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Anatomy and photosystem II activity of *in vitro* grown *Aechmea blanchetiana* as affected by 1-naphthaleneacetic acid

J.P.R. MARTINS[1]*, L.C.A. RODRIGUES[2], E.R. SANTOS[1], B.G. BATISTA[2], A.B.P.L. GONTIJO[2], and A.R. FALQUETO[1]

Plant Ecophysiology Laboratory[1] and Plant Tissue Culture Laboratory[2], Federal University of Espírito Santo, Litorâneo, 29932-540 São Mateus, ES, Brazil

Abstract

Auxins are one of the main regulators of *in vitro* plant growth and development. However, the mechanisms, by which auxins, such as 1-naphthaleneacetic acid (NAA), affect *in vitro* root and leaf anatomy and photosystem function, remain unclear. Accordingly, the aim of the present study was to analyze the effect of different NAA concentrations on the anatomy and photosynthetic performance of *in vitro*-propagated *Aechmea blanchetiana* and to determine whether such a treatment affects micropropagated plants after acclimatization. *In vitro*-established *A. blanchetiana* plants were transferred to culture media that contained 0, 2, 4, or 6 μM NAA, and after 50 d, they were transplanted into plastic seedling trays with a commercial substrate and cultivated for 60 d in a greenhouse. The plants were evaluated after a 50-d *in vitro* NAA exposure (growth traits, chlorophyll *a* fluorescence, and root and leaf anatomy) and after 60 d of acclimatization in the greenhouse (root and leaf growth). Changes induced by NAA in root anatomy might improve uptake of minerals and sugars from the medium, thereby increasing the *in vitro* growth. In the leaves, the lowest chlorenchyma thickness and sclerenchyma area were observed in plants grown without NAA, and NAA exposure also improved photosystem II activity. The highest *ex vitro* growth rate was observed for plants that were propagated with 4 μM NAA. Therefore, the use of NAA during *in vitro* propagation can improve the anatomical and physiological quality of *A. blanchetiana* plants, as well as to improve *ex vitro* transfer.

Additional key words: auxins, chlorophyll fluorescence, photosynthetic apparatus.

Introduction

Micropropagation is the most common method used for large-scale cloning horticultural crops including bulbous plants, fruit trees, and ornamentals. Bromeliads that are grown as flowering potted ornamentals have also a high commercial value (Zhang *et al*. 2012) and their *in vitro* propagation methods have frequently been published (Martins *et al*. 2014, 2015b, 2016b, Corredor-Prado *et al*. 2015, Simão *et al*. 2016).

During *in vitro* propagation, plant growth regulators are frequently used to induce the formation of side shoots or adventitious roots. Auxins, both natural and synthetic auxin analogs, are regularly used for rooting cuttings from a variety of species (Pacurar *et al*. 2014). Auxin responses are highly context-dependent and can involve changes in cell division, cell expansion, and cell fate (Salehin *et al*. 2015). The auxin application is not often a

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Abbreviations: ABS/RC - absorption flux per RC; ETo/RC - electron transport flux (further than Q_A^-) per RC; DIo/RC - dissipated energy flux per RC (at t = 0); F_0 - initial fluorescence; F_M - maximal fluorescence; IBA - indole-3-butyric acid; NAA - 1-naphthaleneacetic acid; PI(total) - overall performance index, which measures the performance up until the final electron acceptors of PS I; PQ - plastoquinone; PS - photosystem; Q_A - primary quinone electron acceptor of PS II; Q_B - secondary quinone electron acceptor; RC - reaction center; TRo/RC - trapping flux (leading to Q_A reduction) per RC; δRo - efficiency/probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PS I acceptor side (RE); φDo - quantum yield of energy dissipation (at t = 0); φEo - quantum yield of electron transport (at t = 0); φPo - maximum quantum yield of primary photochemistry (at t = 0); φRo - quantum yield of reduction of end electron acceptors at the PS I acceptor side (RE); ρRo - efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the PS I end electron acceptors; ΨEo - probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-.

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* Corresponding author; e-mail: jprmartinss@yahoo.com.br

limiting factor for bromeliad rooting, but different auxin types and concentrations could have positive effects on bromeliad growth traits (Chu *et al*. 2010, Martins *et al*. 2013, Viehmannova *et al*. 2016) and 1-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA) belongs to most frequently used (Chu *et al*. 2010, Martins *et al*. 2013). Because IBA has only a local effect, it is commonly used for the induction of adventitious root formation, whereas NAA can be transported and, therefore, induce changes throughout the plant (Ludwig-Müller 2000, Woodward and Bartel 2005).

The relative effects of IBA and NAA on *in vitro* rooting *Aechmea blanchetiana*, an ornamental bromeliad, have already been documented (Chu *et al*. 2010), and NAA treatment was reported to induce the greatest accumulation of root fresh mass. However, shoots propagated in a medium with cytokinins may have a low rate of rooting without auxin supplementation, and studies of *in vitro*-propagated bromeliads have reported higher rates of rooting and plant elongation in NAA-treated plants than in either IBA-treated or control plants (Martins *et al*. 2013, Resende *et al*. 2016).

Plant growth regulators applied during *in vitro* propagation may affect the morphology and anatomy as well as the photosynthetic apparatus function of *in vitro* grown shoots and plantlets (Stefanova *et al*. 2011, Dobránszki and Drienyovszki 2014). Anatomical studies can be used to assess the effect of *in vitro* conditions on the success of the transfer to *ex vitro* conditions (Mohamed and Alsadon 2010, Stefanova *et al*. 2011, Martins *et al*. 2015b).

Chlorophyll *a* fluorescence is a very sensitive, non-invasive, and rapid procedure to evaluate changes in photosynthetic apparatus (Roháček 2002, Baker 2008, Kalaji *et al*. 2014). Many studies have also measured chlorophyll *a* fluorescence in order to determine the photosynthetic apparatus performance of *in vitro* grown plants (Dobránszki and Drienyovszki 2014, Martins *et al*. 2015a, Matysiak and Gabryszewska 2016). Dobránszki and Drienyovszki (2014), for example, have shown that cytokinins can affect the performance and capacity of the photosynthetic apparatus in the apple leaves of *in vitro* propagated plants. This kind of study can improve the rational application of plant growth regulators during micropropagation and potentially decrease acclimatization loss. Indeed, many *in vitro*-propagated plants are characterized by poor photosynthetic apparatus development, nonfunctional roots, and other disorders, which can affect their survival and growth rate during the acclimatization phase (Hazarika 2006).

The addition of auxins to growth mediums can significantly affect the regulation of *in vitro* growth and development and, therefore, the quality and physiological state of micropropagated plants, as well. However, the mechanisms by which auxins affect root and leaf anatomy and photosynthetic apparatus (mainly photosystem (PS) II activity) remain unclear. Therefore, the aim of the present study was to analyze the effect of NAA treatment on the anatomy and photosynthetic performance of *in vitro*-propagated *A. blanchetiana* and to determine the effect of *in vitro* conditions on plant acclimatization.

Materials and methods

Plants and culture conditions: Seeds of *Aechmea blanchetiana* (Baker) L.B.Sm. were germinated *in vitro* and cultivated on Murashige and Skoog (1962; MS) medium for 80 d. Then the plantlets were transferred to glass containers containing 50 cm^3 of liquid MS medium with 10 μM 6-benzylaminopurine and 30 g dm^{-3} sucrose. After 60 d, the shoots were sub-cultivated on liquid MS medium, without plant growth regulators and 30 g dm^{-3} sucrose for further 60 d. The containers were kept in a growth room at a temperature of 26 ± 2° C and a 16-h photoperiod under fluorescent tube lamps (*FT8 HO*, 36W/6400K, *Empalux*, Paraná, Brazil), which provided 90 μmol m^{-2} s^{-1} of photosynthetically active radiation.

Aechmea blanchetiana microshoots of approximately 2.5 cm in length (5 - 8 per seedling obtained from a previous *in vitro* subculture) were cut and transferred to glass containers (five shoots per each) containing 50 cm^3 of MS medium solidified with 5 g dm^{-3} agar (*Vetec*, Darmstadt, Germany) and supplemented with 30 g dm^{-3} sucrose and 0 (control), 2, 4, or 6 μM NAA (*Sigma-Aldrich*, St. Louis, USA). The pH was adjusted to 5.8 before autoclaving at 120°C for 20 min. The plantlets were grown in a growth room under above mentioned conditions. After 50 d, 30 plantlets were randomly sampled from each treatment, and leaf and root numbers, the longest leaf and root length, and plant fresh mass were determined.

Measurement of leaf and root anatomy: The samples were fixed in a 1:1:18 (v:v:v) mixture of formaldehyde, acetic acid, and 70 % (v/v) ethanol for 72 h, followed by storage in 70 % ethanol (Johansen 1940). Cross and paradermal sections were taken from the middle part of the leaf lamina of the first and second completely expanded leaves of each plantlet using a double-edged razor, and cross-sections were also taken at the root base (0.5 cm from the shoot). The sections were cleared using 2.5 % (m/v) sodium hypochlorite, stained with safranin and astra-blue solutions, and assembled on slides using 50 % (v/v) glycerin. The sections were viewed using a light microscope (*Leica DM 1000* combined with a *Leica ICC50 HD* camera; *Leica*, Wetzlar, Germany), and two cross sections and six paradermal section fields from each slide (n = 5) were photographed. The anatomical characteristics were then measured using *UTHSCSA-Imagetool*. For the roots, the cross-sectional area [μm^2] and central cylinder [μm^2], thickness of cell walls in the exodermis [μm] and endodermis [μm], the number of

xylem vessels, and diameter of xylem vessels [μm] were measured. Meanwhile, for the leaves, the stomatal density [mm^{-2}], polar diameter/equatorial diameter ratio, thickness of the adaxial and abaxial epidermis [μm] and chlorenchyma [μm], xylem vessel number, and xylem diameter [μm] were measured.

Measurement of *in vitro* chlorophyll *a* fluorescence: After 50 d, chlorophyll *a* fluorescence transients were measured using a portable *Handy PEA* fluorimeter (*Hansatech*, King's Lynn, Norfolk, UK) between 8:00 and 9:00 and the third completely expanded leaves from eight *in vitro*-cultured plantlets per treatment. The leaves were dark-adapted for 30 min before measurement using a leaf clip (*Hansatech*). The irradiance during measurement was 3 000 μmol (photons) m^{-2} s^{-1}, which was sufficient to generate maximal fluorescence for all treatments. The fast fluorescence kinetics (F_0 to F_M) were recorded from 10 μs to 1 s. The fluorescence intensities at 20 μs (considered F_0), 100 μs, 300 μs, 2 ms (F_J), 30 ms (F_I), and maximum fluorescence (F_M) were recorded and analyzed as described previously (Strasser *et al*. 2004, Stirbet and Govindjee 2011).

***Ex vitro* acclimatization:** After 50 d of *in vitro* culture, the *A. blanchetiana* plantlets were transplanted to plastic seedling trays that were filled with a commercial substrate (*Tropstrato HT*, *Vida Verde*, Mogi Mirim, Brazil) and kept in a greenhouse. In order to maintain a high relative humidity, the plants were covered with transparent polyethylene lids, and the moisture of substrate remained at a full field capacity. After 60 d, 20 plants from each treatment were randomly sampled and the leaf and root numbers, the longest leaf and root, and plant fresh mass were determined.

Statistical analysis: The experiment was performed with four different NAA concentrations (0, 2, 4, and 6 μM) using a completely randomized design. The resulting data were submitted to an analysis of variance (*ANOVA*), and the significance of differences between mean values was determined using Tukey test at a 5 % probability level.

Results

Rooting occurred in all the treatment groups. However, NAA treatment significantly improved the plant growth traits (Fig. 1). The plants grown without exogenous auxin had the fewest and shortest leaves, and NAA supplementation progressively increased the leaf number and length, adventitious root number, and plant fresh mass (Table 1).

The length of unicellular hairs of the root epidermis was affected by NAA treatment, and the roots of the control plants (0 μM NAA) had the shortest hairs (Fig. 1). Treatment with NAA also affected root area, both the total area and central cylinder area increased in the plants cultured with NAA, whereas the lowest values were in the control plants (Fig. 2, Table 1). The thickness of cell walls of the exodermis was lower at all NAA treatments than in the control (Fig. 2, Table 1). Meanwhile, the endodermal thickness was unaffected by NAA treatment. The xylem vessel number was greater in the NAA-treated plants than in the control plants but was similar among the NAA treatment groups. Nevertheless, the diameter of xylem vessels increased with an increasing NAA concentration (Fig. 2, Table 1).

Treatment with NAA had no significant effect on stomatal density, stomatal function, or epidermal thickness. The average stomatal density was 69 ± 11 mm^{-2}, and the stomata of all the treatments were elliptical in shape (Fig. 3) with similar stomatal function characteristics. Meanwhile, the average thicknesses of the adaxial and abaxial epidermis were 20.43 and 13.53 μm, respectively. Nevertheless, the other plant tissues were

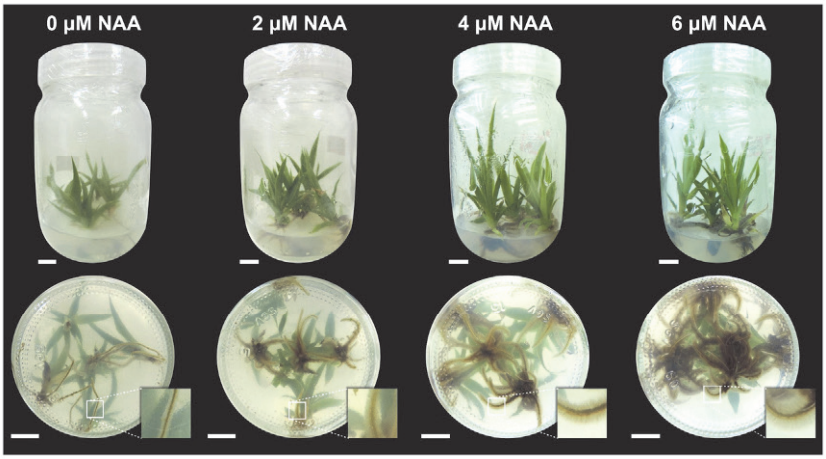

Fig. 1. *Aechmea blanchetiana* plantlets grown in a medium with different 1-naphthaleneacetic acid (NAA) concentrations (0, 2, 4, and 6 μM) for 50 d. *Bars* = 1 cm.

Table 1. Growth traits and anatomical structure of *in vitro* grown *Aechmea blanchetiana* as a function of NAA concentration. Means ± SDs, n = 6 and 5, respectively. Means followed by the same letter in the row are not significantly different according to Tukey test at a probability level > 5 %.

Parameters	NAA [µM] 0	2	4	6
Leaf number	8.51 ± 0.50 ^{c}	11.32 ± 1.19 ^{ab}	10.53 ± 0.33 ^{b}	11.72 ± 0.50 ^{a}
Length of the longest leaf [cm]	3.92 ± 0.23 ^{b}	4.17 ± 0.41 ^{b}	6.03 ± 0.32 ^{a}	6.03 ± 0.53 ^{a}
Root number	2.38 ± 0.20 ^{d}	6.56 ± 0.47 ^{c}	9.84 ± 0.67 ^{b}	12.69 ± 1.51 ^{a}
Length of the longest root [cm]	2.23 ± 0.22 ^{ab}	1.90 ± 0.20 ^{b}	2.43 ± 0.41 ^{a}	2.14 ± 0.38 ^{ab}
Fresh mass [g plant^{-1}]	0.321 ± 0.012 ^{d}	0.556 ± 0.031 ^{c}	0.906 ± 0.026 ^{b}	0.982 ± 0.055 ^{a}
Root total area [µm^2]	99594 ± 12589 ^{b}	124960 ± 19033 ^{ab}	126853 ± 16580 ^{ab}	145441 ± 27505 ^{a}
Root central cylinder area [µm^2]	2834.8 ± 420 ^{b}	5480.7 ± 1467 ^{a}	5175.2 ± 668 ^{a}	6743.4 ± 1031 ^{a}
Cell wall thickness of root exodermis [µm]	2.50 ± 0.32 ^{a}	1.79 ± 0.54 ^{bc}	1.19 ± 0.15 ^{c}	1.88 ± 0.28 ^{ab}
Root endodermis [µm]	8.20 ± 0.13 ^{a}	8.17 ± 0.06 ^{a}	8.21 ± 0.05 ^{a}	8.13 ± 0.10 ^{a}
Root xylem vessel number	7.00 ± 1.29 ^{b}	11.27 ± 0.76 ^{a}	11.93 ± 0.60 ^{a}	12.27 ± 1.52 ^{a}
Root xylem vessel diameter [µm]	7.78 ± 0.16 ^{c}	8.70 ± 0.61 ^{b}	9.46 ± 0.60 ^{ab}	9.67 ± 0.46 ^{a}
Leaf adaxial epidermis [µm]	20.53 ± 0.36 ^{a}	20.37 ± 0.53 ^{a}	20.47 ± 0.37 ^{a}	20.36 ± 0.15 ^{a}
Leaf sbaxial epidermis [µm]	13.55 ± 0.13 ^{a}	13.54 ± 0.21 ^{a}	13.50 ± 0.14 ^{a}	13.53 ± 0.18 ^{a}
Leaf chlorenchyma [µm]	264.25 ± 17.55 ^{c}	286.02 ± 9.42 ^{c}	320.38 ± 13.11 ^{b}	370.25 ± 18.31 ^{a}
Leaf xylem vessel number	3.50 ± 0.35 ^{b}	3.50 ± 0.61 ^{b}	4.30 ± 0.57 ^{b}	5.60 ± 0.55 ^{a}
Leaf xylem vessel diameter [µm]	7.07 ± 0.53 ^{c}	8.69 ± 0.25 ^{b}	10.25 ± 0.57 ^{a}	10.56 ± 0.86 ^{a}
Leaf sclerenchyma area [µm^2]	1506 ± 367 ^{d}	2347 ± 206 ^{c}	3594 ± 327 ^{b}	4839 ± 484 ^{a}

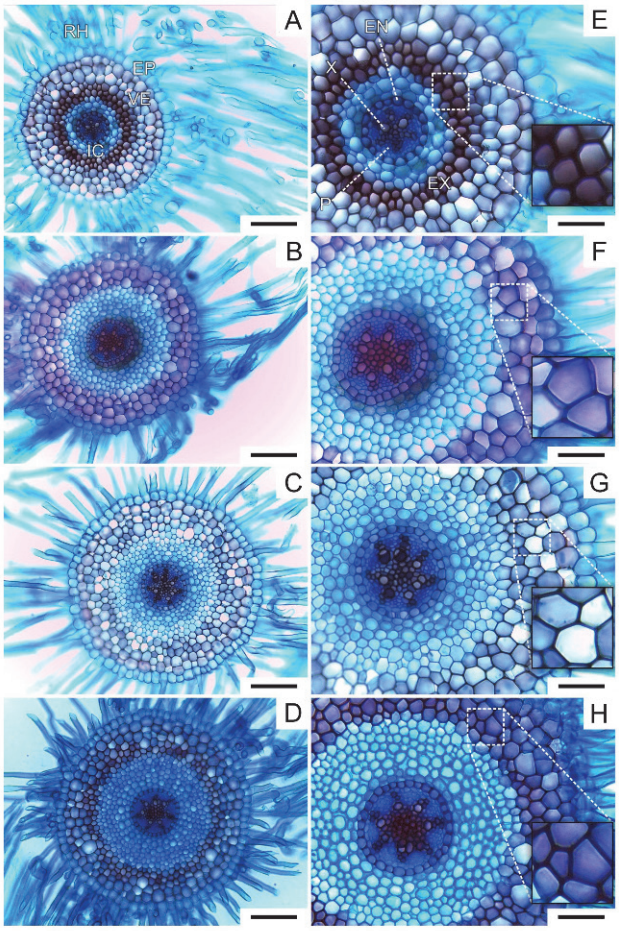

Fig. 2. Root cross sections of *Aechmea blanchetiana* plantlets grown on a medium with different NAA concentrations: 0 (*A*,*E*), 2 (*B*,*F*), 4 (*C*,*G*), and 6 µM (*D*,*H*) for 50 d. RH - root hair, EP - epidermis, VE - velamen, IC - inner cortex, EX - exodermis, EN - endodermis, X - xylem, and P - phloem. *Bars* = 100 µm (*A-D*) and 50 µm (*E-H*).

significantly affected by NAA treatment. Both the chlorenchyma thickness and sclerenchyma area increased linearly as a function of NAA concentration, and the control plants possessed the fewest and thinnest xylem vessels, whereas the xylem vessels with the largest diameter were observed in the plants cultured with 4 and 6 µM NAA (Fig. 3, Table 1).

Treatment with NAA also affected the photosynthetic apparatus after 50 d of *in vitro* culture. All the chlorophyll *a* fluorescence curves possessed typical OJIP shapes from a basal level (F_0) to a maximum level (F_M), and the J and I steps were well defined. The control plants had the highest F_0 values, and the plants cultured with NAA exhibited an increase in the P-step ($P = F_M$; Figs. 4*A* and 6). The relative fluorescence curves [$\Delta V_{OP} = V_{OP(treatment)} - V_{OP(control)}$] included negative bands at the OJ and JI phases in all the treatments, except the control, and a gradual reduction (to more negative values) was observed as a function of NAA concentration (Fig. 4*B*).

To further analyze the effects of NAA treatment, the relative fluorescence between the O and K steps [20 and 300 µs, respectively, $V_{OK} = (F_t - F_0)/(F_K - F_0)$] and between the O and J steps [20 µs and 2 ms, respectively, $V_{OJ} = (F_t - F_0)/(F_J - F_0)$] were normalized and expressed as the kinetic difference [$\Delta V_{OK} = V_{OK(treatment)} - V_{OK(control)}$ and $\Delta V_{OJ} = V_{OJ(treatment)} - V_{OJ(control)}$, respectively]. Both the L-band and the K-band were negative for all the treatment groups when compared to those of the control group, and the values of both bands progressively decreased as a function of NAA concentration (Fig. 5*A,B*).

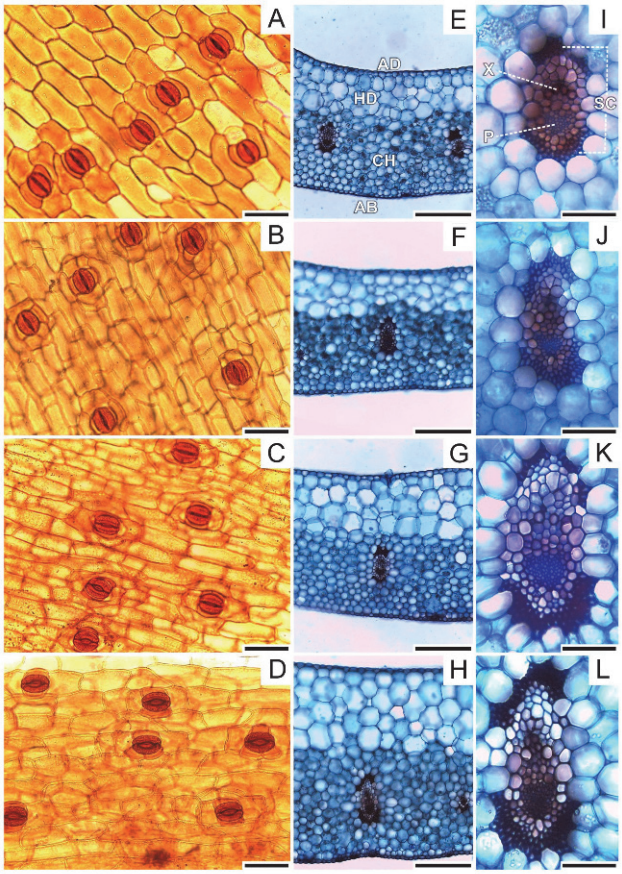

Fig. 3. Paradermal sections (*A-D*) and cross sections (*E-L*) of leaves of *Aechmea blanchetiana* plantlets grown on a medium with different NAA concentrations: 0 (*A,E,I*), 2 (*B,F,J*), 4 (*C,G,K*), and 6 µM (*D,H,L*) for 50 d. AD - adaxial epidermis, AB - abaxial epidermis, HD - hydrenchyma, CH - chlorenchyma, SC - sclerenchyma, X - xylem, and P - phloem. *Bars* = 50 µm (*A-D* and *I-L*) and 200 µm (*E-H*).

The difference between the O (20 µs) and I (30 ms) phases was also evaluated. The chlorophyll fluorescence data were normalized [$V_{OI} = (F_t - F_0)/(F_I - F_0)$] and expressed as kinetic differences [$\Delta V_{OI} = V_{OI(treatment)} - V_{OI(control)}$]. All the NAA treatments showed negative bands, with a negative relationship between the kinetic difference and increasing NAA concentration. The lowest values were observed in the plants cultured with 6 µM NAA (Fig. 5*C*).

All the JIP-test data were normalized relative to the control (0 µM NAA; Fig. 6). The majority of the JIP-test parameters that were based on fluorescence emission kinetics varied significantly as a function of NAA after 50 d of *in vitro* culture. The ABS/RC and DIo/RC values (energy fluxes for absorption and dissipation per reaction center, respectively) were reduced by NAA treatment,

whereas TRo/RC and ETo/RC were unaffected.

The NAA concentration had also a significant effect on quantum yield parameters. The lowest φPo (1 - F_0/F_M = F_v/F_M) was observed in the control plants (0.63), whereas the plants cultured with 2, 4, and 6 μM NAA had φPo values of 0.69, 0.74, and 0.75, respectively.

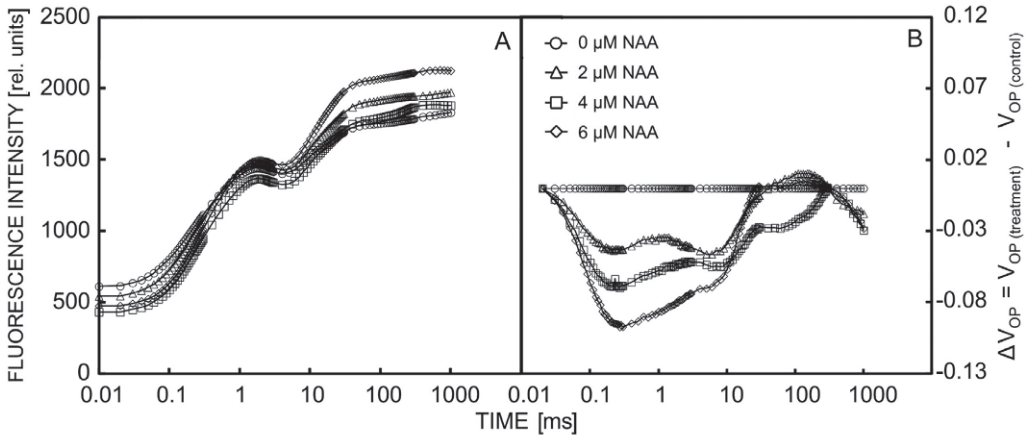

Fig. 4. Chlorophyll *a* fluorescence transients of *Aechmea blanchetiana* plantlets grown on a medium with different NAA concentrations for 50 d. *A* - Fluorescence intensity; *B* - kinetic differences of relative variable fluorescence (ΔV_{OP}).

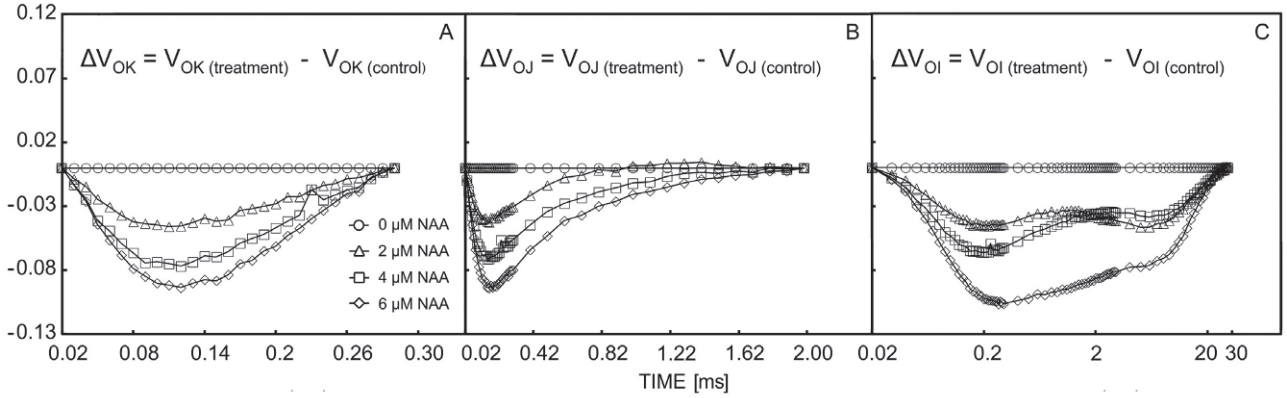

Fig. 5. Chlorophyll *a* fluorescence transient points between the O-J, J-I, and O-I stages of *Aechmea blanchetiana* plantlets grown on a medium with different NAA concentrations for 50 d. *A* - kinetic differences between steps O and K showing the L-band; *B* - kinetic differences between steps O and J showing the K-band; C - kinetic differences between points O (20 μs) and I (30 ms).

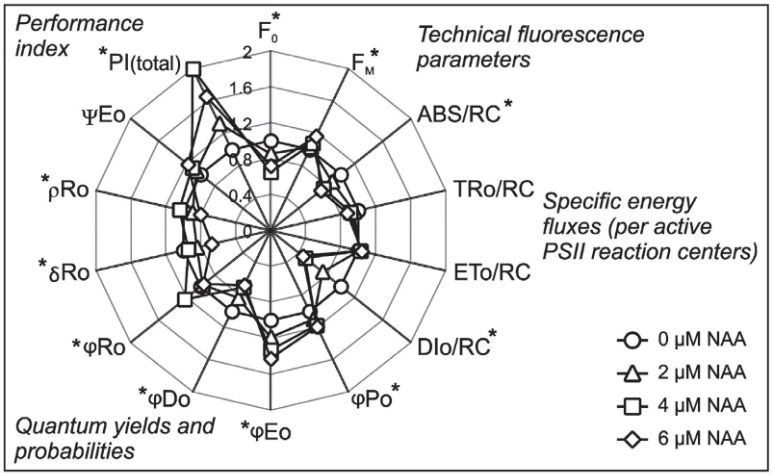

Fig. 6. The JIP test parameters of *Aechmea blanchetiana* plantlets grown on a medium with different NAA concentrations for 50 d. Average values followed by *asterisks* are significantly different according to Tukey test at a probability level < 5 %.

Similarly, φEo values also increased as a function of NAA concentration, but ΨEo was not affected significantly. In contrast, φDo (F_0/F_M) decreased with an increasing NAA concentration, and the highest φRo value was observed in the plants grown with 4 μM NAA. The δRo and ρRo values were lowest in the plants cultured with 6 μM NAA. The performance index [PI(total) = ABS/RC × φPo/(1 - φPo) × ψEo/(1 - ψEo) × δRo/(1 - δRo)] increased by a lower NAA concentration but decreased at concentrations of 4 and 6 μM.

After 60 d of acclimatization, the survival rates of *A. blanchetiana* plants in each treatment group were 100 %. However, the plants cultured with 4 and 6 μM NAA exhibited the greatest biomass accumulation, whereas those cultured without NAA exhibited the lowest fresh and dry masses and the shortest leaves (Table 2).

Table 2. Growth traits of *ex vitro* grown *Aechmea blanchetiana* as a function of NAA concentration. Means ± SDs, n = 4. Means followed by the same letter in the row are not significantly different according to Tukey test at aprobability level > 5 %.

Growth traits	NAA [μM] 0	2	4	6
Length of the longest leaf [cm]	8.49 ± 0.23 ^{c}	9.98 ± 1.37 ^{bc}	13.41 ± 0.49 ^{a}	11.80 ± 1.51 ^{ab}
Shoot fresh mass [g plant^{-1}]	1.72 ± 0.08 ^{c}	2.43 ± 0.67 ^{bc}	4.17 ± 0.45 ^{a}	3.32 ± 0.68 ^{ab}
Shoot dry mass [g plant^{-1}]	0.11 ± 0.02 ^{c}	0.15 ± 0.04 ^{bc}	0.29 ± 0.02 ^{a}	0.24 ± 0.08 ^{ab}

Discussion

The present study represents the first report, to our knowledge, of the effect of NAA on root and leaf anatomy and on the photosynthetic apparatus of a bromeliad during *in vitro* culture. The plants grown without exogenous auxin grew slower and exhibited a poorer photosynthetic performance than those cultured with NAA, and the positive effects of auxin exposure during *in vitro* culture were still evident after 60 d of *ex vitro* conditions.

Even though auxin supplementation was not essential for root induction in the *A. blanchetiana* microshoots, the plantlets grown with NAA had greater growth rates than those grown without NAA. Thus, exogenous auxin may not be a limiting factor for root induction in *in vitro* rooting bromeliad explants, nevertheless, it improves the quality of *in vitro* cultivated plants (Martins *et al*. 2013, Resende *et al*. 2016, Viehmannova *et al*. 2016). In addition, the microshoots may have a low content of endogenous auxins, which could limit the number of adventitious roots (Pacurar *et al*. 2014).

Auxin also promotes cell division and expansion and can be transported actively through vascular bundles or parenchyma (Teale *et al*. 2006). Avramova *et al*. (2015) investigated the distribution of auxin along the leaf growth zone. The authors observed that leaf bases contain the highest auxin content, which probably contributes to the high rates of cellular division and elongation in maize leaf bases, and that auxin content decreases slightly and then stabilizes in the middle zone of the leaf. Similarly, NAA may have been transported from the growth medium to the aerial plant parts by vascular bundles at the base of the cut explant, thereby contributing to the increased growth observed in the *A. blanchetiana* shoots cultured with NAA.

The anatomy of *A. blanchetiana* documented in the present study agrees with that reported by Martins *et al*. (2015b, 2016a) for species of the subfamily *Bromelioideae*. Treatment with NAA had a significant effect on both root anatomy and leaf anatomy of the *A. blanchetiana* plantlets and caused differences that remained important even after the plantlets were transferred to *ex vitro* conditions. The NAA-induced changes in root anatomy could improve mineral and sugar uptake from the medium, thereby increasing *in vitro* growth. Exogenous auxin had also a significant effect on the root hair development of *A. blanchetiana*. Root hairs are a low-cost mechanism for increasing root surface area and, thus, water and nutrient acquisition (Nestler and Wissuwa 2016). As an auxin, NAA may have a positive effect on the length and formation of root hairs (Yu *et al*. 2015) and it may act downstream of pathways that determine the fate of root epidermal cells (Rigas *et al*. 2013).

Exposure to NAA can also reduce the cell wall thickness of the exodermis and might enhance the translocation of minerals between cortex cells. The lignification and suberization of cell walls of the exodermis occur naturally in bromeliads and has already been reported in an *in vitro*-cultured bromeliad (Martins *et al*. 2016a). The lignified cells of the exodermis assume the role of an apoplastic barrier and, in conjunction with the velamen, offer mechanical protection and prevent water from returning from the cortex to external environment, a fundamental strategy of plants with those tissues (Ma and Peterson 2003, Joca *et al*. 2017). The exodermis and endodermis may develop suberin lamellae and thick, tertiary walls, which are collectively known as Casparian bands. Casparian strips function as a barrier to apoplastic flow, thereby regulating the movement of water and mineral elements between the cell layers (Enstone *et al*. 2002). A greater suberization can reduce conductance by reducing the apoplastic inflow of ions

that occurs near the root surface, as verified previously by both Cheng *et al*. (2012) and Martins *et al*. (2016a). Increased exodermis lignification tends to decrease the capacity of roots for metal uptake (Cheng *et al*. 2012). The endodermis also acts as a barrier against the diffusion of metals into the vascular system (Eapen and D'Souza 2005). In the present study, the thickness of the endodermis was unaffected by NAA concentration but was strongly thickened with lignin. A thicker endodermis may not function as an improved apoplastic barrier against mineral uptake. Martins *et al*. (2016a) reported that there is no change in endodermis thickness in an *in vitro*-cultured bromeliad and that thicker exodermal cells can act as an effective first apoplastic barrier.

Variation in the number and diameter of xylem vessels strongly affects axial water conductance. According to Hagen-Poiseuille law, water transport in xylem vessels is proportional to the fourth power of the vessel radius. Therefore, reducing the diameter of the largest xylem vessels would strongly affect the flow of water through the root system by reducing axial conductance (Scholz *et al*. 2013, Lynch *et al*. 2014). From this perspective, even a small increase in vessel number or diameter could have a large effect on a specific hydraulic conductivity, and so NAA could considerably improve the translocation of water, sucrose, and macronutrients and micronutrients.

The main problem for *ex vitro* transfer of micropropagated plants is the insufficient control of transpiration due to poor stomatal function (Dias *et al*. 2014b). An increase in stomata equatorial diameter together with a decrease in polar diameter indicates a reduction of their functionality (Pereira *et al.* 2014). A proper stomatal function is important for avoiding water deficit in *ex vitro* conditions (Dias *et al*. 2014a, Martins *et al*. 2015b). Well-developed epidermal tissue is also crucial for counteracting excess of water loss from leaves during the acclimatization period (Hameed *et al*. 2013). In the present study, the anatomical leaf traits, such as a well-developed epidermis and functional stomata, which were observed in all the treatment groups, can explain the successful acclimatization (100 % survival rate) of the explants, regardless of NAA treatment.

Maintaining a sufficient photosynthetic rate is important for plant growth after transfer to *ex vitro* conditions. A higher photosynthetic capacity may be correlated with a larger mesophyll volume (Freschi *et al*. 2010), as verified in the present study, which showed a positive relationship between the chlorenchyma thickness and some characteristics of photosynthetic apparatus function of *A. blanchetiana*.

Well-developed sclerenchyma cells are important for maintaining plant shape. In response to NAA treatment, the *A. blanchetiana* plants made progressively larger investments into sclerenchyma fibers, provided a mechanical support for plants, and as a component of vascular bundles, supported the transport of water, nutrients, and signaling molecules throughout the plants (Bao *et al*. 2012, Martins *et al*. 2015b).

In the present study, the anatomical changes induced by NAA treatments greatly affected the performance of the photosynthetic apparatus. This could be the result of improved transport of the required minerals to the photosynthetic apparatus, which optimized its performance. The NAA also reduced the F_0 values of treated plants, which is mainly related to energy losses in the pigments of the PS II antenna (Goltsev *et al*. 2016). Reduced F_0 values indicate an improvement in the transfer of electrons from the primary quinone electron acceptor of PS II (Q_A) to the secondary quinone electron acceptor (Q_B). An increased P-step (F_M) level was also observed under NAA treatment. The I-P phase indicates the rapid reduction of the plastoquinone (PQ) pool around PS I (Kalaji *et al*. 2014). Under stress, plants normally inhibit the reduction of both the PQ pool and the electron-transport acceptors around PS I, as indicated by a decreased F_M (Kalaji *et al*. 2014, Martins *et al*. 2015a; Lee *et al*. 2016). Thus, since NAA efficiently induces the reduction of PQ with a lower energy loss by non-photochemical energy dissipation, as indicated by reduced DIo/RC and φDo values (Fig. 6), the NAA-induced increase of F_M level was beneficial to the *in vitro* culture of *A. blanchetiana*. An increase in F_0 and a decrease in F_M may indicate a lower reaction rate or a barrier to the transport of electrons from P_{680} to Q_A^- and the development of non-radiative dissipation of the excited states of PS II antennae chlorophylls (Dabrowski *et al*. 2016).

The negative amplitudes of the O-J and J-I phases in the NAA-treated plants indicate that the *A. blanchetiana* plants had the capacity to photochemically reduce Q_A. However, the kinetic properties required to reduce or oxidize the PQ pool increased as a function of NAA concentration (Fig. 4*B*). The O-J phase indicates the reduction of pheophytin, which is considered the primary electron acceptor in the reaction centers (RCs), to Q_A, whereas the J-I phase indicates the imbalance between Q_A- reduction and reoxidation (Kalaji *et al*. 2014).

The L-band is an indicator of the energy connectivity or grouping among PS II units; the greater grouping PS II, the higher energetic connectivity (Strasser and Stirbet 1998). This grouping is sensitive to thylakoid stacking and de-stacking (Kalaji *et al*. 2014). The L-band may assume positive or negative values depending on the difference between the energetic connectivity of the treatment and control (Yusuf *et al*. 2010, Martins *et al*. 2015a), but when the L-band value is positive, the energetic connectivity is low (Strasser and Stirbet 1998). In the present study, all the NAA treatments yielded negative L-band values indicating a high connectivity. Nevertheless, decreases in the L-band values with an increasing NAA concentration indicate a direct relationship between both the utilization of excitation energy and system stability and auxin concentration.

A positive K-band has been associated with the partial inactivation of the oxygen-evolving complex or an increase in the size of the functional PS II antenna (Yusuf *et al*. 2010, Oukarroum *et al*. 2016). After 60 d of *in vitro*

culture, the plants in the present study had only negative K-band values that, as observed with the L-band, decreased as a function of NAA concentration. This indicates an improved balance between the electrons at the acceptor and donor sides of PS II. These conditions further reduced PQ, which was measured by ΔV_{OI}, *i.e.*, the magnitude of the sequence of events from the exciton capture by PS II to the reduction of PQ (Yusuf *et al*. 2010).

The results of the present study indicate that NAA can enhance the efficiency of photosynthesis per RC. A decrease in absorbed energy flux (ABS/RC) failed to reduce electron trapping flux (TRo/RC) or energy transport flux (ETo/RC), although a sharp decrease in energy dissipation flux (DIo/RC) was observed. The ABS/RC, *i.e.*, the effective antenna size of an active reaction center, is calculated as the mean number of photons absorbed by chlorophyll molecules of the RCs (Mehta *et al*. 2010). However, photons are absorbed by chlorophyll molecules associated with both active RC and inactive RC (Öz *et al*. 2014). The increased ABS/RC of the control plants might indicate a decrease in antenna size or, rather, PS II inactivation and the transfer of excitation energy from inactive PS II to active PS II units. The value of F_0, which is an indication of the number of functional chlorophyll molecules, is not related to the RCs of PS II since they both may increase concomitantly (Öz *et al*. 2014) as observed in the present study. The DIo/RC describes the ratio of the total dissipation of untrapped excitation energy from all RCs to the number of active RCs. Dissipation refers to the loss of energy as heat, fluorescence emission, and energy transfer to systems other than electron transport (Strasser *et al*. 2000, Eullaffroy *et al*. 2009). Decreases in the dissipation indicators (DIo/RC and φDo) analyzed in the present study suggest that NAA reduces the loss of trapped energy that does not go to the RC and could be dissipated.

The φPo values represent the conversion and capture efficiency of primary light energy and are an excellent measure of the quantum yield of the primary PS II photochemistry (Strasser *et al*. 2004). The φEo values are a direct measure of electron transport and represent the quantum yield of electron transport at t = 0 (Mathur *et al*. 2016). Improved φPo and φEo values suggest that NAA increases the quantum efficiency of PS II photochemistry either by causing an increase in the connection between antennae and PS II, or by increasing the efficiency of photosynthetic electron transport.

A higher φRo with no changes in δRo or ρRo was observed in the plants grown with 4 μM NAA. In contrast, the plants cultured with 6 μM NAA exhibited decreased δRo and ρRo values. The increased φRo suggests that a greater proportion of energy is transferred into the PS I end electron acceptors, thereby improving the efficiency of electron transport from trapped electrons or plastoquinol to the PS I end electron acceptors *via* cytochrome *b6/f* (Yan *et al*. 2013, Zivcak *et al*. 2014). Meanwhile, reductions in δRo decrease the ability of PS I to transport electrons to its final acceptors and their lower relative pool sizes (Nikiforou and Manetas 2011).

The PI(total) is a performance index that is closely related to the overall growth and survival of plants under stressful conditions and has been described as a sensitive parameter for the JIP test (Yusuf *et al*. 2010, Martins *et al*. 2015a). In the present study, the NAA-treated plants had higher PI(total) values than the control plants. However, the PI(total) values did not increase as a function of NAA concentration. At NAA concentrations higher than 4 μM NAA, δRo decreased, which subsequently caused a slight decrease in the PI(total).

In vitro culture history can have a strong effect on plant growth after transfer to *ex vitro conditions* (Martins *et al*. 2015b). In the present study, NAA-treated *A. blanchetiana* plants exhibited greater growth rates under *ex vitro* conditions owing to the enhanced translocation of nutrients from the medium throughout the plants during *in vitro* culture. This allowed the plants to develop an efficient photosynthetic apparatus probably due to the improved uptake of minerals and sugars.

Conclusions

The addition of NAA to the *in vitro* growth medium of *A. blanchetiana* plants affected both *in vitro* growth and *ex vitro* growth as well as plant anatomy and physiology. The use of NAA during *in vitro* culture improved the quality of *A. blanchetiana* plantlets which positively affected their *ex vitro* growth. The greatest *ex vitro* growth rate was observed for plants that were propagated *in vitro* with 4 μM NAA. The results of the present study demonstrate that the rational application of auxins (*e.g.*, NAA) can improve photosynthetic apparatus of micro-propagated plants.

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