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Morphometric parameters and photosynthetic performance of *in vitro* propagated pineapple

Parâmetros morfométricos e desempenho fotossintético de abacaxis propagados *in vitro*

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Abstract

Pineapple (*Ananas comosus*) is a horticultural species of the Bromeliaceae family of high socioeconomic interest, widely cultivated around the world. The multiplication of pineapple seedlings in the field can be time-consuming, requiring a significant labor investment. The objective of this study was to evaluate the performance of continuous and temporary immersion systems in the micropropagation scale-up of the species. Shoots were obtained from explants subcultured in flasks with gelled culture medium and without gas exchange. The shoots were transferred to liquid MS medium supplemented with 2mM NAA, and 4mM BAP, and cultivated in four different devices: sealed flasks, flasks with semipermeable gas membranes, RITA®, and twin-flasks. After 45 days of cultivation, plant growth, fresh mass increment, the stomatal density of the abaxial surface of the leaves, the maximum quantum yield of the photosystem II, and contents of chlorophyll and carotenoids were analyzed. Significant differences were observed in plant growth, stomatal density, and contents of chlorophyll and carotenoids. The twin-flasks and RITA® devices revealed better results in morphological parameters, such as plant growth and stomatal density, while the treatments in sealed flasks and with membrane stood out in the chlorophyll content.

Keywords: *Ananas comosus*, bioreactor, micropropagation, photosynthesis, in vitro culture

Resumo

O abacaxi (*Ananas comosus*) é uma espécie hortícola da família Bromeliaceae de alto interesse socioeconômico, amplamente cultivada em todo o mundo. A multiplicação de mudas de abacaxi no campo pode ser demorada, exigindo um investimento significativo de mão-de-obra. O objetivo deste estudo foi avaliar o desempenho de sistemas de imersão contínua e temporária na ampliação da escala de micropropagação da espécie. As brotações foram obtidas de explantes sub-cultivados em frascos com meio de cultura gelificado e sem troca gasosa. Os brotos foram transferidos para meio líquido MS suplementado com 2mM ANA e 4mM BAP e cultivados em quatro diferentes dispositivos: frascos selados, frascos com membranas gasosas semipermeáveis, RITA® e frascos duplos. Após 45 dias de cultivo, foram analisados o crescimento das plantas, o incremento de massa fresca, a densidade estomática da superfície abaxial das folhas, o rendimento quântico máximo do fotossistema II e os teores de clorofila e carotenoides. Diferenças significativas foram observadas no crescimento das plantas, densidade estomática e teores de clorofila e carotenóides. Os frascos duplos e os dispositivos RITA® revelaram resultados superiores em parâmetros morfológicos, como crescimento vegetal e densidade estomática, enquanto os tratamentos em frascos selados e com membrana se destacaram no teor de clorofila.

Palavras-chave: *Ananas comosus*, biorreator, micropropagação, fotossíntese, cultura in vitro

INTRODUCTION

Ananas comosus L. Merril., popularly known as pineapple, is a bromeliad fruit species native to Central and South America, currently cultivated in several countries around the world (Crestani, 2010). The multiplication of this species is generally carried out by sectioning and replanting the young shoots (suckers, slips, or hapas) in the field. The crown of the infructescence can also be planted, but in a commercial scenario, it does not occur, as the structure follows the fruit in commercialization. Such propagation practices are laborious, time-consuming, and may promote the propagation of unhealthy plants. As an alternative, plants can be micropropagated, with the advantage of obtaining higher amounts of genetically uniform plants with high phytosanitary quality and increased productive potential (Escalona et al., 1999; Scherer et al., 2015).

Micropropagation can be achieved in gelled medium or liquid medium. Liquid medium culture, in turn, can be performed in permanent immersion system (PIS) or temporary immersion system (TIS). Cultivation in liquid immersion systems has advantages over the conventional gelled culture such as accelerating the production process, reducing cost, and increasing plant uniformity. On the other hand, liquid culture has drawbacks such as the occurrence of explants asphyxia, hyperhydricity, and physiological disorders in the plants (Watt, 2012; Hwang et al., 2022).

Several advances have been reported for the pineapple micropropagation protocols in the last decades (e.g., Escalona et al., 1999; Scherer et al., 2013; 2015) aiming at increasing efficiency and reducing the costs of the propagation process. Usually, such studies have evaluated the number and size of the developed shoots, the dry and fresh mass, amount of some biochemical compounds, and survival rate at the acclimatization step. In this study, morphometric parameters and photosynthetic performance were evaluated in pineapple shoots propagated in four different liquid medium systems. Evaluating morphometric and photosynthetic parameters of micropropagated pineapple plants, we intended to compare four different cultivation systems concerning plant growth, stomata development, and photosynthetic parameters aiming at contributing to the choice of the most pertinent device for commercial propagation of pineapple in small, medium, or large scale.

MATERIAL AND METHODS

PLANT MATERIAL

Shoots used for the experiment originated from buds introduced and subcultured in agar-gelled MS (Murashige & Skoog, 1962) culture medium supplemented with 30 g L^{-1} sucrose, 2.0 mL L^{-1} Morel's vitamins, 2.0 µM 1-naphthaleneacetic acid (NAA), and 4.0 µM 6-benzylaminopurine (BAP). Shoots of similar size and weight were selected and transferred to PIS or TIS devices containing liquid culture media with the same composition they were previously cultivated. All treatments were maintained in a growth room at 25 ± 2 °C with a 16 h photoperiod (72 µmol m^{-2} $^{-1}$ white LED lamp irradiance).

Shoots were cultivated in four different immersion devices (Figure 1, Table 1): sealed glass flasks (T1), glass flasks with semipermeable gas membranes (passive gas exchange; T2), RITA® (Vitropic, Saint-Mathieu-de-Tréviers, France; T3), and twin-flasks (T4). Treatments T1 and T2 were built using transparent glass flasks with 350 mL capacity. Treatment T4 was built using polyethylene flasks of 5.0 L capacity. Considering the different sizes of the used immersion devices, 30 mL of culture medium and three shoots were placed for treatments T1 and T2; 250 mL and five shoots for the treatment T3; and 500 mL and 11 shoots for the treatment T4 (Table1). Despite the difference in the internal capacity of the systems, we intended to compare the devices most frequently employed in propagation laboratories, as they are implemented.

In treatments T3 and T4, the shoots were immersed in culture medium for 3 min every 3 h. Air was injected into the TIS through silicone hoses at a pressure of 0.28 bar. The injected air was sterilized through 0.2-µm filters.

MORPHOMETRIC PARAMETERS

The fresh mass increment was obtained by weighing the plants of each repetition on a semi-analytical scale before and after 45 days of culturing in PIS and TIS devices. The increment was calculated in grams

based on the difference between these measurements. The shoot growth was estimated based on the length of the longest leaf of each shoot. The length of the leaves was determined with aid of a ruler, measured from the base to the apices of the leaf.

The analysis of the stomatal density of the abaxial leaf surface was performed using the technique of epidermal printing on a glass slide, as proposed by Weyers & Johansen (1985). The second and third largest leaves of the plants were used for the analysis. The impression was performed with a drop of cyanoacrylate ester (super glue) on the surface of the slide, pressing the leaf onto it for about 30 seconds. The leaf was then removed, and the slide was observed under an Olympus® BX-40 light microscope and photographed using a DP-71 image capturing system. Twenty-four samples were analyzed for treatments T1 and T2 and 48 samples for treatments T3 and T4. Subsequently, the counting of stomata was performed from the photographic records, and the average number of stomata per mm^2 was calculated.

Figure 1

Systems of in vitro culture with permanent and temporary immersion in liquid culture medium. (A) sealed flasks (permanent immersion system), (B) RITA® (temporary immersion system), (C) glass flask with semipermeable gas membranes (permanent immersion system), and (D) twin-flasks (temporary immersion system).

Table 1

Treatments for the cultivation of pineapple shoots in liquid culture medium in permanent immersion system (PIS) or temporary immersion system (TIS).

Immersion system	Treatment	Device	Volume of medium	Flask Capacity	Number of shoots
PIS	T1	Sealed glass flasks	30 mL	300 mL	03
	T2	Glass flasks with semipermeable gas membrane	30 mL	300 mL	03
TIS	T3	RITA®	250 mL	500 mL	05
	T4	Twin-flasks	500 mL	5 L	11

PHOTOSYNTHETIC PERFORMANCE

The maximum quantum yield of the photosystem II (Fv/Fm) was obtained with the aid of the MINI-PAM Chlorophyll Fluorometer (Waltz, Effeltrich, Germany). Plants were acclimated in the dark for 60 minutes. The largest leaves of each explant were "clipped" at their middle third and the value of the maximum quantum yield was recorded.

The content of photosynthetic pigments (chlorophylls and carotenoids) was measured by collecting three 0.3 mm diameter discs (totaling 21.20 mm² of area for each repetition) from the middle third of the second-largest leaf of each sample. Leaf discs were incubated in a test tube with 3 mL of dimethylsulfoxide (DMSO) and stored in the dark wrapped in aluminum foil for 24 hours (Shinano, 1996; Baldotto et al., 2009). For treatments T1 and T2, three tubes were used for each repetition, and treatments T3 and T4, five tubes were placed per repetition. To calculate the absorbance, a Spectramax spectrophotometer (Molecular Devices, San Jose, CA, USA) was set at wavelengths 665 nm, 649 nm, and 480 nm. The contents of the pigments were estimated according to Wellburn (1994):

Chlorophyll *a* = $12.47 \times A_{665} - 3.62 \times A_{649}$

Chlorophyll *b* = $25.06 \times A_{649} - 6.5 \times A_{665}$

Total carotenoids: $(1000 \times A_{480} - 1.29 \times Ca - 53.78 \times Cb) / 220$

Total chlorophyll = chlorophyll *a* + chlorophyll *b,*

where A_{665} is the absorbance at 665 nm, A_{649} the absorbance at 649 nm, A_{480} the absorbance at 480 nm, C*a* is the content of chlorophyll *a*, and C*b* is the content of chlorophyll *b.*

The occurrence of inter-conversion of chlorophyll *b* to chlorophyll *a* was evaluated through the estimation of the chlorophyll *a/b* ratio. This inter-conversion plays an important role in the establishment of the necessary chlorophyll *a/b* ratio during the adaptation to stresses (Martins et al., 2018).

EXPERIMENTAL DESIGN

The experiments were performed in a completely randomized block design, with four replications for each treatment. All treatments were placed in a growth room with homogenous light, humidity, and temperature conditions. After 45 days of cultivation, morphometric parameters (plant growth and stomatal density) and photosynthesis performance (maximum quantum yield of photosystem II, content of chlorophyll *a*, content of chlorophyll *b*, chlorophyll *a/b* ratio, total chlorophyll content, and total carotenoids content) were evaluated.

STATISTICAL ANALYSIS

The data obtained were subjected to analysis of variance and, when significant, the means were compared using the Mann-Whitney pairwise test at 5% significance, in the Past 4.02 software (Hammer et al., 2001).

RESULTS

The fresh mass increment was 1.87 g/shoot in T1, 1.79 g/shoot in T2, 1.46 g/shoot in T3, and 2.37 g/shoot in T4. There was no significant difference among treatments, although shoots cultivated in the twin-flasks device presented a higher mass increase.

Regarding plant growth, the twin flasks stood out in comparison to treatments without gas exchange and with passive gas exchange, not significantly differing from the RITA® device (Figure 2A). The stomatal density was significantly higher in treatments with active exchange of the internal atmosphere (T3 and T4; Figures 2B-F).

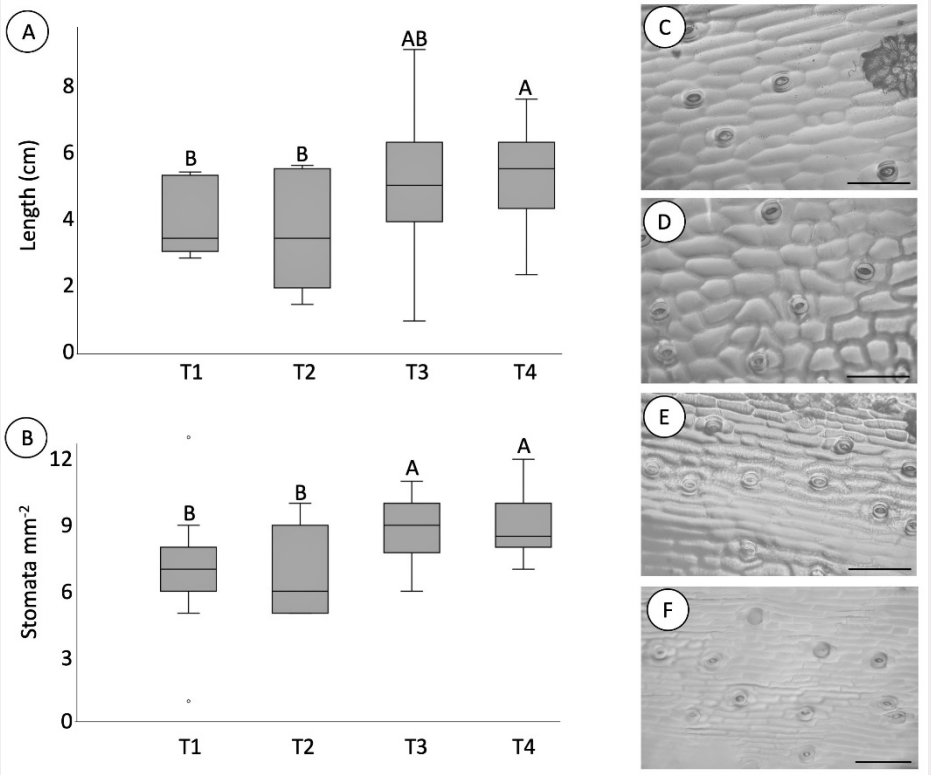

Figure 2

Morphometric parameters of Ananas comosus plants after 45 days of cultivation in four different treatments. (A) Length of the longest leaf, in cm. (B) Stomatal density per mm² on the abaxial surface. (C-F) Stomata at the abaxial surface were recorded by light microscopy from plants cultivated in (C) sealed flasks (T1); (D) flasks with semipermeable membrane (T2); (E) RITA® (T3); (F) twin-flasks (T4). In A and B, data represent the mean ± standard error (SE) of four or five biological replicates (see text for details). Different letters indicate significant differences at p < 0.05 (Mann-Whitney pairwise test). In C-F, bars = 100 µm.

The mean maximum quantum yield of the photosystem II (*Fv/Fm*) measured for each treatment was 0.706 electron quantum^{-1} for treatment T1, 0.715 electron quantum^{-1} for treatment T2, 0.732 electron quantum^{-1} for treatment T3, and 0.729 electron quantum^{-1} for treatment T4. These measurements did not differ statistically at 5% significance.

In the analyses of photosynthetic pigments, the highest amounts of chlorophyll *a* (Figure 3A), chlorophyll *b* (Figure 3B), total chlorophyll (Figure 3C), and carotenoids (Figure 3D) were observed in the PIS devices without gas exchange (T1), followed by the flasks with passive gas exchange (T2). The RITA® device (T3) revealed the lowest content of chlorophyll *a*, total chlorophyll, and carotenoids. Regarding chlorophyll *b* contents, the RITA® device did not significantly differ from treatments without gas exchange and with passive gas exchange (Figure 3B). Shoots cultivated in the twin flasks (T4) did not differ from the RITA® system for the content of chlorophyll *a*, chlorophyll *b*, and total chlorophyll. Concerning the carotenoid content, twin flasks did not differ from both PIS devices (Figure 3D). The chlorophyll *a/b* ratio was 1.68 (S.E. 0.16) for T1, 1.36 (S.E. 0.13) for T2, 0.80 (S.E. 0.06) for T3, and 1.41 (S.E. 0.13) for T4. This ratio for treatment T3 was statistically different from all other treatments *($p < 0.029$).*

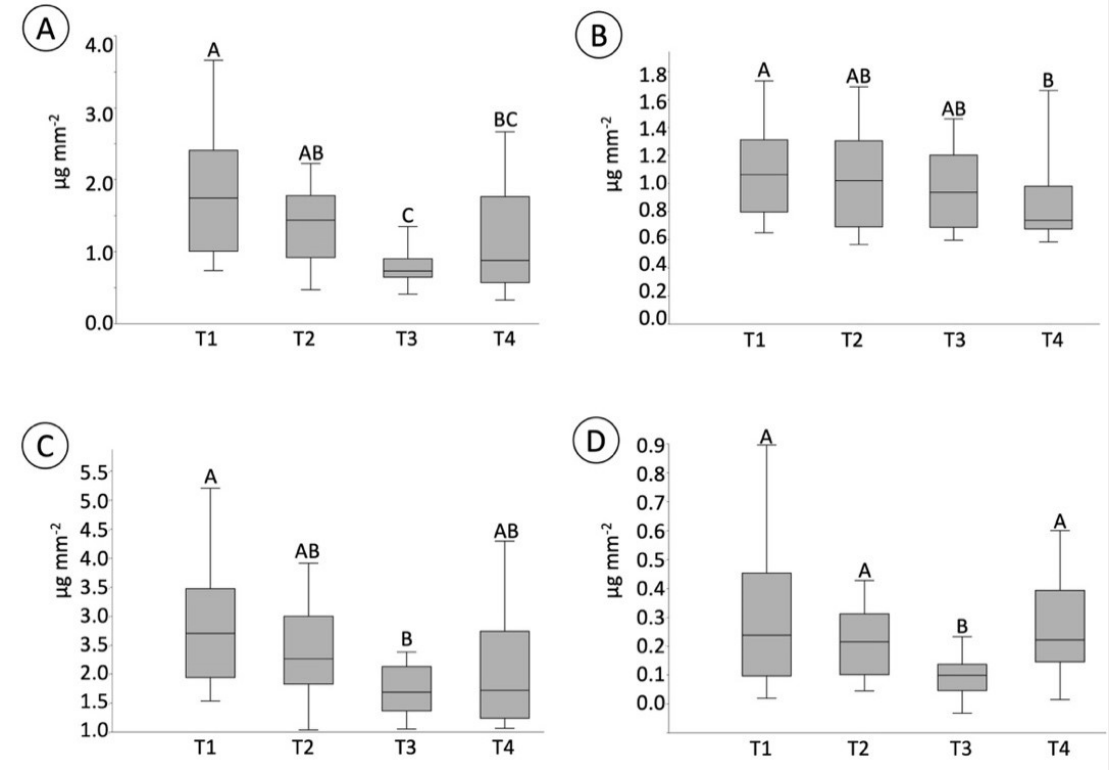

Figure 3

Photosynthetic pigments in Ananas comosus shoots after 45 days of cultivation in four different devices. (A) Content of chlorophyll a in leaves. (B) Content of chlorophyll b in leaves. (C) Content of total chlorophyll in leaves. (D) Content of total carotenoids in leaves. Data represent the mean ± standard error (SE) of four or five biological replicates (see text for details). Different letters indicate significant differences at $p < 0.05$ (Tukey test).

DISCUSSION

It has been observed that the exchange of the internal atmosphere of the culture flasks through TIS enhances regeneration and growth (Steinmacher et al., 2011; Kim et al., 2020), optimizing *in vitro* production and adaptation of the shoots to the *ex vitro* environment. Unlike PIS devices, TIS allows for active gas exchange, promoting greater oxygenation in the internal environment and the temporary permanence of plants in contact with the culture medium.

The superior plant growth observed in the twin flasks in this study supports the hypothesis that the constant renewal of the internal atmosphere in TIS favors the growth of pineapple plants, as suggested by Scheidt et al. (2009) and Silva et al. (2007). Moreover, changing the internal atmosphere in temporary immersion systems promotes the removal of ethylene and a higher respiratory rate, which boosts an acceleration of cell division and, consequently, greater plant size.

The higher stomatal density in the leaves of shoots cultivated in RITA® and twin-flasks devices are probably related to the reduction of relative humidity inside the temporary immersion systems, stimulating the formation of new stomata, as reported by Escalona et al. (1999).

Besides evaluating morphometric aspects, assessing photosynthetic parameters is crucial in plant micropropagation. Chlorophyll fluorescence parameters are sensitive to stress and are good markers for comparing different *in vitro* culture systems. Values of *Fv/Fm* of 0.80 are expected for plants without stress and tend to decrease when plants are subjected to stressful conditions (Jiang et al., 2020). The values of *Fv/Fm* observed for all treatments in this study ranged from 0.706 to 0.732, suggesting that the photosynthetic apparatus of the micropropagated plants was not highly affected by the cultivation conditions.

The results for chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents are likely associated with the intensity of light crossing through the flasks' walls since the walls of the sealed flasks and the flasks with membranes were more transparent and allowed a greater intensity of light to cross. On the other hand, the RITA® devices were already opaque due to the time of use, and the twin flasks commonly accumulated water droplets on their walls, likely promoting light refraction. The isolated analysis of the efficiency of photosynthetic activity in gas exchange treatments of pineapple showed that the effect of the renewal of the internal atmosphere was not sufficient to increase the chlorophyll contents and the luminous intensity is directly related to the production of these pigments (Couto et al., 2014).

The RITA® device did not significantly differ from treatments without gas exchange and with passive gas exchange regarding chlorophyll *b* contents. This feature may be linked to the fact that chlorophyll *b* is associated with light capture of different wavelengths than chlorophyll a, with the possibility that the opaque wall of the device caused less interference in the capture of this light spectrum. This result plays an important role in the development of post-acclimatization plants since chlorophyll *b* is also related to the efficiency of energy transfer to the photosynthetic mechanism of the plant.

The chlorophyll *a/b* ratio can reflect the change of photosystem, relating to photosystem II (Xu et al., 2020). The relatively higher level of chlorophyll *a/b* ratio in treatment T1 and T2 indicates greater light adaptability, higher electron transport ability of chlorophyll, and higher activity of Calvin cycle enzymes (Evans, 1988). An increase in chlorophyll *a/b* ratio may occur due to the conversion of chlorophyll b to chlorophyll *a*, but also indicates less emphasis on light-harvesting concerning the rates of photosystem II photochemistry under stress (Martins et al., 2018). Thus, it suggests that the PIS devices promoted a somewhat more stressful culture environment for the pineapple shoots, reflected in the higher chlorophyll *a/b* ratio.

It is noteworthy that, although the content of photosynthetic pigments is closely related to the plant's ability to carry out photosynthesis through the capture and transposition of light energy, this does not mean that plants will necessarily perform better in the field. Other factors are extremely important for the full development of the plant organism, such as greater adaptation to the acclimatization of the transpiration route and the full availability of nutrients in *in vitro* cultivation, favored, among other things, by the renewal of the internal atmosphere in cultivation systems (Escalona et al., 2007).

CONCLUSION

In summary, the active gas exchange in the temporary immersion systems favored the plants' growth and stomata development in the leaves, while the transparency of the flasks used in the continuous immersion

systems favored the production of chlorophyll and carotenoid. Thus, the use of *in vitro* culture in temporary immersion systems with flasks that allow high light incidence across the walls may be recommended for producing healthy and vigorous plants for the establishment and/or renovation of commercial plantations of pineapple. Moreover, the twin-flasks system using 5 L polyethylene flasks is easy to build and use, demands low monetary input, and allows the production of up to 300 shoots in each device (data not shown), reducing the cost per unity of seedling produced.

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