival rate of 80–100% during acclimatization in the substrate in a greenhouse. These results are contradictory with those obtained in the current study with the same species and culture medium, in which the in vitro-rooted plantlets did not survive to acclimatization in the greenhouse with controlled temperature regulated using the pad-fan cooling system. However, the authors (Oliveira et al. 2010) also reported lower survival rates from other rooting treatments in media with auxins or other concentrations of sucrose, suggesting that *Melaleuca* plantlets exhibit a certain degree of recalcitrance to acclimatization.

Plants grown in the conventional in vitro micropropagation system had poorly developed shoots and roots, non-functional stomata, thin and small leaves, high water content and limitations of photosynthesis (Cha-um et al. 2010; Xiao et al. 2011). Therefore, due to the differences between the in vitro and ex vitro conditions, which lead to these physiological and morphological disorders in the in vitro plants, there may be a great decrease in plant survival rates during acclimatization (Cha-um et al. 2010; Hazarika 2006).

Osmotic agents affected in vitro plantlet development and reduced dehydration rate in *Melaleuca*

The osmotic agents, sorbitol and mannitol affected the in vitro shoot and root development of *Melaleuca*; however, osmotic potential did not affect these parameters. Similar results were observed for pitaya (Tisarum et al. 2018b) and apricots (*Prunus armeniaca*), as sorbitol affected in vitro shoot development but not due to the osmotic effects (Marino et al. 1993).

The highest increase in shoot height, percentage of rooting shoots, root length and total fresh weight was recorded with sucrose, while the combination of sucrose with sorbitol or mannitol had negative effects on in vitro shoot and root development (Table 5; Fig. 3); total fresh weight was reduced up to 53% compared to that in sucrose treatment (Table 5).

Moreover, these osmotic agents can also contribute to the hardening of in vitro plantlets during acclimatization as the micropropagated plantlets are very susceptible to changes in the abiotic and biotic factors, such as a reduction in relative humidity, a wider range of temperature, higher light intensity and lower water, nutrient uptake and contact with microorganisms, when transferring from in vitro to ex vitro environments (Teixeira da Silva et al. 2017). This susceptibility is also the consequence of morphological, anatomical and physiological characteristics of in vitro micropropagated plantlets, such as poorly developed cuticle and reduced capacity of stomatal closure, leading to uncontrolled water loss from these plants in the new environment (Kumar and Rao 2012; Teixeira da Silva et al. 2017).

The inhibitory effect of sorbitol or mannitol on the dehydration rate of *Melaleuca* plantlets compared to that in the sucrose-containing medium was observed in the present study (Fig. 4b, c). Furthermore, with the use of these osmotic agents, a positive correlation ($y = 0.0666x + 23.576$, $r = 0.911^{**}$) was found between the reduction in stomatal density in leaves and decrease in the dehydration rates in micropropagated *Melaleuca* plantlets; however, the same correlation was not detected for sucrose treatments. As an example, the addition of sucrose at 30.0 g L^{-1} (control) resulted in the lowest stomatal density (121 stomata mm^{-2}) but the highest dehydration rate (53% of initial rate) (Fig. 4a).

It can be suggested that sorbitol and mannitol not only caused a reduction in dehydration rate partially due to the lower stomatal density, but also they provide better control of stomatal closure under ex vitro conditions as the reduction in stomatal density because of the addition of sucrose at 30 g L^{-1} was not sufficient to decreases the dehydration rate. Sorbitol was used as an osmotic agent and proved to be highly efficient to evoke slightly stomatal closure in *Arabidopsis* compared to abscisic acid (ABA) (Leshem et al. 2010).

The results of the present study showed that sorbitol affected both the plant development in terms of stomatal density of newly forming leaves and its metabolism by reducing the rapid dehydration rate of micropropagated plantlets during acclimatization (Table 6; Fig. 4). However, it was not enough to result in a successful

acclimatization of *Melaleuca alternifolia*. The death of in vitro-derived *Melaleuca* plantlets during acclimatization would be the consequence of other undesirable effects of sorbitol to plant metabolism that limited shoot development since under in vitro conditions. Although there was observed a reduction in dehydration rate, other unexpected effects of sorbitol plus sucrose treatment were low rooting rate of shoots (Table 6) and poor in vitro shoot and root development, with similar results observed for other tree species, such as *Prunus armeniaca* (Marino et al. 1993). In *Olea europea*, osmotic stress using sorbitol at 0.2 M increased tissue osmolality and lipid peroxidation, and decreased water, soluble protein, and chlorophyll contents, as well as rooting was completely inhibited by sorbitol (Brito et al. 2003).

Conclusion

The results of the present study showed that it is possible to obtain an efficient protocol for the in vitro micropropagation of *Melaleuca* on a culture medium containing BA at a concentration of 1.11 µM. Considering a period of 180 days (five subcultures) in the multiplication medium, it would be possible to obtain more than 52,000 plantlets from a single apical shoot in a period of six months and around 48,000 plantlets at the acclimatization stage. However, acclimatization was the most challenging stage and had the highest impact with great losses of the micropropagated *Melaleuca* plantlets due to excessive and rapid dehydration of these plantlets. The combination of sucrose and sorbitol in rooting medium, both at 30.0 g L^{-1} significantly reduced dehydration rates of *Melaleuca* plantlets under acclimatization conditions but strongly affected rooting development and was not sufficient to obtain the successful acclimatization of the plantlets. Further studies are needed to improve acclimatization of woody species as *Melaleuca,* with the aim of discovering the main factors affecting plant losses and to provide successful acclimatization of micropropagated *Melaleuca*.

Author contribution statement Material preparation, data collection and analysis were performed by CMI. The first draft of the manuscript was written by CMI. JCC provided critical revision of the article, provided final approval of the version to publish. Both contributed substantially to the conception and design of the study and data analysis and interpretation.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflicts of interest.

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