Scientific Article

Bioreactor in the micropropagation of ornamental pineapple[1]

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ABSTRACT

Ornamental pineapple is a hard plant with significant landscaping value. Typically, conventional propagation is performed by clump division with low yields, and may even spread diseases. Plant tissue culture is viable, yielding plants with a high phytosanitary and genetic quality over a short time period. This study aimed to verify the *in vitro* multiplication of ornamental pineapple plants (*Ananas comosus* var. bracteatus L.) in different micropropagation systems, in association with BAP concentrations. Plants with about 2 cm were used, transplanted to the different treatments: bioreactor, natural ventilation and conventional micropropagation, combined with 3 BAP concentrations (0, 1 and 2 mg L^{-1}). The basic medium used consisted of MS salts. The highest number of shoots and *in vitro* culture growth were obtained with the use of bioreactor and culture medium containing 2 mg L^{-1} BAP. The temporary immersion bioreactor allows air renewal inside the bottles, leading to a better performance of *in vitro* cultivation of ornamental pineapple, when compared to conventional micropropagation.

Keywords: *Ananas comosus* var. bracteatus L., in vitro multiplication, temporary immersion, natural ventilation system.

RESUMO

Biorreator na micropropagação de abacaxi ornamental

O abacaxi ornamental é uma planta rústica e de alto valor comercial. Normalmente, a propagação convencional é realizada por divisão de touceira apresenta baixo rendimento e ainda podendo causar disseminação de doenças. A cultura de tecidos da espécie mostra-se viável, produzindo plantas com alta qualidade genética e fitossanitária em curto espaço de tempo. O presente trabalho teve por objetivo avaliar a multiplicação *in vitro* de plantas de abacaxi ornamental (*Ananas comosus* var. bracteatus L.) em diferentes sistemas de micropropagação em associação com concentrações de benziloaminopurna (BAP). Foram utilizadas plantas com cerca de 2 cm de comprimento, transplantadas para os diferentes tratamentos: biorreator, ventilação natural e convencional, combinados com 3 concentrações de BAP (0, 1 e 2 mg L^{-1}). O meio básico utilizado foi o composto pelos sais do meio MS. Maior número de brotos e crescimento da cultura *in vitro* foram obtidos com o emprego do biorreator e meio de cultura contendo 2 mg L^{-1} de BAP. O biorreator de imersão temporária permite a renovação do ar no interior dos frascos possibilitando melhor desempenho do cultivo *in vitro* do abacaxi ornamental quando comparado à micropropagação convencional.

Palavras-chave: *Ananas comosus* var. bracteatus L., multiplicação *in vitro*, imersão temporária, sistema de ventilação natural.

1. INTRODUCTION

Ornamental pineapple (*A. comusus* var. bracteatus L.) belongs to the Bromeliaceae family, and it is the most economical and the third most commercialized species in the world, grown in Thailand, Costa Rica, Brazil, the Philippines, Indonesia and India (FAO, 2013).

A. comosus var. bracteatus is a native species widely used in landscape compositions to delineate areas or beds (OLIVEIRA et al., 2010); it is perennial and easily cultivated, appreciated for the beauty of its leaves and inflorescence. This species is vegetative propagated by clump division, which can spread diseases such as fusariosis (SILVA et al., 2007).

In plant propagation, each plant produces up to ten seedlings per year, and the demand for healthy seedlings is a limiting factor for crop expansion (CORREIA et al., 2000). Zepeda and Sagawa (1981) studied pineapple and estimated that an axillary bud can yield about 5,000 plants per year through micropropagation. However, plants coming from tissue culture techniques are still very expensive and difficult to acquire. This can be basically attributed to the production costs in the laboratory, especially with the intense use of labor and losses during acclimatization (MOREIRA et al., 2013).

Bioreactors are systems used in plant micropropagation, and can be a viable alternative for process optimization and reduction in production costs. This system allows air renewal in the *in vitro* environment, increasing the production of propagated plant biomass, reducing the time required for *in vitro* propagation and labor (MALLÓN et al., 2012; ZHAO et al., 2012; MOREIRA, 2013).

Another way to enable photoautotrophic micropropagation would be *in vitro* cultivation, with the use of caps or flasks that allow gas exchange, which is called the natural ventilation system (MOHAMED and ALSADON,

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2010). The beneficial effects of natural ventilation are due to the reduction in *in vitro* relative humidity (IVANOVA and VAN STADEN, 2010; SILVA et al., 2016), to the increase in gas exchange with the external atmosphere (MOHAMED and ALSADON, 2010; SILVA et al., 2016) and the reduction in water availability, besides producing more rustic *in vitro* plants, reducing seedling losses during the acclimatization phase (SILVA et al., 2016).

Therefore, this study aimed to evaluate the *in vitro* multiplication of *A. comosus* var. bracteatus L. in conventional micropropagation systems, natural ventilation and temporary immersion bioreactor, in association with different BAP concentrations.

2. MATERIAL AND METHODS

A. comosus var. bracteatus L. plants were obtained by the cultivation of axillary buds. The basic culture medium consisted of MS salts (MURASHIGUE and SKOOG, 1962), added with 1.0 mg L^{-1} BAP and 30 g L^{-1} sucrose, solidified with 6 g L^{-1} agar and pH adjusted to 5.8 before autoclaving at 121 °C for 20 min. The *in vitro* cultivation was maintained in a growth room at a temperature of 24 ± 2 °C, 12-hour photoperiod and luminous intensity of 36 µmol photons m^{-2} s^{-1} (PAR).

After 60 days of cultivation, plants set in the previous phase were standardized at about 2 cm in length and inoculated in the different treatments (Figure 1). The treatments consisted of micropropagation systems (conventional, natural ventilation and bioreactor), combined with different concentrations of benzylaminopurine - BAP (0, 1 and 2 mg L^{-1}). The experimental design was completely randomized (CRD), in a factorial scheme consisting of 3 (micropropagation systems) x 3 (BAP concentrations), totaling 9 treatments, with 4 replicates and 5 plants as an experimental unit.

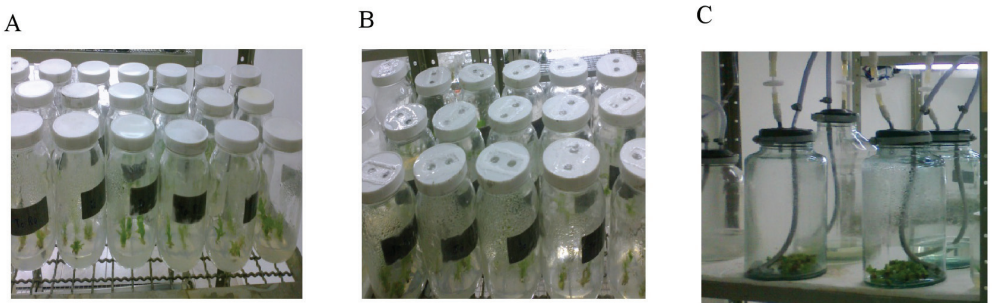

Figure 1. Conventional Micropropagation; (B) Natural Ventilation System; Temporary Immersion Bioreactor (C) in *A. comosus* var. *bracteatus* L. plants.

In conventional micropropagation (CM), normal caps were used to close the flasks, which did not allow gas exchange (Figure 1A). In the natural ventilation system (NVS), filter membranes (Milli Seal, Millipore, Tokyo, Japan - 0.5µm) were used to cover two holes (10 mm diameter) in culture flasks (Figure 1B). For CM and NVS, 500 mL bottles were used; 70 mL of culture medium were distributed into each flask and inoculated with 5 explants per flask.

The bioreactor consisted of two vials connected by silicone tubes, so that one flask served as a culture medium reserve and the other for *in vitro* plant growth (Figure 1C), similar to the BIT® twin-flask system, as described by Silva et al. (2007). Culture medium (700 mL) was placed in a 5 L flask and 20 explants were inoculated per flask. In the temporary immersion, aeration was performed every 180 min for a period of 3 min. In all micropropagation systems, the pH of the culture medium was adjusted to 5.8 before autoclaving at 121 ºC for 20 min and a pressure of 1 atm.

After 90 days of *in vitro* cultivation, the plants were evaluated according to the following variables: shoot length, number of shoots, number of shoots greater or equal than to 1 cm, plant fresh and dry matter. Data were submitted to analysis of variance (ANOVA) and the means were compared by the Scott-Knott test at 5% probability, using the SISVAR statistical software (FERREIRA, 2011).

3. RESULTS AND DISCUSSION

The multiplication of *A. comosus* var. bracteatus L. *in vitro* were significantly affected ($p \leq 0.05$) by the interaction of the factors under study (micropropagation systems and BAP). It can be observed that, in the absence of BAP, the conventional micropropagation (CM) and the natural ventilation system (NVS) were superior (22.5%) in relation to shoot length (SL), when compared to the plants maintained in the temporary immersion bioreactor (TI) (Table 1). However, with the increase in BAP concentration in the culture medium, there was a reduction of 31.6% in SL in the CM (Table 1). Plants growing in NVS showed a reduction in SL, only with the use of BAP at the highest dosage (Table 1). In the TI, no differences were observed for SL with increasing BAP concentrations of 1 and 2 mg L^{-1} (Table 1).

Table 1. Shoot length (SL), number of shoots (NB) and number of shoots larger or equal than 1 cm (NS ≥ 1) of in vitro *A. comosus* var *bracteatus* L. growing in different micropropagation systems.

MS*	BAP (mg L^{-1})								
	0	1	2	0	1	2	0	1	2
	----------SL(cm)------			----------NS----------			---------NS ≥1 (cm)-------		
CM[1]	4.50Aa	3.22Bb	3.62Bb	0.40Bc	4.12Bb	6.25Ca	0.50Bb	2.20Ba	1.80Ca
NVS[2]	4.32Aa	4.52Aa	3.57Bb	1.17Ac	8.07Aa	8.87Ba	0.90Bc	2.72Bb	4.92Ba
TI[3]	3.60Ba	4.30Aa	4.22Aa	1.62Ac	5.47Cb	1.,12Aa	2.87Ac	5.07Ab	7.07Aa

Averages followed by the same capital letter vertically or lower case horizontally do not differ by the Scott-Knott test at 5% probability. (*) Micropropagation System; (1) Conventional Micropropagation (CM); (2) Natural Ventilation System (SVN); (3)Temporary Immersion Bioreactor (IT).

The use of TI, in combination with BAP (2 mg L^{-1}), yielded the best results for number of shoots (NS) and number of shoots larger or equal than 1 cm (NS ≥ 1), when compared to the other treatments (Table 1). NB was positively affected by NVS in combination with BAP (1 or 2 mg L^{-1}) and NS ≥ 1 by NVS along with BAP (2 mg L^{-1}) (Table 1). The increase in BAP concentrations led to an increase in NS, mainly at a concentration of 2 mg L^{-1}, for all MS (micropropagation system) under study (Table 1). However, CM was the system with the lowest NS, when compared to NVS and TI (Table 1), showing a reduction of 42% and 77.9%, respectively.

The benefits of air injection in liquid cultivation systems (TI), as well as gas exchange provided by the use of porous membranes (NVS), are directly related to the multiplication and promotion of *in vitro* growth of different species, such as orchid (MOREIRA et al., 2013), pineapple (SCHEIDT et al., 2009), grape (JIN et al., 2013) and medicinal plants (PIATCZAK et al., 2014), among others. The benefits of air injection and the promotion of gas exchange in the *in vitro* multiplication of *A. comosus* var. bracteatus L. were also observed in this study (Table 1).

TI and SVN yielded better results for LS and NS≥1, even at the highest BAP concentrations (Table 1). These results are in agreement with the great majority of studies conducted with these micropropagation systems. Moreira et al. (2013) and Ayenew et al. (2013) worked with orchid and pineapple, respectively, and found that the use of temporary immersion bioreactor and natural ventilation system showed better results for shoot length.

Micropropagation systems that allow gas exchange (TI and NVS) led to the *in vitro* budding of ornamental pineapple (Table 1). However, the addition of BAP in the culture medium had a synergistic effect, which was necessary for the increase and satisfactory induction of *in vitro* ornamental pineapple shoots. Cytokinins have an action in several plant development processes, including cell division, differentiation of cell cultures and sprout induction (PIATCZAK et al., 2014). *Ananas comosus* micropropagation studies have yielded efficient results in sprout induction with cytokinin BAP (SILVA et al., 2007; SCHEIDT et al., 2009; AYENEW et al., 2013).

Despite the high investment in equipment acquisition, the use of bioreactors has been justified by this micropropagation system, since it yields a significant increase in *in vitro* multiplication rates, which was observed in this study (Table 1). Bioreactor cultivation systems can yield large-scale sprouting, which allows the automation of the micropropagation process and work reduction (ETIENNE and BERTHOULY, 2002). Piatczak et al. (2014) observed, in a temporary immersion system, a multiplication rate of 21 shoots per explant in 60 days, three times higher than the conventional protocol for the medicinal plant *Rehmannia*. Silva et al. (2007) and Scheidt et al. (2009) reported that temporary immersion yielded multiplication rates up to three times higher, when compared to conventional pineapple micropropagation systems. In this study, TI yielded multiplication rates 1.78 times higher than CM (Table 1).

There was a significant effect of the micropropagation system ($p \leq 0.05$) and BAP doses ($p \leq 0.05$) for plant fresh and dry matter, with no interaction between these factors. BAP doses directly affected plant fresh and dry matter. The use of BAP (2 mg L^{-1}) led to the greatest accumulation of fresh (88.8%) and dry (57.1%) matter, when compared to CM (Figure 2 BD). According to Oliveira et al. (2011), the mass and number of shoots showed the same increasing trend, that is, the higher the shoot proliferation, the higher the mass. This behavior was also observed in this study, that is, the larger the number of shoots produced, the greater the *in vitro* cultivation mass.

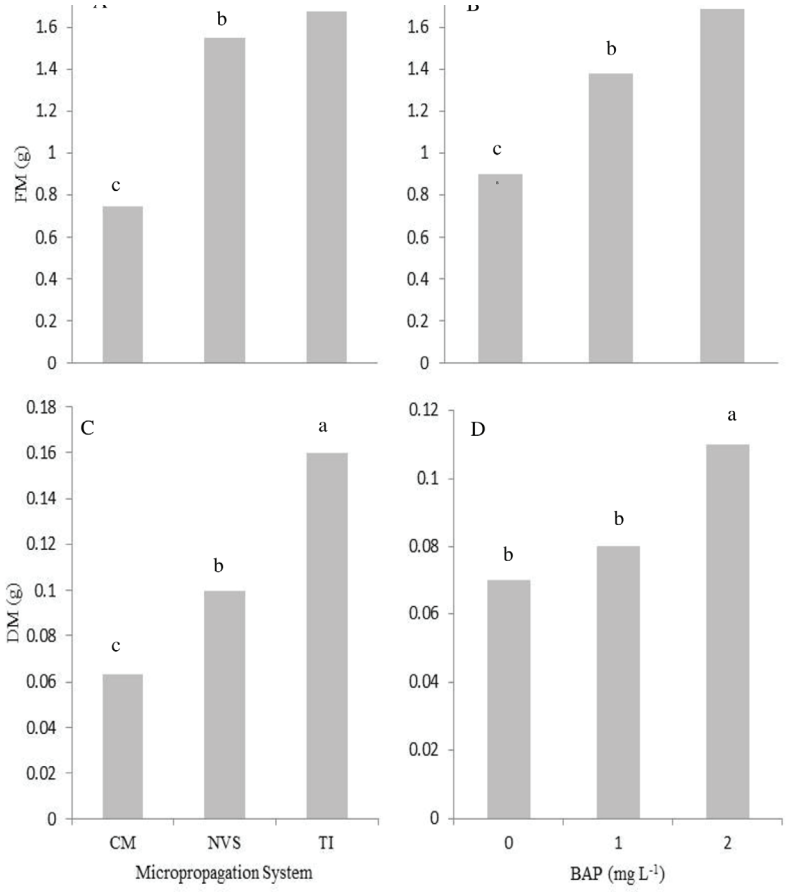

Figure 2. Fresh mass in function of different micropropagation systems and concentrations of BAP (B); Dry mass in function of different micropropagation systems (C) and concentrations of BAP (D) in *A. comosus* var *bracteatus* plants. Averages with same letters do not differ by Scott-Knott test at 5% probability. Conventional Micropropagation (CM); Natural ventilation system (NVS); Temporary Immersion Bioreactor (TI).

The highest fresh and dry matter was found in plants growing in TI (Figure 2 AC), showing an increase of 112.5% and 283.3%, respectively, when compared to the growth of plants in CM. Plant dry matter expresses the real growth, since it is related to the accumulation of proteins and other substances that are the direct results of photosynthesis, with values of 0.16 g, respectively for NVS and TI (Figure 2C). Similar results were found by Moreira et al. (2013), who compared micropropagation systems in orchid cultivation and found that plants growing in a temporary immersion bioreactor had a higher dry matter, reaching 0.032 g, followed by the natural ventilation system, which were superior to conventional micropropagation.

Silva et al. (2016) verified a greater dry matter accumulation in orchid plants growing in natural ventilation systems, when compared to conventional micropropagation. This result is in agreement with those observed in this study, where it was observed that the plants maintained in NVS had 66% greater dry mass accumulation than the plants in CM (Figure 2C). The use of a membrane in natural ventilation yields greater gas exchange, which leads to increased photosynthetic rates for plants grown *in vitro*, resulting in increased plant growth and mass accumulation, when compared to the closed system, without permeable membrane air passage (SALDANHA et al., 2012).

The use of temporary immersion bioreactors is a promising method for the production of *Digitalis purpurea* biomass through *in vitro* shoot multiplication (ALONSO et al., 2009). Jova et al. (2011) and Aragón et al. (2010) reported higher photosynthesis rates and better growth for plants grown in temporary immersion bioreactors, when compared to other micropropagation methods. The efficiency of the temporary immersion bioreactor in plant micropropagation is directly related to the greater availability of nutrients (due to the use of liquid medium), associated with gas exchange, which increases *in vitro* plant growth (ETIENNE and BERTHOULY, 2002).

4. CONCLUSIONS

The use of a temporary immersion bioreactor, in association with BAP (2 mg.L^{-1}), is efficient in the multiplication and in vitro growth of ornamental pineapple.

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AUTHORS CONTRIBUTIONS

C.O.R: Installation and conduction of the experiment, evaluation of plant growth and data tabulation. **A.B.S**.: Conception of the study, statistical analysis and writing of the manuscript. **P.R.C.L**.: Writing of the manuscript, interpretation of the results and critical revision. **J.A.B**.: Installation and conduction of the experiment, evaluation of plant growth and data tabulation. **G.A.R.J**.: Conduction of the experiment, evaluation of plant growth and data tabulation.

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