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IN VITRO GROWTH OF *MELALEUCA ALTERNIFOLIA* CHEEL IN BIOREACTOR OF IMMERSION BY BUBBLES

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Abstract

Development of new techniques for *in vitro* culture is necessary to optimize and reduce the production costs. The aim of this work was to introduce the bioreactor BIB® (Bioreactor of Immersion by Bubbles) and compare it with conventional culture and bioreactor RITA® (Recipient with Automatized Temporary Immersion) for *In vitro* growth of *M. alternifolia* plantlets. Plantlets with 1.0 cm height from the fifth *In vitro* subculture were used. Treatments were: (1) conventional culture (control) using 200 mL glass flasks covered with polypropylene lids; (2) bioreactor RITA® and (3) bioreactor BIB®. Continuous immersion and aeration was used in bioreactors. It was used liquid MS medium supplemented with 30 g.L^{-1} sucrose and 1 drop.200 mL^{-1} Tween 20® (i.e. detergent for bubbles formation). BIB® obtained best results for total fresh mass (0.39 g) and shoot fresh mass (0.27 g), fresh mass increment (3.1), total dry mass (0.036 g) and shoot dry mass (0.033 g) than RITA® and conventional culture. In conclusion, BIB® is more efficient to the biomass accumulation of *M. alternifolia* plantlets.

Introduction

Plant *In vitro* culture using bioreactors enables the production of genetically identical individuals from cells and tissues extracted from an elite plant, that allows plant multiplication in large scale and free of pathogens, in less time as compared to conventional method and also reduced the per seedling cost (Soccol *el at*., 2008). The use of liquid culture media also decrease the production cost (Scheidt *et al*., 2009). Moreover, bioreactors increases multiplication rate using liquid culture media, which automate the process and decreases labor (Roels *et al*., 2006). Several bioreactors have been used for tissue culture, such as RITA (Recipient with Automatized Temporary Immersion) (Alvard *et al*., 1993), bubble column bioreactor (Takayama *et al*., 1991), temporary immersion systems (Teixeira, 2002) and TRI-bioreactor (Afreen *et al*., 2002).

We introduce a new bioreactor for use in tissue culture, the BIB® (Bioreactor of Immersion by Bubbles), this bioreactor consists of Blindex® glass cylinder, and it has two chambers divided transversely by a porous plate (porosity from 170 to 220 µm). The lower chamber has 3.5 cm tall and contains an opening to the air. The higher chamber has 24.5 cm tall, and all the internal components are made of stainless steel. The bioreactor has 28 cm tall and 9 cm in diameter. This equipment utilizes a system interlinked by hoses of flexible rubber for air flow and plant tissues receive culture medium by bubbles (i.e. formed by detergent), this bioreactor is protected by brazilian patents (Soccol *et al*., 2008).

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). The main product of the tea tree is an essential oil possessing antimicrobial and anti-inflammatory properties (Kiong *et al*., 2007) therefore, used in pharmaceutical industry, cosmetics and toiletries. *In vitro* growth of plantlets facilitates the research of physiological effects of nutrients, elicitors and plant growth regulators.

The aim of this work was to introduce the bioreactor BIB® and compare it with conventional culture and bioreactor RITA® for *in vitro* growth of *M. alternifolia* plantlets.

Materials and Methods

Melaleuca alternifolia Cheel was *in vitro* established and propagated via direct organogenesis (Oliveira *et al*., 2010). Plantlets with 1.0 cm height were used. Treatments were: (1) conventional culture (control) using 200 mL glass flasks covered with polypropylene lids; (2) bioreactor RITA® and (3) bioreactor BIB® (Soccol *et al*., 2008). Continuous immersion and aeration were used in bioreactors (Fig. 1). It was used liquid MS medium (Murashige & Skoog, 1962) supplemented with 30 g.L^{-1} sucrose and 1 drop Tween 20® (i.e. detergent for bubbles formation) per 200 mL^{-1} culture medium, and the pH was adjusted to 5.8. It was used 200 mL of the culture medium in the bioreactors and 20 mL per flask (control). Media were sterilized at 1 atm and at 120 °C for 20 minutes.

Total fresh mass (g), shoot fresh mass (g), root fresh mass (g), fresh mass increment (i.e. relation between the final fresh mass and initial fresh mass), shoot dry mass (g), root dry mass (g), total dry mass (g), root total length (cm), root volume (cm^3), root number, shoot height (cm) and root mean length (cm) (i.e. relation between the root total length and root number) were evaluated after 21 days of *in vitro* culture. Root length was measured with aid of WINRHYZO® equipment and dry mass was determined after treatment in stove at 80° C for 24h.

The cultures were kept at 25 ± 2ºC under white fluorescent light (32 µM m^{-2} s^{-1}) with a 16 h photoperiod. The experimental design was completely randomized with four replicates of six plantlets. The data were submitted in a normality analysis for the Lilliefors´s test and, analysis of variance (ANOVA) followed by Duncan's test at a P< 0.05. All statistical analyses were done following the procedures of the software GENES (Cruz, 2001). Variables from counting were transformed to $\sqrt{x+0.5}$.

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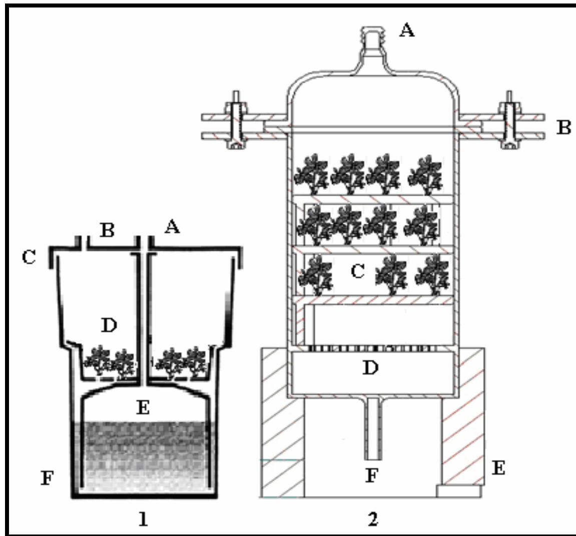

Fig. 1. Schematic representation of the bioreactors (1) A: Air Entrance; B: Air Exit; C: Kit Fixing; D: Steps; E: Internal Base; F: Base and (2) A: Air Exit; B: Kit Fixing; C: Steps; D: Porous Plates; E: Base; F: Air Entrance.

Results and discussion

Hyperhydricity was not observed in any plant, hyperhydricity induce somewhat abnormalities in the developing plantlets (Haq & Dahot, 2007). Various factors can be involved with the hyperhydricity occurrence; however, the most common is the liquid culture. It is possible that this tolerance can be associated with the plant habitat; usually it grows in swamp environment.

Values obtained for root volume and root dry mass did not show statistical differences for all treatments (Table 1). These values vary from 0.025 to 0.038 cm^3 and from 0.022 to 0.026 g for root volume and root dry mass, respectively. Bioreactors (BIB® and RITA®) did not show statistical differences for root fresh mass, on the other hand, conventional culture (control) was significantly lower for root fresh mass than bioreactors. Similar results were obtained with many species using bioreactors (Ziv, 2005).

Root number and root total length did not show statistical differences among BIB® and control, and also among BIB® and RITA®, although control was higher than RITA® for root number and root total length (Table 1). Shoot height varies from 4.4 to 5.9 cm and there are statistical differences for RITA® and BIB®, while control was not significant with RITA® and BIB®. Root mean length was not significant for control and BIB®, however BIB® and control was significant with relation to the RITA® (Table 1). It may be associated with the fact that roots in conventional culture are in direct contact with the culture medium, allowing greater access to water and nutrients (Etienne-Barry *et al*., 1999).

BIB® obtained best results for total and shoot fresh mass, fresh mass increment, total and shoot dry mass (Table 1). These results can associated with larger internal space of the BIB® with relation to the RITA® and flasks used for conventional culture. Moreover, larger internal space can allow inside greater concentration of the exogenous CO_2 came from out air. When air is fed into the bioreactor, it provides CO_2 that can be fixed during photosynthesis, consequently promoting a greater accumulation of the dry mass.

Table 1. *In vitro* growth of *Melaleuca alternifolia* plantlets in different culture systems after 21 days. Total fresh mass (TFM), shoot fresh mass (FMS), root fresh mass (FMR), fresh mass increment (FMI), root total length (TLR), root volume (VR), shoot dry mass (DMS), root dry mass (DMR) total dry mass (TDM), root number (NR), shoot height (HS) root mean length (MLR).

Treatments	TFM (g)	FMS (g)	FMR (g)	FMI	TLR (cm)	VR (cm^3)
BIB®	0.390 a[1]	0.267 a	0.035 a	3.1 a	7.07 ab	0.035 a
RITA®	0.273 b	0.142 b	0.036 a	2.4 b	4.61 b	0.028 a
Control	0.217 c	0.132 b	0.032 b	1.9 c	8.39 a	0.035 a
Treatments	**DMS (g)**	**DMR (g)**	**TDM (g)**	**NR**	**HS (cm)**	**MLR (cm)**
BIB®	0.033 a	0.022 a	0.036 a	4.6 ab	5.9 a	1.5 a
RITA®	0.023 b	0.023 a	0.025 b	4.1 b	4.4 b	1.1 b
Control	0.019 c	0.026 a	0.022 b	5.1 a	4.6 ab	1.6 a

[1]Treatments with the same letters at the columns did not differ by the Duncan´s test at the level of 5% of error

Gaseous conditions, in particular those of CO_2 and O_2 affect culture growth, conventional culture is practically closed system in which gas exchange is slow and as the culture grows, O_2 diminishes and CO_2 builds up. In contrast, bioreactor is supplied with a constant flow of air, which can keep the dissolved oxygen level high and thus, growth of plant cultures in bioreactor increases in biomass to increase productivity (Sajid & Pervaiz, 2008).

Traditionally, it has been considered that *in vitro* plants present low photosynthetic rates and use carbohydrates from culture medium as carbon source (Kozai *et al*., 1997). Nevertheless, *heliconia* plantlets cultured in bioreactors with aeration have their multiplication rate elevated (Rodrigues *et al*., 2006). Another factor can have contributed with larger biomass accumulation in the plantlets cultured in BIB® was higher transparency than RITA®. High transparency allow larger light intensity on the plantlets what increases photosynthetic rate. This idea already was demonstrated in *Cymbidium in vitro* cultured, where the environment was enriched with 2000 $mg.L^{-1}$ CO_2 and two light intensities was tested, 35 $\mu M.m^{-2}.s^{-1}$ and 200 $\mu M.m^{-2}.s^{-1}$, nevertheless, the higher photosynthetic rate was with 200 $\mu M.m^{-2}.s^{-1}$ of light intensity (Kozai *et al*., 1987). In conclusion BIB® is more efficient to the biomass accumulation of *M. alternifolia* plantlets.

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