Rodriguésia 72: e01242019. 2021
http://rodriguesia.jbrj.gov.br
DOI: http://dx.doi.org/10.1590/2175-7860202172018

Original Paper

Impact of saline solution on growth and photosystem II during *in vitro* cultivation of *Bromelia antiacantha* (Bromeliaceae)

Rosiane Cipriano[1,3,7], *João Paulo Rodrigues Martins*[1,4], *Luiz Carlos de Almeida Rodrigues*[2], *Antelmo Ralph Falqueto*[1,5] & *Andreia Barcelos Passos Lima Gontijo*[1,6]

Abstract

In vitro cultivation is a technique with wide application for micropropagation. However, each species has specific mineral needs for this type of cultivation. The objective was to assess the impacts of the saline solution culture medium on the performance of the photosynthetic apparatus and growth of *Bromelia antiacantha* during *in vitro* cultivation, and thus to elucidate the mitigation of the nutritional imbalance that can interfere in the electron transport in the plants. Plants were cultivated in a salt concentration gradient of MS medium (0%, 25%, 50%, 75% or 100%). The growth traits and fluorescence *a* chlorophyll were analyzed. Intermediate concentrations of MS medium resulted in plants with a larger number of leaves and longer root length. The OJIP curves and results of the JIP test showed that the plants grown without MS salts presented less efficient photosystem II (PSII), as indicated by the performance index [$Pi_{(TOTAL)}$]. In contrast, the intermediate concentrations (MS 25% and 50%) had a positive effect on the performance of the photosynthetic apparatus. The MS 25% medium can be used for *in vitro* cultivation of *B. antiacantha*, enabling the development of plants with suitable physiological qualities for planting in the field.

Key words: bromeliad, chlorophyll *a* fluorescence, plant physiology, tissue culture.

Resumo

Cultivo *in vitro* é uma técnica de grande aplicabilidade para micropropagação. Porém, cada espécie tem uma necessidade mineral mais adequada para o seu cultivo. O objetivo foi verificar os impactos da solução salina do meio de cultivo no desempenho do aparato fotossintético e crescimento de *Bromelia antiacantha* durante o cultivo *in vitro*, e dessa forma, elucidar a mitigação do desbalanço nutricional que poderia interferir na cadeia de transporte de elétrons nas plantas. Plântulas foram cultivadas em um gradiente de concentração do meio MS (0%, 25%, 50%, 75% ou 100%). Foram analisadas as variáveis de crescimento e a fluorescência da clorofila *a*. Concentrações intermediárias de sais do meio MS resultaram em plantas com maior número de folhas e comprimento de raiz. As curvas OJIP e a análise do teste JIP mostraram que as plantas cultivadas na ausência dos sais MS apresentaram menor eficiência do fotossistema II (FSII), como evidenciado por meio do índice de performance [$Pi_{(TOTAL)}$]. Contrariamente, concentrações intermediárias (MS 25% e 50%) atuaram positivamente na performance do aparato fotossintético. O meio MS 25% pode ser utilizado para o cultivo *in vitro* de *B. antiacantha*, possibilitando o desenvolvimento de plantas com qualidades fisiológicas adequadas para o cultivo.

Palavras-chave: bromélia, fluorescência da clorofila *a*, fisiologia vegetal, cultura de tecidos.

[1] Universidade Federal do Espírito Santo, Depto. Ciências Agrárias e Biológicas, Bairro Litorâneo, São Mateus, ES, Brazil.

[2] Universidade Federal de Alfenas, Bairro Centro, Alfenas, MG, Brazil. ORCID: <https://orcid.org/0000-0001-6336-0247>.

[3] ORCID: <https://orcid.org/0000-0001-7107-8719>.

[4] ORCID: <https://orcid.org/0000-0003-0554-6793>.

[5] ORCID: <https://orcid.org/0000-0002-0488-7441>.

[6] ORCID: <https://orcid.org/0000-0003-3422-4398>.

[7] Author for correspondence: bio.rosiane@gmail.com

Introduction

Ornamental bromeliads, native to the tropical and subtropical zones of Central and South America, are among the most commercially important ornamental plants in the world, occupying a valuable position in horticulture and the flower industry (Zhang *et al.* 2012).

Among the most prevalent methods for large-scale propagation of horticultural species is tissue culture, mainly of ornamental species like orchids and bromeliads (Silva *et al*. 2017a; Lembrechts *et al*. 2017; Rosa *et al*. 2018). The *in vitro* multiplication of bromeliads can be accomplished by direct or indirect organogenesis (Martins *et al.* 2014; Simão *et al.* 2016). Studies of the growth and physiology of bromeliads *in vitro* also have been conducted (Martins *et al.* 2014, 2016, 2018; Simão *et al.* 2016; Corredor-Prado *et al.* 2019). Martins *et al.* (2015) observed that the saline gradient of the culture medium developed by Murashige & Skoog (1962), better known as MS, can have a strong impact on the morphogenetic responses of bromeliads during *in vitro* cultivation.

The mineral nutrition is directly involved in plants' metabolism, affecting their growth and development. This aspect needs to be observed in micropropagation programs, since plants need to absorb nutrients from the culture medium until they can be transplanted to *ex vitro* conditions (Akin *et al.* 2017; Poothong *et al.* 2017). The benefits of optimizing the nutrients in culture media are well documented for a wide range of species and applications (Hand & Reed 2014; Tavares *et al.* 2015, 2017; Wada *et al.* 2015; Leljak-Levanic *et al.* 2016; Akin *et al.* 2017; Dias *et al.* 2017; Poothong *et al.* 2017; Silva *et al*. 2017b; Assis *et al.* 2018; Pérez-Alonso *et al.* 2018). The success of micropropagation can depend on the nature and concentration of mineral nutrients in the medium, and this can vary and have different effects depending on the species, genotype or technique employed (Akin *et al.* 2017; Poothong *et al*. 2017). These differences have led to adaptations of the existing culture media and even development of new formulations (Greenway *et al.* 2012; Poothong & Reed 2014; Andrade & Tamaki 2016). Besides this, physiological disturbances of plants grown *in vitro* due to abiotic factors have often been reported (Magyar-Tábori *et al.* 2010; Martins *et al.* 2015, 2016).

Evaluations by the chlorophyll *a* fluorescence technique can enable verifying the physiological state of plants based on the detection of alterations in some components of photosystem II (PSII), components of the electron transport chain, and light-dependent photochemical reactions (Lotfi *et al.* 2018). By means of the technique's measurements, it is possible to detect changes in the bioenergy status of plants' general photosynthetic apparatus (Borawska-Jarmułowicz *et al.* 2014), with numerous advantages. It is a very precise and nondestructive method that allows analyzing a large number of samples in a brief time interval (Zivcak *et al.* 2013; Goltsev *et al.* 2016). By applying the OJIP test, it is possible to obtain qualitative and quantitative visual information about the entire photosynthetic apparatus or about specific aspects of the PSII, intersystem and photosystem I (PSI). Besides this, the curves obtained from the OJIP test can be quantified by the JIP test, which supplies quantitative information about the productivity and efficiency of the photosynthetic apparatus (Kalaji *et al.* 2016; Rosa *et al.* 2018).

Among the bromeliad species, *Bromelia antiacantha* Bertol. has strong potential for medicinal, nutritional, ornamental and industrial uses (Krumreich *et al.* 2015; Vallés & Cantera 2018). *In vitro* propagation techniques have been widely studied and used for rapid multiplication of various economically important plant species, including other bromeliads such as *Ananas comosus* var. *comosus (*Scherer *et al*. 2015), *Billbergia zebrina* (Herb.) Lindl. (Martins *et al.* 2015), *Vriesea cacuminis* L.B. Sm. (Resende *et al.* 2016), *Ananas comosus* var. *ananassoides* (Baker) Coppens & F. Leal (Silva *et al.* 2017b), *Vriesea reitzii* Leme & Costa (Corredor-Prado *et al.* 2019), *Aechmea ramosa* Mart. *ex* Schult. f. (Faria *et al.* 2018), *Vriesea incurvata* Gaudich (Sasamori *et al.* 2016, 2018; Pulido-Rueda *et al.* 2018), and *Aechmea blanchetiana* (Tavares *et al.* 2015, 2017; Martins *et al.* 2018, 2019; Rosa *et al.* 2018), among others. In particular, Mercier & Yoshida (1998) studied the activity of bromelain in the leaf tissues of *B. antiacantha* plants grown *in vitro*, but they did analyze the physiological quality of the plants.

Although complete MS medium (100%) is most often used for *in vitro* cultivation, for some species a dilution of macronutrients can produce better results (Sasamori *et al.* 2016). Dilutions MS medium salts have been used for *in vitro* cultivation of several bromeliad species, such as *Ananas comosus* var. *ananassoides* (Baker) Coppens & F. Leal (Silva *et al.* 2017b). The demand for mineral nutrients accompanies the specific life

forms found within the group of bromeliads. In this context, knowing the effect of modifications of the traditional MS culture medium on the *in vitro* cultivation of *B. antiacantha* can support the development of a more suitable protocol for *in vitro* cultivation of this species. Therefore, the aim of this study was to observe the impacts of the saline solution of MS culture medium on the performance of the photosynthetic apparatus and growth of *B. antiacantha* during *in vitro* cultivation, and thus to elucidate the mitigation of nutritional imbalance, which can interfere in the electron transport chain of plants.

Material and Methods

Plant material

Bromelia antiacantha seeds were removed from ripe fruits collected from 15 matrix plants from areas in sandbank forest in the municipality of São Mateus, Espírito Santo state, Brazil. They were washed in tap water to remove the mucilage, dried on paper towels, placed in paper envelopes and stored at a temperature of 4 ºC.

For performance of the experiment, a batch in which seeds from two or more fruits from each plant were mixed with seeds from other plants, to obtain a sample composite representative of the population diversity of *B. antiacantha* present in the collection areas.

In vitro cultivation

The seeds were disinfested in 70% ethanol for five minutes, followed by a 1% (v/v) sodium hypochlorite solution with three droplets of Tween 20 for five minutes. Then the seeds were washed three times in sterile distilled water. After disinfestation, the seeds were placed in flasks containing 50 ml of different concentrations (v/v) of MS medium salts (0%, 25%, 50%, 75% or 100%), obtained by serial dilution of the original composition proposed by Murashige & Skoog (1962). All the media were supplemented with 30 g L^{-1} sucrose, solidified with 8 g L^{-1} agar, and the pH was adjusted to 5.8 before autoclaving at 120 ºC for 20 minutes. After inoculation, the plant material was kept for 90 days in a growth room with 16:8 hour photoperiod, under light intensity of 80 $mmol.m^{-2}.s^{-1}$ and temperature of 26 ± 1 ºC.

Plant growth

After cultivation for 90 days, the number of leaves, number of roots, aerial part length, root length and leaf area were recorded, in the last case using a leaf area meter (LI-COR L1-3100C). For analysis of the growth variables, 24 plants were collected at random and divided into six portions, thus composing six repetitions per treatment. For leaf area, five plants were used per treatment.

Chlorophyll *a* fluorescence analysis

The photosynthetic efficiency was analyzed between 8:00 and 10:00 a.m. by measurements of the chlorophyll *a* fluorescence using a Handy-PEA continuous excitation fluorometer (Hansatech, UK), according to the recommendations of Strasser *et al.* (2004). Before the readings, the leaves were adapted to the dark using leaf clips for 30 minutes, sufficient for complete oxidation of the photosynthetic system. Then a flash of light was emitted with saturation irradiance of 3,000 mmol photons $m^{-2}s^{-1}$ on the leaves, with duration of 1 second. The fluorescence intensity was measure at 50 ms, 100 ms, 300 ms, 2 ms, 30 ms and 1 s. Based on the OJIP fluorescence transient, the parameters were calculated as established by the JIP test. The interpretation and normalization of the parameters measured and calculated using this test were performed according to Strasser *et al.* (2004) and Stirbet & Govindjee (2011). The chlorophyll *a* fluorescence measurements were carried out with eight plants per treatment.

Statistical analysis

The experimental design was completely randomized and the data obtained were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test at 5% probability, using the SISVAR 5.4 software (Ferreira 2011).

Results

In vitro growth

No differences were observed in relation to the control (MS 100%) in the treatments with different salt concentrations for number of roots. In MS 25% and MS 50% favored greater root length (Fig. 1). For all the variables related to growth of the aerial part, the treatment without addition of salts (MS 0%) presented the smallest values, mainly in relation to the plants grown in MS 50% medium.

Chlorophyll *a* fluorescence

The polyphasic OJIP curves of the plants presented typical polyphasic behavior, with

increasing magnitude of the fluorescence signals from the basal level (called F_0) to the maximum level (called F_m), with well-defined intermediate points J and I (Fig. 2a). In the O-J phase, there was a rise in the curves in all the treatments, a pattern that was most pronounced in the treatment with MS 0%. Starting at the J-I phase, the curve of MS 0% began to decrease.

The relative variable fluorescence between points O and P (V_{OP}) was greatest in plants grown in MS 0% (Fig. 2b). The kinetic difference curves of the transient fluorescence related to the control [$DV_{OP(treatment)} - V_{OP(control)}$] presented negative bands in phases O-J, J-I, and I-P in the treatments with MS 25% and MS 50%. Virtually no variations were observed of the parameter DV_{OP} for the plants grown in MS 75% in relation to the control (MS 100%), while the MS 0% treatment was the only one that presented more pronounced positive bands.

The relative fluorescence between steps O (50 ms) and K (300 ms) [$V_{OK} = (Ft - F_0) / (F_K - F_0)$] was normalized and presented kinetic difference of DV_{OK} [$DV_{OK} = V_{OK(treatment)} - V_{OK(control)}$] also called L-band (Fig. 3a). The L-bands appeared at approximately 0.12 ms. The relative fluorescence between points O (50 ms) and J (2 ms) [$V_{OJ} = (Ft - F_0) / (F_J - F_0)$] was normalized and is shown as kinetic difference [$DV_{OJ} = (V_{OJ\ (treatment)} - V_{OJ\ (control)}$] (Fig. 3c), revealing K-band. Positive L- and K-bands were observed for the plants grown in MS 0%, which differed from the other treatments (Fig. 3b and 3d, respectively). On the other hand, negative L- and K-bands were observed in the other treatments, with the L-bands being most pronounced in the plants cultivated in MS 25% and MS 50%, which differed from the other treatments (Fig. 3d).

The lowest values of F_0 were observed for the plants grown in MS 100% and MS 75% with differences only occurring in relation to MS 0%. The maximum photochemical efficiency values of PSII ($jP_0 = F_V/F_M = TR_0/ABS$) were lowest in MS 0% and differed from the other treatments (Fig. 4).

In general, the specific energy flux values per reaction center (RC) decreased with declining concentration of the MS medium, until the concentration of 25%, and the highest values were obtained in MS 0% (Fig. 4). For the energy transport flux per reaction center (ET_0/RC) and the reaction center density per cross section (RC/CS), no differences were noted between the treatments. The photochemical performance index [$Pi_{(TOTAL)}$] was lowest in MS 0%, and was significantly different from the other treatments. The S_M/Tf_{max} (average fraction of open RCs in time period 0; Tf_{max} = time of maximum fluorescence production) did not differ between the different concentrations of salts in the MS medium.

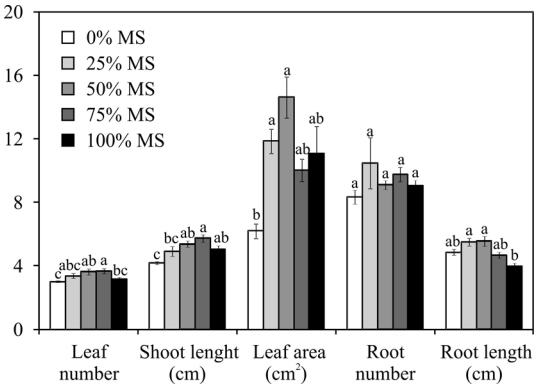

Figure 1 – Growth traits of *Bromelia antiacantha* plants grown *in vitro* for 90 days at different concentrations of MS medium salts. Means followed by the same letter, in each growth trait, do not different by the Tukey test at 5% significance.

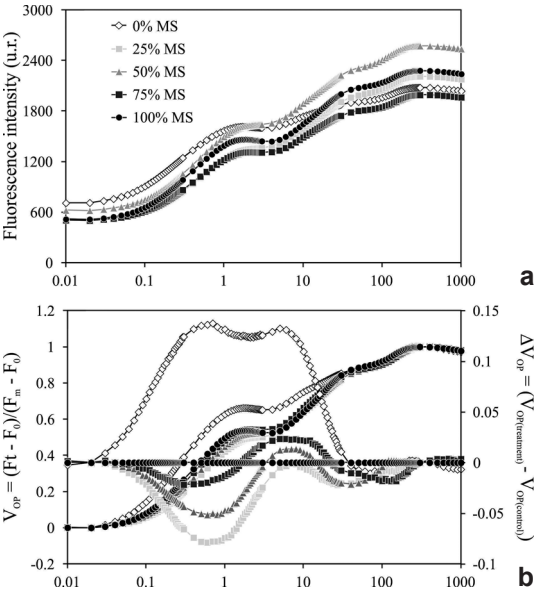

Figure 2 – a-b. Chlorophyll *a* fluorescence transients *Bromelia antiacantha* during *in vitro* cultivation in function of different concentrations of MS medium salts – a. fluorescence intensity; b. relative variable fluorescence [$V_{OP} = (Ft-F_0) / (Fm-F_0)$] and kinetic differences of V_{OP} [$DV_{OP[treatment]} - V_{OP[control]}$].

Discussion

For *Bromelia antiacantha*, the concentrations of MS 25% and MS 50% favored longer root length, showing the advantage of using these dilutions to promote this growth trait. Dilution of the MS medium was observed to be advantageous for other species of bromeliads cultivated *in vitro*, such as *Ananas comosus* var. *ananassoides* (Baker) Coppens & F. Leal (Silva *et al.* 2017b). The dilution of the MS medium, and especially of the nitrogen compounds, increased the rooting and number of leaves in *Vriesea incurvata* cultivated *in vitro* (Sasamori *et al.* 2016). Smaller concentrations of nitrogen (15 mM) in the MS medium were suggested to optimize multiplication of *A. comosus* var. *ananassoides* (Silva *et al.* 2017b).

The observation of the difference of the relative variable fluorescence between F_0 and F_M (DV_{OP}) revealed a large variation of fluorescence in the MS 0% medium, indicating that the plants submitted to this treatment suffered damage to the intersystem electron carriers, thus requiring a minimum concentration of salts in the MS medium. Nutrients such as Cu (micronutrient), Mg and Ca (macronutrients) are essential to plants as components of the photosynthetic apparatus and of enzymes that act during photosynthesis (Poothong *et al.* 2017; Kwano *et al.* 2017). The absence of these nutrients limits the electron transfer process (Purohit 2018), as observed in the results of DV_{OP} during the *in vitro* cultivation of *B. antiacantha*.

Point J is directly involved in the constant changes in the transfer of electrons from quinone A (Q_A) to quinone B (Q_B) (Mehta *et al.* 2010). This inflection in point J observed in the curve of MS 0% represents a double reduction of the pheophytin electron carriers, Q_A and Q_B (Chen *et al.* 2014), which possibly indicates damage caused by the absence of basic nutrients for this process. For point I, no substantial alterations were observed. This can be attributed to the distinct dissipative pathways that lead to complete closing of the PSII reaction center at point I, in which the alterations are related to events that occur before reduction of the pool of plastoquinones (PQ) (Chen *et al.* 2014).

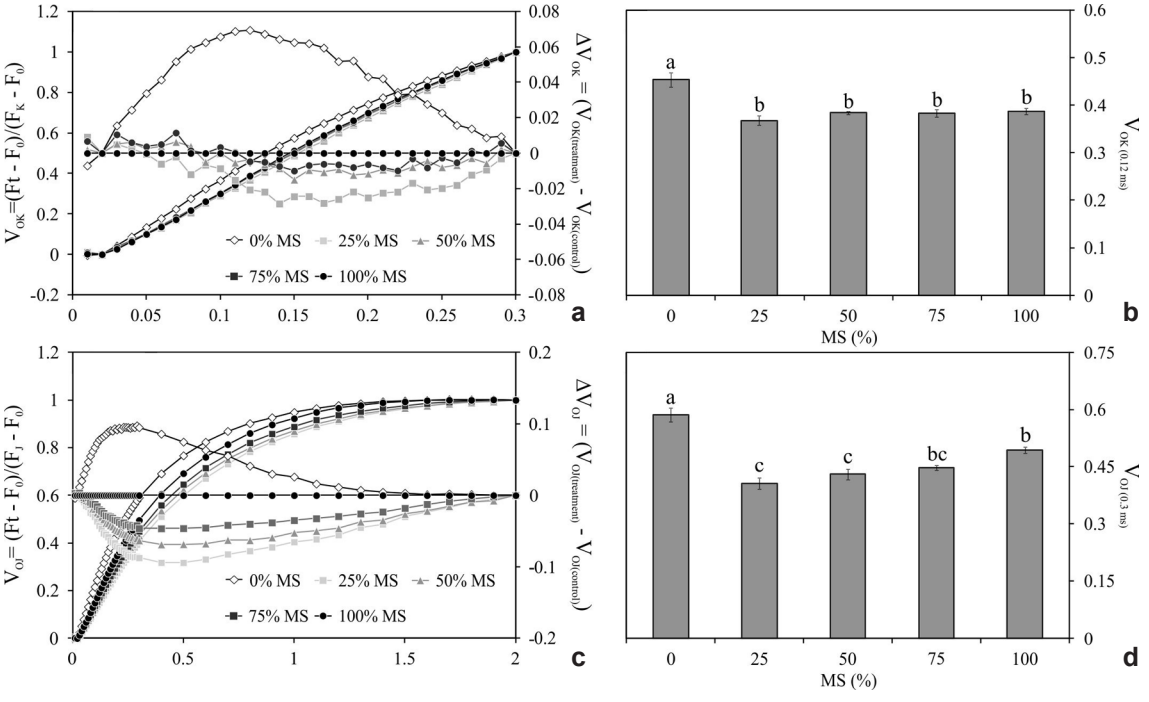

Figure 3 – a-d. Chlorophyll *a* fluorescence transients *Bromelia antiacantha* during *in vitro* cultivation in function of different concentrations of MS medium salts – a. variable fluorescence between steps 0 and K [$V_{OK} = (Ft\text{-}F_0) / (F_K\text{-}F_0)$] and kinetic difference of V_{OK} [$DV_{OK} = V_{OK(treatment)} - V_{OK(control)}$]; b. means of $V_{OK(0.12ms)}$ followed by the same letter are not significantly different according to the Tukey test at 5%; c. variable fluorescence between steps O to J [$V_{OJ} = (Ft\text{-}F_0) / (F_J\text{-}F_0)$] and kinetic difference of V_{OJ} [$DV_{OJ} = (V_{OJ(treatment)} - V_{OJ\ (control)}$]; d. means of $V_{OJ(0.3ms)}$ followed by the same letter are not significantly different according to the Tukey test at 5%.

Positive amplitudes of L- and K-bands are an indicator of a reduction of the plastoquinone pool in plants cultivated in MS 0%. These bands can be considered potential markers of disturbances before the appearance of visible signs of response to stress (Meng *et al.* 2016). The appearance of L-band is an indicator of energy connectivity or grouping of units of PSII, which presents a positive deviation when the connectivity is low (Yusuf *et al.* 2010). The positive L-band in plants grown in MS 0% indicates some level of disturbance in the membranes of the thylakoid membranes, reducing the connectivity between the reaction centers (RCs) of PSII (Rosa *et al.* 2018). Therefore, the absence of nutrients can impair the stability of the subunits of PSII, causing disturbances in the energy connectivity (Chen & Cheng 2010). A positive L-band can appear in plants suffering from nutritional deficiency, which suggests that the photosynthetic system increases the dissipation to

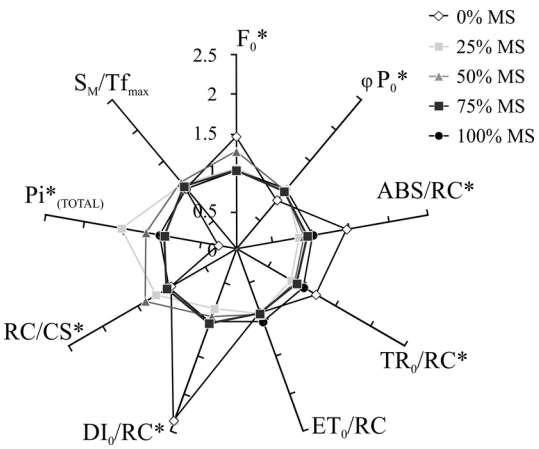

Figure 4 – JIP test parameters as a function of MS concentrations obtained based on chlorophyll *a* fluorescence of *Bromelia antiacantha*. Means accompanied by an asterisk are significantly different by the Tukey test at 5% significance. F_0 = initial fluorescence; φP_0 = maximum quantum yield of primary photochemistry at ($t = 0$); ABS/RC = absorption flux per RC; TR_0/RC = trapping flux (leading to Q_A reduction) per RC; ET_0/RC = electron transport flux (further than Q_A^-) per RC; DI_0/RC = dissipated energy flux per RC (at $t = 0$); RC/CS = density of reaction center per cross section; $Pi_{(TOTAL)}$ = overall performance index, which measures the performance up until the final electron acceptors of PS I; S_M/Tf_{max} = average fraction of open RC in the period of 0 to TFmax (time of maximum fluorescence production).

improve the use of excitation energy. On the other hand, the treatment involving MS 25% presented negative L-bands, demonstrating better use of the excitation energy and improved connectivity between the reaction centers, and consequently better stability of the system.

Likewise, the presence of the K-band also indicates disturbances in PSII, which are related to the imbalance between the donation of electrons from the oxygen evolution complex (OEC) and the Q_A^- electron acceptors (Kalaji *et al.* 2016). These are linked to dissociation of the OEC, which uses manganese (Mn) as an essential cofactor in the oxidation of water. The presence of a positive K-band reflects the inactivation of the OEC and/or increase in the size of the functional antenna of PSII (Yusuf *et al.* 2010). The larger the amplitude of this band, the greater will be the inactivation of the OEC (Adamski *et al.* 2011). Therefore, the appearance of a positive K-band in the plants grown in MS 0% can be related to low manganese content in the leaves, the result of the absence of mineral salts in the MS medium. However, the presence of negative K-bands with greater amplitude in the plants grown in MS 25% and MS 50% suggests the maintenance of activity of the OEC. This indicates better balance between the electron acceptor and donor sides of PSII.

The increase of F_0 in the plants grown in MS 0% is related to the reduced energy capture rate by PSII and can be attributed to the smaller number of active reaction centers, in turn caused by the smaller energy transfer from the light-harvesting complexes of PSII (LHCII) to the reaction centers. That effect was probably caused by the limitation of nutrients essential for the functioning of the photosynthetic apparatus. This behavior is a consequence of the dissociation of the LHCII from the nucleus of PSII or the inactivation of the oxygen evolution complex (Mathur *et al.* 2011; Ghotbi-Ravandi *et al.* 2014). The results obtained for K-band and L-band suggest that the increased fluorescence intensity observed in F_0 can be attributed both to the inactivation of the OEC and the reduction of the energy connectivity between the subunits of PSII. This increase of F_0 was documented as one of the most direct signs of photoinhibition in plants (Lotfi *et al.* 2018).

Besides this, the increase of F_0 together with the decrease of F_M causes a lower value of jP_0 in MS 0%. The reduction of jP_0 caused by stress or limitation of nutrients in the MS medium is considered to be a marker of damages in PSII (Martins *et al.* 2015; Falqueto *et al.* 2017). However,

the photodamage observed can be considered a positive adaptation caused by the regulation of the photochemical and photoprotective mechanisms, such as dissipation of non-photochemical energy, state transition, cyclic electron flow around PSI or also the water oxidation cycle (Miyake 2010; Rochaix 2011; Keren & Krieger-Liszkay 2011; Raven 2011; Duffy *et al.* 2013). These mechanisms prevent the super-reduction of the electron transport chain of photosynthesis and diminish the potential for damages caused by oxidative stress (Carvalho 2008; Nishiyama *et al.* 2011; Kalaji *et al.* 2011; Campos *et al.* 2014; Ali *et al.* 2018).

The increase in the absorption flux (ABS/RC) and trapping flux of energy per reaction centers (TR_0/RC) complemented by the increased flux of energy dissipated per active reaction center (DI_0/RC) suggests existence of a mechanism to protect the plants, as also observed in *Solanum nigrum* L. during acclimation (Swain *et al.* 2010). The energy transport flux represents the rate of reoxidation of Q_A^- and its increase indicates that the plant is using light energy combined with a lower energy dissipation rate (DI_0/RC) (Lotfi *et al.* 2018). The lower values of DI_0/RC observed in the treatments with MS 25%, MS 50%, MS 75% and MS 100% indicate these were the treatments when greater light energy use occurred. Another study demonstrated that the light reactions and electron transport during photosynthesis can be altered when plants face shortage of nutrients (Wagner *et al.* 2016). This can explain the need for nutrients for better functioning of the photosynthesis process.

According to the values of DI_0/RC observed, the energy from the increases of ABS/RC and TR_0/RC did not contribute to the energy transport flux (ET_0/RC), and instead was dissipated in the form of heat or emission of fluorescence (DI_0/RC), as also observed by Araújo & Deminicis (2009) and Falqueto *et al.* (2017). The increase of DI_0/RC observed in the plants grown in MS 0% medium, which differed from the other treatments, suggests activation of an energy dissipation mechanism in response to reduced assimilation of CO_2 (Falqueto *et al.* 2017). The efficiency with which absorbed light energy is used also is modulated during deprivation of nutrients (Wagner *et al.* 2016).

The total performance index [$Pi_{(TOTAL)}$] is a plant vitality indicator and has also been considered a sensitive parameter to detect stress in plants (Yusuf *et al.* 2010; Gururani *et al.* 2017; Lotfi *et al.* 2018), because its calculation includes parameters related to conservation of energy from photons absorbed by PSII (ABS), trapping of excitation energy (TR), conversion of excitation energy for the transport of electrons to the intersystem (ET) and reduction of the final acceptors of PSI (RE) (Redillas *et al.* 2011). This parameter responds not only to losses in the activity of PSII, but also to damages related to PSI (Xiang *et al.* 2013). Therefore, the lower value of $Pi_{(TOTAL)}$ found in the plants cultivated in MS 0% medium can cause loss of the structure and function of PSI, inhibiting the donation of electrons and reduction of the final acceptors of PSI, consequently contributing to the reductions of $Pi_{(TOTAL)}$.

Conclusion

I. Use of the MS 0% medium impaired the photosynthetic apparatus, while MS 25% produced the best results compared to the other concentrations of salts tested.

II. The MS 25% medium can be used for *in vitro* cultivation of *B. antiacantha*, enabling the development of plants with suitable physiological qualities for *ex vitro* cultivation.

Acknowledgements

The authors would like to acknowledge the scholarship awarded by CAPES (Coordination for the Improvement of Higher Education Personnel).

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Area Editor: Dr. Nelson Augusto Santos Jr
Received in July 08, 2019. Accepted in March 09, 2020.