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Zinc and selenium as modulating factors of the anatomy and physiology of *Billbergia zebrina* (Bromeliaceae) during *in vitro* culture

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Abstract

Deleterious effects induced by high zinc (Zn) concentrations can be alleviated with selenium (Se) co-exposure. Therefore, we analyzed the morphophysiological changes of *Billbergia zebrina* in response to Zn and Se co-exposure. Plants were cultured in media containing three Zn concentrations (0, 30, and 300 μM) combined with two Se concentrations (0 and 4 μM), for a total of six treatments. At 75 d of culture, the leaf anatomy, chlorophyll (Chl) *a* fluorescence, and contents of photosynthetic pigments (Chl) and nutrients were analyzed. The total Chl content declined with rising Zn concentrations. Plants cultured with Se presented a decrease in the Chl *a*/*b* ratio and greater total Chl content. Positive L- and K-bands were verified under Se absence and with 30 and 300 μM Zn. Plants showed bioaccumulation capacity and tolerance to excess Zn. Se acted as a modulator to alleviate the Zn stress.

Additional keywords: biomonitors; bromeliad; JIP test; plant anatomy.

Introduction

In recent decades, anthropogenic activities have caused the accumulation of heavy metals at toxic concentrations in the environment (Hou *et al.* 2019, Kumar *et al.* 2019). Heavy metals can accumulate in the soil or be transported as dust particles by the wind (Abbasi *et al.* 2017, Khademi *et al.* 2019, Kumar *et al.* 2019). Their increased concentration in the environment is a serious concern because the areas contaminated by them can directly and/or indirectly harm living beings. Therefore, many countries have established programs to monitor and control heavy metal contents (Sidhu *et al.* 2017, Chen *et al.* 2019). Heavy metals, such as cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) can influence the condition of plants in different ways (Sidhu 2016, Sidhu *et al.* 2017, Georgiadou *et al.* 2018, Singh *et al.* 2018). Nevertheless, some of them are vital elements for cell metabolism, such as Cu, Ni, and Zn.

Zinc is a micronutrient required by plants, essential for enzymatic activities, electron transport chain, and growth and development, especially of higher plants. Consequently, Zn deficiency induces various severe symptoms, in particular reducing crop yields (Roosta *et al.* 2018, Paradisone *et al.* 2020). On the other hand, high concentrations of these micronutrients may cause negative effects due to oxidative stress (Tiecher *et al.* 2017, Sidhu *et al.* 2020). Exposure to high concentrations of Zn can increase contents of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) as well as higher superoxide dismutase (SOD) and peroxidase (POD) activity (Jain *et al.* 2010, Tiecher *et al.* 2017). The toxic effects of higher amounts of Zn also include inhibition of growth and photosynthesis, altered water balance and nutrient assimilation, senescence, and cause plant death (Singh *et al.* 2016).

Excess Zn can impair the photosynthetic activity of the leaves, causing a reduction in the yield of PSII photochemistry and photodamage (Monnet *et al.* 2005). In contrast, some plant species have the ability to grow in Zn-contaminated areas due to high photosynthetic capacity

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Abbreviations: Chl – chlorophyll; F_v – variable fluorescence; OCE – oxygen-evolving complex; $PI_{(ABS)}$ – performance index based on absorption; $PI_{(total)}$ – overall performance index, which measures the performance up until the final electron acceptors of PSI; RC – reaction center; RC/CS_0 – density of the active reaction centers; $SFI_{(ABS)}$ – structure–function index; total Chl – total chlorophyll (= Chl *a* + Chl *b*); V_I – relative variable fluorescence at 30 ms (point I); V_J – relative variable fluorescence at 2 ms (point J); V_K – relative variable fluorescence at 0.3 ms (point K); W_K – represents the damage to OEC; W_L – represents the changes in the fluidity of the thylakoid membrane and damages to its function and structural integrity; φ_{D0} – quantum yield of energy dissipation; φ_{E0} – quantum yield of electron transport; φ_{P0} – maximum quantum yield of primary photochemistry; φ_{R0} – quantum yield of reduction of end electron acceptors at the PSI acceptor side; ψ_0 – probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-.

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in the presence of excessive Zn concentrations (Andrejić *et al.* 2018).

The negative effects induced by heavy metals can be alleviated by a co-exposure to some mineral elements, such as selenium (Se) and silicon (Si) (Pereira *et al.* 2018, Paradisone *et al.* 2020). The use of Se, at low concentrations, can play a positive role in plants by promoting growth, increasing mineral nutrition and photosynthetic pigment content, and inducing morphological changes against heavy metal toxicity (Handa *et al.* 2018, Yin *et al.* 2019a, Zhao *et al.* 2019). Although this element is considered nonessential for plants, it is considered beneficial to many species, including bromeliads (Haghighi *et al.* 2016, Souza *et al.* 2019). A previous report demonstrated the beneficial effects of Se at low concentrations on the photosynthetic apparatus performance of the bromeliad *Billbergia zebrina* (Souza *et al.* 2019). This can be attributed to the nutritional modulations induced by Se exposure.

Pollution levels can be monitored by analyzing plants that assimilate the heavy metals dispersed in the environment or present clear morphophysiological changes. The results of the analysis provide information about the quality of their environment. Some studies have already shown potential use of bromeliad species as biomonitors (Cardoso-Gustavson *et al.* 2016, Martins *et al.* 2016, Marques Junior *et al.* 2018, Benítez *et al.* 2019). A study carried out under *in vitro* conditions showed that the species *B. zebrina* and *Aechmea blanchetiana*, both belonging to the Bromeliaceae family, have the potential for biomonitoring of Cu (Martins *et al.* 2016, 2020). Therefore, their use for monitoring other heavy metals is promising.

In vitro culture techniques offer benefits in studies related to plant physiology since they permit isolating the effects of the heavy metal stress without any external environmental interference. Several studies have reported morphophysiological alterations of plants submitted to heavy metals under *in vitro* conditions (Martins *et al.* 2016, 2020; Rodrigues *et al.* 2017, Muszyńska *et al.* 2018). These authors verified such changes through histological analysis (plant anatomy), measurement of enzymatic activity of the antioxidant system, or performance of the photosynthetic apparatus. Chlorophyll (Chl) *a* fluorescence measurements can be used to learn the stress level of plants through the performance of the photosynthetic apparatus, especially regarding PSII (Kalaji *et al.* 2017, 2018). Determining the nutrient content can also assist in this type of study (Aydoğan *et al.* 2017, Tiecher *et al.* 2017, Wu *et al.* 2020). The nutrient status can affect the photochemical process of photosynthesis as well as plants' growth and development (Kalaji *et al.* 2018).

In view of the foregoing, we hypothesized that: (*1*) the species under study is tolerant to excess Zn, but Zn exposure impairs its photosynthetic capacity; (*2*) Zn induces anatomical and physiological changes in *B. zebrina* plants; (*3*) Se can modulate the nutritional status; and (*4*) Se acts as a modulator to alleviate Zn stress. Thus, our aim was to analyze the effects of Zn and Se co-exposure during *in vitro* cultivation of *B. zebrina* plants. In addition, we also checked the biomonitoring potential of this species.

Materials and methods

Plant material and Se and Zn exposure: *Billbergia zebrina* plants were previously propagated *in vitro* through the procedures described by Martins *et al.* (2015). To analyze the co-exposure effects of Se and Zn during *in vitro* culture, side shoots of *B. zebrina* were used as explants. The shoots were transferred to 268-ml glass containers holding 50 ml of Zn-modified MS culture medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose and solidified with 3.5 g L^{-1} agar. The treatments consisted of three concentrations of Zn (0, 30, and 300 μM) combined with two concentrations of Se (0 and 4 μM), totaling six treatments. The treatment with no addition of either Se and Zn was considered the control. The Se concentrations were defined according to Souza *et al.* (2019). As Zn and Se sources, $ZnSO_4$ and Na_2SeO_3 were chosen. The pH of the culture medium was adjusted to 5.8 before autoclaving at 120°C for 20 min. After inoculation in a laminar flow chamber, the plant material was kept in a growth room for 75 d at a temperature of 26 ± 2°C and a photoperiod of 16 h.

Growth analysis: To infer the growth of *in vitro* plants, 50 plants from each treatment were collected randomly, divided into ten samples, and weighed on a precision scale. Then the fresh mass (FM) of the shoots (aerial part) and roots was determined in grams per plant.

Photosynthetic pigments content: For the quantification of photosynthetic pigments, eight plants were used. The pigments were extracted with 30–39 mg of leaf material (the third fully expanded leaf). The plant material was placed in test tubes containing 5 ml of 80% acetone, kept at a temperature of 4°C in the dark for 48 h, followed by spectrophotometric analysis (*Genesys*™ *10S* UV-Vis spectrophotometer, *Thermo Fisher Scientific*, West Palm Beach, FL, USA). The absorbances were read at $\lambda = 665$ (Chl *a*) and 645 nm (Chl *b*) (Arnon 1949). The photosynthetic pigment contents were expressed in μg g^{-1}(FM of leaf tissue).

Chl *a* fluorescence analysis: The transients of Chl *a* fluorescence were measured after 75 d of co-exposure to Zn and Se. The analysis was performed in 12 plants per treatment using a portable fluorometer (*Handy PEA*, *Hansatech Instruments Ltd.*, King's Lynn, Norfolk, UK) and the measurement procedures were based on Souza *et al.* (2019). The emission of transient fluorescence was then recorded from 1 μs to 1 s with 120 fluorescence points. The fluorescence intensities were expressed as F_0 (0.02 ms), F_L (0.15 ms), F_K (0.3 ms) F_J (2 ms), F_I (30 ms), and F_m (300 ms). The analyses of OJIP transients and the JIP test parameters were according to Strasser *et al.* (2004).

Leaf anatomy: The leaf anatomy was analyzed in five plants chosen randomly from each treatment. The samples were fixed/stored in 50% ethanol. The cross sections and paradermal sections were obtained and stained and the measurement procedures were performed as described by Martins *et al.* (2019). The sections were viewed using a light microscope (*L-2000A-Fluor*, *Bioval*, São Paulo, Brazil), and images were captured with a *Leica EC3* camera (Wetzlar, Germany). The stomatal density [0.1 mm^2] and the number and diameter of xylem vessels [µm] were determined in leaves.

Mineral nutrient content: The mineral nutrient content in *B. zebrina* plants was analyzed at 75 d of growth. There were three repetitions per treatment, using only the aerial part (previously washed in distilled water and dried by forced air circulation at 70°C). The contents of N, K, Ca, Mg, Cu, Mn, S, and Zn were determined. The dried plant material was ground in a Wiley mill with 20 mesh sieve and digested in nitro-perchloric solution 2:1 (v/v) for the determination of K, Ca, Mg, Cu, Mn, S, and Zn; and sulfuric acid solution for the determination of nitrogen (N) by the micro Kjeldahl method (distillation). The contents of N, Ca, Mg, Cu, Mn, and Zn were then determined by atomic absorption spectroscopy (*AA-7000*, *Shimadzu*, Tokyo, Japan); S content by UV-visible spectrophotometry (*Biospectro SP-22* spectrophotometer, São Paulo, Brazil); and K content by flame photometry (*B262*, *Micronal*, São Paulo, Brazil) (Malavolta *et al.*1997).

Statistical analysis: The experimental design was completely randomized with a 2 × 3 factorial scheme: two Se concentrations (0 and 4 µM) and three Zn concentrations (0, 30, and 300 µM). The resulting data were submitted to analysis of variance (*ANOVA*) and the averages were compared using the *Tukey*'s test ($p<0.05$). All analyses were performed using the *SISVAR* software.

Results

Biomass accumulation: After 75 d of *in vitro* culture with excess Zn, irrespective of Se supplementation, the plants did not show any mortality or visible physiological disturbances, such as chlorosis, necrosis, or leaf discoloration. However, both variation factors influenced biomass accumulation, but they acted independently. The fresh mass of roots increased as a function of Zn concentrations (R^2 = 0.98). In contrast, the tested concentration range of Zn did not influence the shoot fresh mass. When comparing the Se treatments, irrespective of Zn, the biomass of roots decreased and shoots increased with 4 µM of Se supplementation (Fig. 1).

Photosynthetic pigments: The contents of pigments were also influenced by the treatments, but the factors (Zn and Se concentrations) did not have significant interaction. The total Chl content decreased as a function of Zn concentrations, but there were no changes in the Chl *a*/*b* ratio. On the other hand, Se exposure induced a decrease in the Chl *a*/*b* ratio as well as increased the total Chl content (Fig. 2).

Chl fluorescence analysis: All plants remained photosynthetically active, irrespective of the applied treatment. Nevertheless, the relative variable fluorescence between the O- and P-steps presented a decrease at the K-step and J-step in all treatments with 4 µM Se compared to Se absence. The V_I did not change as a function of Se, but it increased when plants were exposed to Zn (Fig. 3*A*,*B*). Kinetic differences were also observed between steps O–K and O–J. Positive L- and K-bands were verified under Se absence and with 30 and 300 µM Zn. In addition, W_L [$W_L = (F_L - F_0)/(F_K - F_0)$] and W_K [$W_K = (F_K - F_0)/(F_J - F_0)$] were lower when plants were cultured with Se (Fig. 3*C*,*D*).

The JIP test showed changes in the photosynthetic apparatus as a function of both Zn and Se. This influence of both factors was independent (without a significant interaction of the variation factors) for most parameters. When the plants were cultured with 300 µM Zn, they presented reduced F_v and RC/CS_0 values, independently of the Se concentration. In contrast, plants grown in

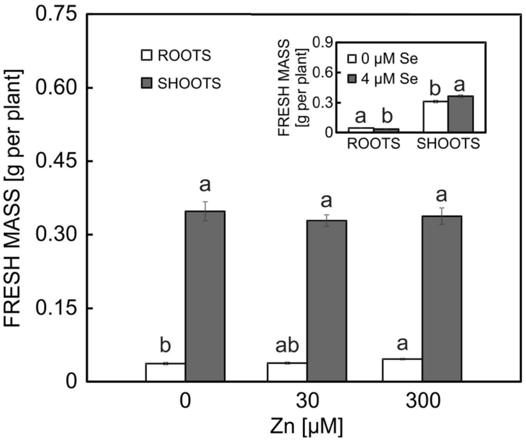

Fig. 1. Fresh mass [g per plant] of shoots (aerial parts) and roots of *Billbergia zebrina* plants at 75 days of *in vitro* growth as a function of Zn [µM] and Se [µM] concentrations. For each growth trait, means ± SE (n = 10) followed by *the same letter* do not differ significantly according to the *Tukey*'s test ($p<0.05$).

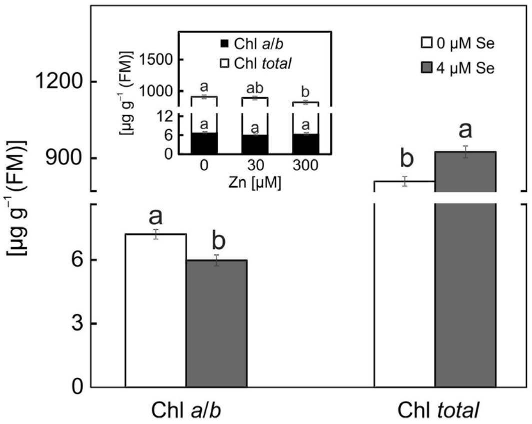

Fig. 2. Chlorophyll content and Chl *a*/*b* ratio [µg g^{-1}(FM)] of *Billbergia zebrina* plants as a function of Zn [µM] and Se [µM] concentrations during *in vitro* culture. For each trait, means ± SE (n = 8) followed by *the same letter* do not differ significantly according to the *Tukey*'s test ($p<0.05$). FM – fresh mass.

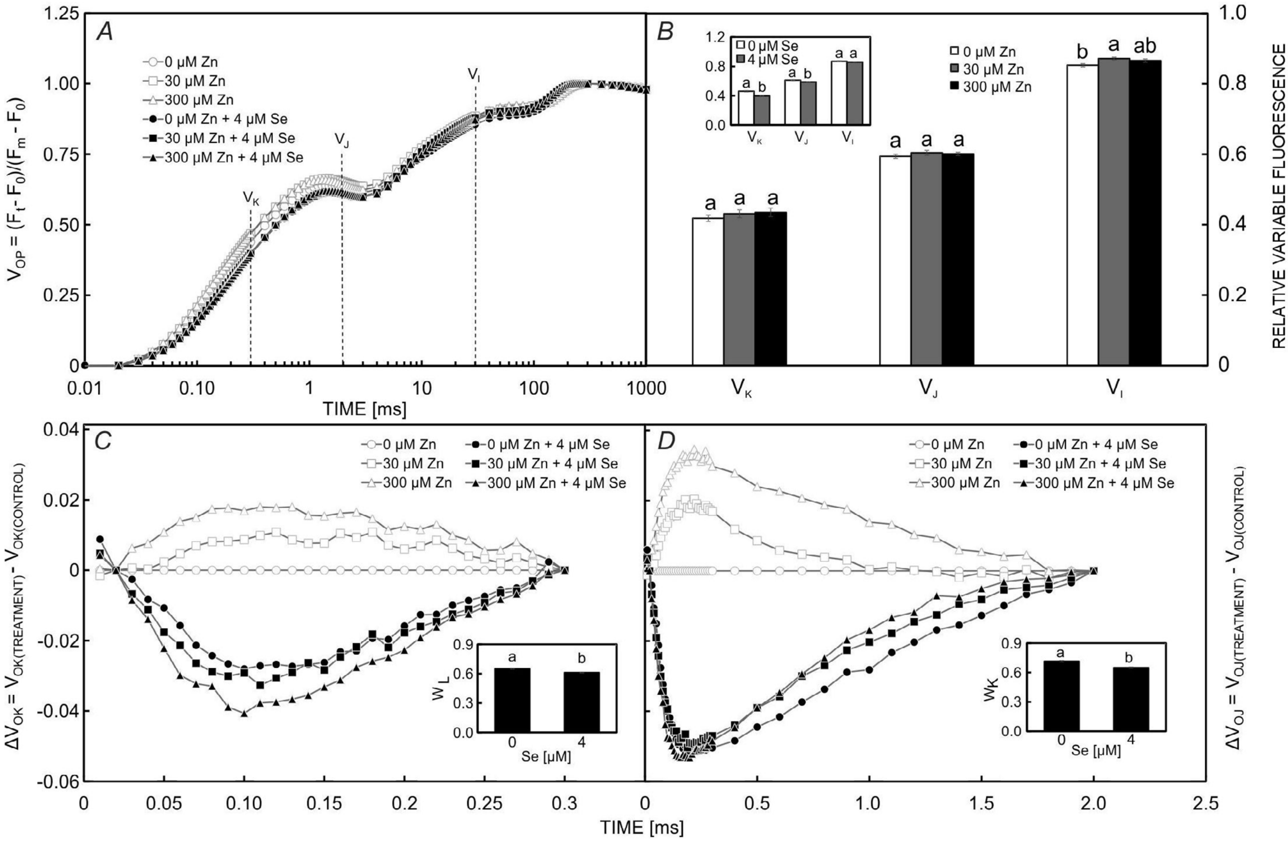

Fig. 3. Relative variable fluorescence and kinetic differences of *Billbergia zebrina* plants grown in media with different Zn [µM] and Se [µM] concentrations. Relative variable fluorescence between F_0 and F_M (V_{OP}) (*A*); relative variable fluorescence at phases K, J, and I (*B*); kinetic differences between steps O and K [$V_{OK} = (F_t - F_0)/(F_K - F_0)$], showing the L-band (*C*); kinetic differences between steps O and J [$V_{OJ} = (F_t - F_0)/(F_J - F_0)$], showing the K-band (*D*). For each parameter, means ± SE ($n = 12$) followed by *the same letter* do not differ significantly according to the *Tukey*'s test ($p<0.05$). V_I – relative variable fluorescence at 30 ms (point I); V_J – relative variable fluorescence at 2 ms (point J); V_K – relative variable fluorescence at 0.3 ms (point K); W_K – damages to OEC; W_L – changes in the fluidity of the thylakoid membrane and damages to its functional and structural integrity.

medium supplemented with 4 µM Se, irrespective of Zn concentration, had higher values of ψ_0, φ_{E0}, φ_{R0}, $SFI_{(ABS)}$, and $PI_{(ABS)}$ compared to those cultured in Se-free medium (Fig. 4, Table 1).

The parameters related to quantum yield of primary photochemistry (φ_{P0}) and dissipation (φ_{D0}), as well as overall performance index [$PI_{(total)}$] interacted significantly to both Zn and Se concentrations. Plants cultured without Se had similar values of φ_{P0} and φ_{D0}. On the other hand, when the media were supplemented with Se, plants showed increased φ_{P0} and decreased φ_{D0} as a function of Zn concentrations. When comparing the two Se concentrations at each Zn concentration, plants grown with Se exhibited higher φ_{P0} and lower φ_{D0}. Likewise, under 4 µM Se exposure, the $PI_{(total)}$ values were similar for the Zn concentrations and higher than that under Se-absence treatment. Also, plants cultured without Se showed decreased $PI_{(total)}$ as a function of rising Zn concentrations (Fig. 5).

Leaf anatomy: All anatomical traits are shown in Fig. 6*A–F*. Stomatal density was influenced by both factors. In media without Se addition, the leaves had a larger number of stomata, while in the presence of Se, the number of stomata per area was similar, regardless of the Zn concentration. Among the concentrations of Zn, only 0 µM Zn + 0 µM Se (control) produced a lower stomatal density (Fig. 6*G*). Concerning the xylem traits, the number of vessels was similar in all treatments, but the diameter of xylem vessels was larger in leaves of Se-treated plants (Fig. 6*H*).

Nutrient content: The contents of nutrients analyzed were influenced by the treatments, but Se and Zn acted separately (Table 2). The high Zn supplementation showed a clear negative effect regarding N, Mn, and Cu contents. The contents of these nutrients decreased at 300 µM Zn. In contrast, the content of Zn increased as a function of Zn concentrations in the media. When analyzing the nutrient contents as a function of Se concentrations, the contents of N, K, Ca, Mg, Mn, and Zn were statistically similar, but the contents of S and Cu were lower in plants cultured with 4 µM Se in comparison with plants cultured in Se-free media.

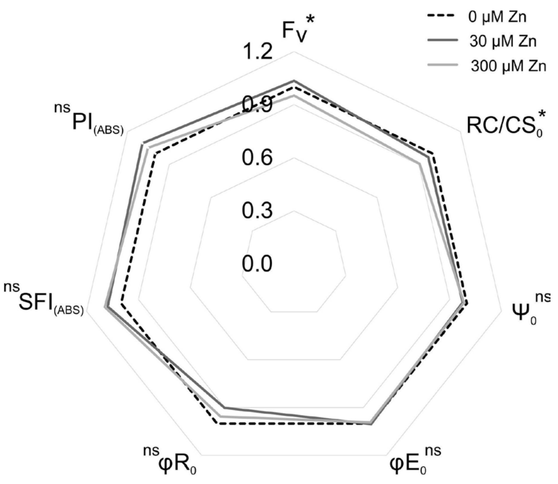

Fig. 4. The JIP test parameters of *Billbergia zebrina* plants grown as a function of Zn [μM] concentrations. Means ± SE ($n = 12$) followed by an asterisk (*) are significantly different according to the *Tukey*'s test ($p<0.05$). F_v – variable fluorescence; $PI_{(ABS)}$ – performance index based on absorption; RC/CS_0 – density of the active reaction centers; $SFI_{(ABS)}$ – structure–function index; φ_{E0} – quantum yield of electron transport; φ_{R0} – quantum yield of reduction of end electron acceptors at the PSI acceptor side; ψ_0 – probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-.

Table 1. JIP test parameters as a function of Se [μM] concentrations obtained based on chlorophyll *a* fluorescence of *in vitro* grown *Billbergia zebrina* plants. Means ± SE ($n = 12$) accompanied by *different letters* in the same row are significantly different by the *Tukey*'s test ($p<0.05$). F_v – variable fluorescence; $PI_{(ABS)}$ –performance index based on absorption; RC/CS_0 – density of the active reaction centers; $SFI_{(ABS)}$ – structure–function index; φ_{E0} – quantum yield of electron transport; φ_{R0} – quantum yield of reduction of end electron acceptors at the PSI acceptor side; ψ_0 – probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-.

Parameter	Se concentration [μM] 0	4
F_v	1,758.14 ± 28.37^a	1,727.64 ± 26.40^a
RC/CS_0	234.98 ± 3.54^a	232.23 ± 3.05^a
ψ_0	0.387 ± 0.004^b	0.413 ± 0.004^a
φ_{E0}	0.253 ± 0.004^b	0.284 ± 0.004^a
φ_{R0}	0.085 ± 0.003^b	0.091 ± 0.001^a
$SFI_{(ABS)}$	0.061 ± 0.002^b	0.072 ± 0.001^a
$PI_{(ABS)}$	2.29 ± 0.27^b	3.70 ± 0.14^a

Discussion

In this study, we reported the effects of excess Zn and Se during the *in vitro* growth of *B. zebrina*. Both elements individually acted as modulating factors of the plants' anatomy and physiology. However, we also found that Se can alleviate the physiological stress of *B. zebrina* plants under excessive Zn exposure.

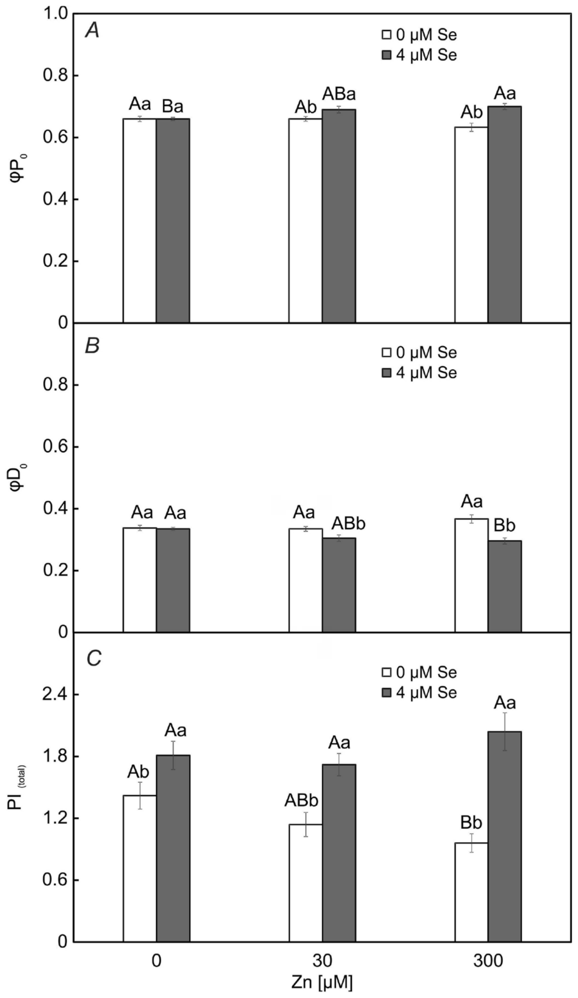

Fig. 5. Photochemical parameters derived from the analysis of the JIP test of *Billbergia zebrina* plants grown in media with different Zn [μM] and Se [μM] concentrations. Means ± SE ($n = 12$) followed by *the same letter* (*uppercase* at each Se concentration – comparing the Zn concentrations; and *lowercase* at each Zn concentration – comparing the two Se concentrations) do not differ significantly according to the *Tukey*'s test ($p<0.05$). $PI_{(total)}$ – overall performance index, which measures the performance up until the final electron acceptors of PSI; φ_{D0} – quantum yield of energy dissipation; φ_{P0} – maximum quantum yield of primary photochemistry.

The conditions imposed during *in vitro* cultivation altered the plants' morphology. The exposure to excess Zn induced plants with a higher root fresh mass. Similarly, Giampaoli *et al.* (2012) and Zampieri *et al.* (2012) verified an increase of biomass accumulation of roots in a bromeliad species under *in vitro* conditions. In our work, this response was linked to a higher number of roots per plant, even though the roots were shorter. Plants can increase the number of roots to allow them to explore a larger area in the medium (Martins *et al.* 2016). Changes in the root architecture may be a response to metals, acting as a heavy-metal 'escape' strategy aimed at recruiting metal-free fragments (Bochicchio *et al.* 2015). On the

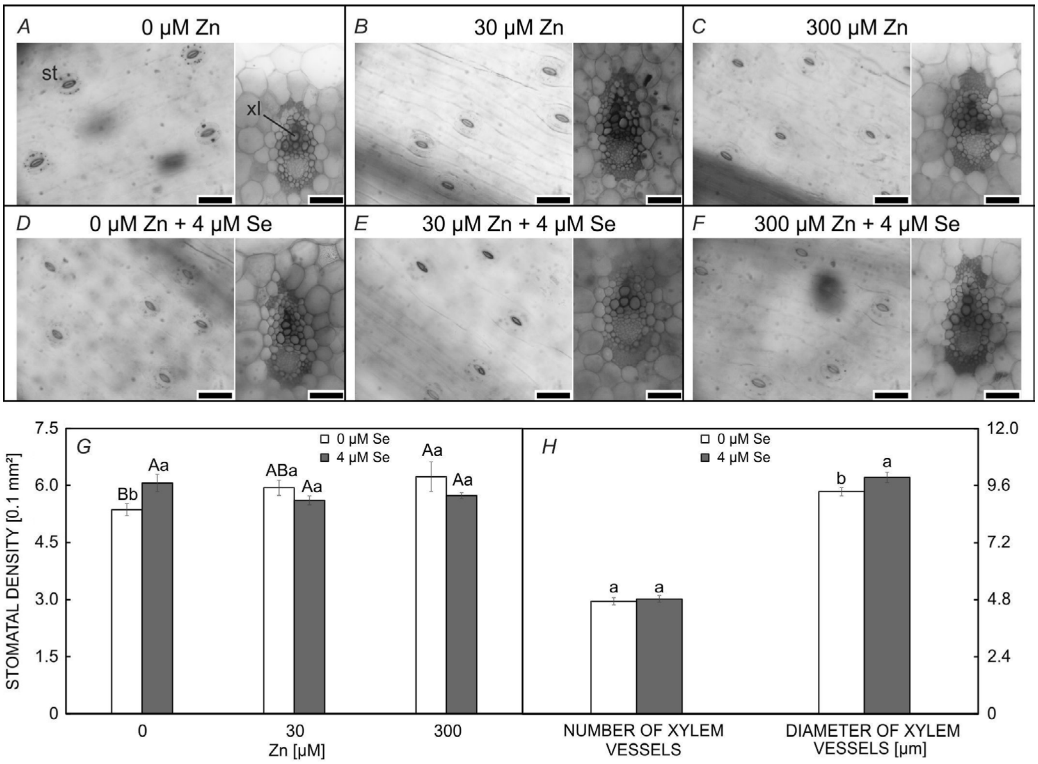

Fig. 6. Anatomical traits of *Billbergia zebrina* leaves of plants grown *in vitro* as a function of Se [μM] and Zn [μM] concentrations. Paradermal sections and cross-sections of *Billbergia zebrina*'s leaves as a function of the Se (0 and 4 μM) and Zn (0, 30, and 300 μM) concentrations during *in vitro* culture (*A–F*). Bar = 100 μm. st – stoma; xl – xylem. Stomatal density as a function of Se and Zn treatments (*G*). Number and diameter of xylem vessels as a function of two Se levels (*H*). For each trait, means ± SE (*n* = 5) followed by *the same letter* do not differ significantly according to the *Tukey*'s test ($p<0.05$). Means ± SE (*n* = 5) followed by *the same letter* (*uppercase* at each Se concentration – comparing the Zn concentrations; and *lowercase* at each Zn concentration – comparing the two Se concentrations) do not differ significantly according to the *Tukey*'s test ($p<0.05$).

Table 2. Nutrient contents of *Billbergia zebrina* plants as a function of Se [μM] or Zn [μM] concentrations during *in vitro* growth. Means ± SE (*n* = 3) accompanied by *different letters* in the same row are significantly different by the *Tukey*'s test ($p<0.05$). Ca – calcium; Cu – copper; K – potassium; Mg – magnesium; Mn – manganese; N – nitrogen; S – sulfur; Zn – zinc.

Nutrients	Se concentration [μM]		Zn concentration [μM]		
	0	4	0	30	300
N [%]	4.47 ± 0.11^{a}	4.46 ± 0.14^{a}	4.57 ± 0.16^{ab}	4.70 ± 0.12^{a}	4.13 ± 0.07^{b}
K [%]	4.09 ± 0.09^{a}	4.01 ± 0.06^{a}	3.96 ± 0.04^{a}	4.20 ± 0.12^{a}	3.98 ± 0.07^{a}
Ca [%]	0.57 ± 0.02^{a}	0.56 ± 0.01^{a}	0.53 ± 0.01^{a}	0.60 ± 0.02^{a}	0.56 ± 0.02^{a}
Mg [%]	0.21 ± 0.01^{a}	0.21 ± 0.01^{a}	0.22 ± 0.01^{a}	0.21 ± 0.01^{a}	0.20 ± 0.02^{a}
S [%]	0.34 ± 0.01^{a}	0.30 ± 0.01^{b}	0.30 ± 0.01^{a}	0.33 ± 0.01^{a}	0.32 ± 0.02^{a}
Cu [ppm]	2.52 ± 0.27^{a}	1.86 ± 0.14^{b}	1.90 ± 0.18^{b}	2.87 ± 0.31^{a}	1.81 ± 0.16^{b}
Mn [ppm]	230.03 ± 7.64^{a}	214.15 ± 9.89^{a}	243.70 ± 4.86^{a}	224.98 ± 7.93^{ab}	197.59 ± 10.55^{b}
Zn [ppm]	135.36 ± 37.87^{a}	132.30 ± 35.11^{a}	39.58 ± 2.45^{c}	85.48 ± 3.95^{b}	276.43 ± 8.41^{a}

other hand, Se exposure had a positive effect on the fresh mass of *B. zebrina* shoots. The Se application can increase the biomass accumulation of many plant species, as well as alleviate abiotic stress, such as that caused by salt and heavy metals (Ashraf *et al.* 2018, Souza *et al.* 2019, Yin *et al.* 2019a). According to Souza *et al.* (2019), low concentrations of Se can increase plant growth due to enhanced photosynthetic apparatus performance. Our findings corroborate that observation.

The present study revealed the physiological stress induced by excess Zn on photosynthetic pigment contents. The stress level of plants can be inferred by analyzing the photosynthetic pigment content because it usually decreases under stress conditions (Martins *et al.* 2018, Souza *et al.* 2020). A reduction of Chl content may be associated with degradation due to oxidative stress (Santos *et al.* 2020, Sidhu *et al.* 2020). Indeed, under Zn stress, alterations of the enzymatic and nonenzymatic status of the antioxidant system have been reported (Bernardy *et al.* 2020, Santos *et al.* 2020, Sidhu *et al.* 2020). Nevertheless, the different Zn concentrations did not change the Chl *a*/*b* ratio. The adjustments in the anatomy and/or physiology can counteract the deleterious effects of excess heavy metals, which is confirmed by the absence of changes in the Chl *a*/*b* ratio (Martins *et al.* 2020). Normally, under heavy metal stress plants show signs of physiological damages induced by the metals' toxicity, such as reduced Chl *a*/*b* ratio along with the reduced total Chl content, indicating an imbalance in the contents of photosynthetic pigments (Aydoğan *et al.* 2017). This response indicates that the heavy metals have a negative effect on Chl *a* content (Houri *et al.* 2020). Still concerning the photosynthetic pigments, Se had a positive effect on Chl contents. The lower Chl *a*/*b* ratio along with the increased total Chl led to improved photosynthetic apparatus performance, as evidenced by the $PI_{(ABS)}$ and $PI_{(total)}$ values.

Further analysis confirmed the positive effects of Se on physiological status, as well as that excess Zn can influence the photosynthetic apparatus of *B. zebrina*. The improvement in the electron transport chain was pronounced in plants cultured with Se. The V_K, W_K, and V_J values can characterize the performance of the water-splitting system of PSII and electron flow from quinone A (Q_A) to quinone B (Q_B) (Chen *et al.* 2015, Zhang *et al.* 2018, Yin *et al.* 2019b). The lower V_K, W_K, and V_J values observed in plants cultured with Se indicated enhanced oxygen-evolving complex (OEC) activity at the electron donor side of PSII and an optimized rate of electron transport between Q_A and Q_B, which means an increase in primary photochemical activity.

Although the positive L- and K-bands were observed in plants under Se absence and with 30 and 300 μM of Zn, the W_L and W_K values indicated a clear distinction between Se treatments (0 and 4 μM Se). Higher W_L values denote lower functional and structural integrity of the thylakoid membranes (Zhang *et al.* 2018). Likewise, increased W_K values may indicate partial damage to electron transport from OEC to P_{680}^+ (Wang *et al.* 2016). In this study, *B. zebrina* plants cultured without Se had a lower performance of thylakoid membranes and OEC efficiency on the donor side of PSII. In addition, the negative L- and K-bands appeared in plants in medium with Se and it pointed to improved energy absorption and connectivity between PSII units.

The higher F_v values of plants also confirmed the enhanced dynamic structure of thylakoid architecture. A decline in F_v values can indicate damage to the thylakoid structure and direct impairment in the reaction centers (RCs) (Kalaji *et al.* 2016, Sha *et al.* 2019). Thus, the decreased values of F_v and RC/CS_0 verified in plants cultured with 300 μM Zn mean that excess Zn can cause damages, leading to the interconversion of active RCs into inactive ones. Inactivation of a part of the RCs of PSII leads to higher energy dissipation ('energy sinks'), thus transforming the excitation energy of the antenna chlorophylls into heat energy (Paunov *et al.* 2018). The heat dissipation through this photoprotective mechanism induced by excess Zn stress was indicated by the φ_{D0} value. There was a reduction of PSII efficiency, demonstrated by φ_{P0} values, which can be attributed to the insufficient energy transfer from the light-harvesting complex to the reaction center of Chl molecules (Sha *et al.* 2019). In this study, Se acted against the stress caused by excess Zn, efficiently assuring better photosynthetic performance (higher values of φ_{P0} and $PI_{(total)}$) due to lower energy loss by nonphotochemical energy dissipation (φ_{D0}).

The parameters related to electron transport were influenced by Se exposure. The increased values of ψ_0 and φ_{E0} as well as φ_{P0} in plants cultured with 4 μM Se pointed to enhanced electron flow from PSII to PSI and enhanced Q_A^- reoxidation due to higher diffusion of plastoquinone (PQ) across the thylakoid membrane (Singh *et al.* 2018), as already indicated by the lower W_L values, pointing to higher functionality of the thylakoid membranes. The improved structure and function of PSII in plants cultured with Se were confirmed by the structure–function index [$SFI_{(ABS)}$]. Increments in $SFI_{(abs)}$ can indicate a reduction in energy dissipated as heat and fluorescence, which may be linked to an increased number of active RCs of PSII and a higher φ_{P0} in plants (Stirbet *et al.* 2018). This high vitality induced an improvement of PSII performance, which was confirmed by the $PI_{(ABS)}$ values.

Alterations were also observed in the I-step (V_I). Increased V_I values can indicate changes in the efficiency/probability of electron movement from PSII to the PSI acceptor side. This is because V_I values lead to decreased values of ψ_{R0} (= 1 – V_I). This parameter is sensitive to the effect of heavy metals and is associated with the significant decrease of PSI activity (Rastogi *et al.* 2019, Faseela *et al.* 2020). According to Küpper *et al.* (2019), a decline in ψ_{R0} under heavy metal exposure can denote a decrease in the efficiency or probability of trapped electron transfer from PSII to PSI. In the present study, Zn exposure only caused a slight increase (~ 2.3%) of V_I values in relation to the plants cultured without Zn. Therefore, Zn in the concentration range tested did not induce a drastic decrease in PSI activity. In contrast, Se acted positively on the PSI activity, as shown by the φ_{R0} values. Souza *et al.* (2019) suggested that Se mainly influences PSI functionality, and this element can enhance the electron transport dynamics from the intersystem to the PSI.

Besides the effects of Zn and Se on *B. zebrina*'s physiology, these mineral elements also influenced the plant leaf anatomy. The plants grown with Zn exposure presented morphological traits indicating a higher potential for mineral uptake. The presence of heavy metals (at moderate concentrations) can stimulate an increase of stomatal density, as already reported for some plant species (Pereira *et al.* 2016, Pires-Lira *et al.* 2020), including other bromeliads (Martins *et al.* 2016, 2020). These authors observed quadratic behavior in relation to stomatal density, that is, the number of stomata increased as a function of heavy metal concentrations, followed by a decrease with supraoptimal concentrations. The number of stomata per area can regulate the transpiration rate and consequently the stomatal conductance (Pereira *et al.* 2016). Increased stomatal density induced by heavy metal exposure can increase stomatal conductance, enhancing CO_2 uptake, possibly indicating tolerant plants. In addition, it can favor nutrient uptake by mass flow from the medium (Martins *et al.* 2019).

The traits of xylem vessels can also modulate hydraulic conductance and metal translocation under heavy metal stress conditions (Silva *et al.* 2012, Rodrigues *et al.* 2017, Cao *et al.* 2020). A larger number and/or diameter of xylem vessels can allow greater translocation and accumulation of heavy metals in the aerial part of plants (Cao *et al.* 2020, Martins *et al.* 2020). In contrast, a decrease in these xylem traits can avoid high uptake and translocation of metals (Rucińska-Sobkowiak 2016, Martins *et al.* 2019). Therefore, it was possible to note anatomical mechanisms that regulated the uptake and transport of Zn from the culture medium, allowing the plants to accumulate this metal. This also confirms that Zn (in the tested concentration range) did not negatively affect the *B. zebrina* plants. In addition, Se can enhance the modulation of those anatomical characteristics.

The nutritional status was associated with the plants' physiological responses. Plants grown under supraoptimal concentrations of Zn can present nutritional imbalance due to interference with other essential microelements (*e.g.*, Cu and Mn) by displacing other vital metallic ions located at the active sites of proteins, thus promoting physiological disorders (Sidhu 2016). This can occur because Zn has a similar ionic radius as bivalent cations, for instance Cu. Hence, it can be absorbed by the same transporters and subsequently be translocated to the aerial part of plants (Tiecher *et al.* 2017). Still in relation to the Cu content, plants grown with Se supplementation had lower contents of this micronutrient. This response was correlated with Se source since under selenite supplementation, plants can present a decrease in Cu contents in their aerial parts (Pazurkiewicz-Kocot *et al.* 2008, Boldrin *et al.* 2013).

Nutrients such as Mn and N are structural components of Chl molecules, so their deficiency can negatively affect the photosynthetic apparatus (Mao *et al.* 2018, Roosta *et al.* 2018, Martins *et al.* 2019). Zinc excess had an antagonistic relationship with N and Mn contents. This influenced the Chl contents as well as the photosynthetic performance. Excess or deficiency of Zn can influence the nitrogen metabolism, leading to inefficient N assimilation and consequently inhibition of Chl formation (Sidhu 2016). Indeed, in this work, the lowest Chl content corroborated the lower N content. Moreover, Mn is associated with the production of oxygen from water during photosynthesis (Mn_4CaO_5 complex) (Haider *et al.* 2006, Gururani *et al.* 2012). So, the decrease of Mn content may have minimized the PSII functionality and diminished the OEC integrity. In the present study, this was expressed as a lower functionality of the OEC, as inferred by the K-bands and W_K values.

The lower S content observed in *B. zebrina* plants cultured with Se may be due to competition by using the sulfate pathway of the plants. Both S and Se share the same absorption site, besides having a competitive relationship for biochemical processes, such as assimilation into amino acids of essential proteins (Drahoňovský *et al.* 2016, Gupta and Gupta 2017). Those elements can decrease heavy metal stress by increasing the activity of glutathione, thus, in turn, reducing oxidative stress (Khan *et al.* 2015).

The co-exposure of Se with heavy metal has often been reported, and Se can reduce the effects of oxidative stress (Pereira *et al.* 2018, Chauhan *et al.* 2019, Wu *et al.* 2020). In the present study, the *B. zebrina* plants showed bioaccumulation capacity and tolerance to excess Zn, irrespective of Se co-exposure; although only exhibiting slight physiological disturbances (*e.g.*, total Chl and RC/CS_0). This response could be related to alterations in H_2O_2 and MDA contents, as reported by Jain *et al.* (2010) and Tiecher *et al.* (2017). The results clearly showed that Se relieved all stress levels induced by excess Zn, in addition to improving plant performance. We suggest this occurred due to the protective role of Se by improved activation of the antioxidant system (Lin *et al.* 2012).

Conclusion: Both Zn and Se, alone or applied concurrently, affected the growth, anatomy, and physiology of *B. zebrina* plants during *in vitro* culture. High concentrations of Zn in the culture medium caused reduced biomass accumulation, besides physiological disorders, such as decreased photosynthetic pigments and the number of active RCs of PSII (RC/CS_0). Se did not have a pronounced impact on nutritional status. Instead, Zn acted to modulate the nutritional status of *B. zebrina* plants. The exposure to Se enhanced the growth and photosynthetic apparatus performance. Moreover, Se also acted as a modulator to alleviate Zn stress. *Billbergia zebrina* plants can be used for biomonitoring due to their tolerance for excess Zn and the ability for bioaccumulation of this metal.

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