Analysis of tolerance to copper and zinc in *Aechmea blanchetiana* grown *in vitro*

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Abstract

The aim of this study was to evaluate the growth and development of *Aechmea blanchetiana* Baker L.B. Sm. *in vitro* on medium with 0.0, 0.145, 1.45 and 14.5 µM Cu and 0.0, 2.75, 27.5 and 275 µM Zn. Significant accumulation of Cu and Zn occurred at 14.5 µM Cu and 27.5 and 275 µM Zn, respectively, and there were no significant changes in contents of the other macro- and micronutrients. Superoxide dismutase (SOD) activity significantly changed in the presence of both metals. Spermine content increased as Zn concentration increased and decreased with increasing concentrations of Cu. There was an accumulation of H_2O_2 in the leaf tissue of plants grown in 1.45 and 14.5 µM Cu and 27.5 and 275 µM Zn. *A. blanchetiana* was found tolerant to the Cu and Zn in concentrations used in this study and displays the capacity to accumulate these metals.

Additional key words: ascorbate peroxidase, ascorbic acid, *Bromeliaceae*, heavy metals, hydrogen peroxide, polyamines, superoxide dismutase.

Introduction

Copper and zinc are naturally present in the environment and in low concentrations essential for plants and animals (Fernandes and Henriques 1991, Broadley *et al.* 2007). However, they are also present in some sewage sludge, organic and inorganic fertilizers and fungicides and they are emitted by various human activities in amounts that could be toxic (Mantovi *et al.* 2003, Figueiredo *et al.* 2007). Some plant species can withstand the excess of heavy metals from the environment compartmentalizing, chelating with metallothionein or excluding them (Cheng 2003, Usha *et al.* 2011). Cu and Zn may accumulate in plant tissues, causing many physiological and biochemical changes, growth reduction and yield loss particularly after chronic exposure (Wannaz *et al.* 2003, Mazen 2004). One of consequences of metal accumulation is an increase in reactive oxygen species (ROS) contents, which are destructive if protective antioxidant mechanisms do not operate efficiently (Halliwell and Gutteridge 1989, Bray *et al.* 2000). Many antioxidant substances such as ascorbate and the enzymes ascorbate peroxidase (APX) and superoxide dismutase (SOD) are increased under heavy metal stress (Zhang and Kirkham 1996). Also the enhancements of contents of carotenoides and poly-amines have also been correlated with plant tolerance (Alcázar *et al.* 2006).

Some species of the *Bromeliaceae* family have developed strategies to obtain efficiently water and nutrients from the atmosphere through specialized structures on the leaf surface. As a consequence, *Tillandsia* species are considered to be excellent

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Abbreviations: AA - ascorbic acid; APX - ascorbate peroxidase; CAT - catalase; DAB - 3,3'-diaminobenzidine; MS - Murashige and Skoog medium; Put - putrescine; ROS - reactive oxygen species; SOD - superoxide dismutase; Spd - spermidine; Spm - spermine.
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biomonitors of atmospheric pollution (Elias *et al.* 2008, Wannaz and Pignata 2006, Bermudez *et al.* 2009). Elias *et al.* (2008) concluded that some other Brazilian native bromeliads, such as *Aechmea coelestis*, *Nidularium innocentii*, *Nidularium krisgreeniae*, *Vriesea carinata*, *Billbergia amoena* and *Canistropsis billbergioides*, are able to accumulate high concentrations of different metals. These bromeliads possibly have some, still unknown, efficient mechanisms for increasing their tolerance to the harmful effects of heavy metals on their metabolism. It is still beneficial to continue the search for new bromeliad species suitable for environmental monitoring purposes and *Aechmea blanchetiana* is supposedly one good alternative. This bromeliad is widely used in landscaping and has adapted to all regions of Brazil (Lorenzi and Souza 1998). Its *in vitro* propagation is well known (Galvanese *et al.* 2007). It is responsive to different concentrations of salts under *in vitro* cultivation (Kanashiro 2009) and tolerant to acclimatization (Tavares *et al.* 2008).

The technique of *in vitro* culture is advantageous because it enables to isolate the effects of heavy metals on plant metabolism from the effects of other stresses. The aim of this work was to evaluate the tolerance of *Aechmea blanchetiana* cultivated *in vitro* to increasing concentrations of copper and zinc.

Materials and methods

Seeds of *Aechmea blanchetiana* Baker L.B. Sm. were washed thoroughly and sowed in growth medium consisting of half strength Murashige and Skoog (1962; MS) medium supplemented with 30.0 g dm^{-3} of sucrose, 6.0 g dm^{-3} agar (*Sigma*, St. Louis, USA), pH 5.8 ± 0.2. After 210 d, ten shoots (6.0 cm length, 7 leaves, 0.12 g of leaves and 0.023 g of roots) were transferred to half strength, MS liquid medium containing 30.0 g dm^{-3} of sucrose (pH 5.2) and supplemented with 0.0, 0.145*, 1.45 and 14.5 µM of copper ($CuSO_4 . 5 H_2O$) and 0.0, 2.75*, 27.5 and 275 µM of zinc ($ZnSO_4 . 7 H_2O$). The values followed by asterisks were the maximum limits of Cu and Zn accepted in water for human consumption in Brazil. The cultures were incubated for 130 d in a growth room at temperature of 25 ± 2 °C and 16-h photoperiod with irradiance of 32 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

The analysis of mineral elements was performed on whole dried plants (forced air oven at 70 °C) that were macerated with a pestle and pulverized in an agate ball mill according to Malavolta *et al.* (1997). Samples were subjected to nitric-perchloric acid digestion and phosphorus contents were determined by the metavanadate colorimetric method, potassium by flame spectrophotometry (*600plus*, *Femto*, São Paulo, Brazil), calcium, magnesium, zinc, iron, copper and manganese by atomic absorption spectrometer (*2380*, *Perkin-Elmer*, Norwalk, CT, USA) and sulfur by turbidimetry of barium sulphate. Nitrogen contents were evaluated by Kjeldahl method and boron was assessed by the azomethine-H method. Assays were conducted for each of the three replicated treatments.

The activities of SOD and APX and the AA content were determined using a UV spectrophotometer (*UV 160A*, *Shimadzu*, Tokyo, Japan). SOD activity was assayed as described by Osswald *et al.* (1992) by determining its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). APX activity was analyzed according to Ramachandra-Reddy *et al.* (2004) by monitoring the decrease of ascorbate at 290 nm for 1 min. Assays were conducted for each of the three replicated treatments.

Ascorbic acid (AA) contents were determined for five samples of 0.7 g of fresh leaf according to the method described by Keller and Schwager (1977). Samples were mixed in extraction solution and centrifuged at 31 700 *g* for 30 min. The supernatant was spectophotometrically analyzed at 520 nm. After the first reading, 0.05 cm^3 of 1 % ascorbic acid was added and AA content was estimated based on the differences in the absorbances.

Putrescine (Put), spermidine (Spd) and spermine (Spm) contents were determined by thin layer chromatography (TLC) according to Flores and Galston (1982) and optimized according to Lima *et al.* (2006). Polyamines were quantified by fluorescence emission spectroscopy (*Cliniscan2*, *Helena Laboratorie*, Beaumont, TX, USA; excitation at 350 nm and emission at 495 nm) with the video documentation system, using the program *Image Master Software 2.0*.

The histochemical analysis of H_2O_2 was performed in 4 samples of fragments (1.0 cm^2) from the third expanded leaf. They were detached with a razor blade, immediately placed into a solution of 1.0 mg cm^{-3} 3.3'-diaminobenzidine (DAB), adjusted to pH 5.6 with NaOH or HCl, and incubated in the dark for 24 h. Fragments were cleared in 96 % boiling ethanol and a DAB solution supplemented with 10 mM of ascorbic acid was used as a negative control. H_2O_2 accumulation was visualized as a reddish-brown coloration observed by light microscopy according to Iriti *et al.* (2003) with some modifications.

After 130 d of *in vitro* culturing, growth parameters were evaluated, including the numbers and the lengths of leaves and roots, the stem diameters and the fresh and dry masses of leaves and roots.

The results were subjected to *ANOVA* (*F*-test) followed by the Tukey test to check for significant differences between means ($P < 0.05$) using the software *Sisvar* (Ferreira 2000).

Results

A significantly higher content of Cu was observed in plant tissues of *A. blanchetiana* treated with 14.5 µM Cu than in the others treatments, whereas zinc accumulation increased with increasing Zn concentrations (Table 1). Contents of macro-nutrients (N, P, K, Ca, Mg and S) and micronutrients (B, Fe and Mn) were similar in all treatments of both elements (data not shown).

The highest activities of SOD were measured in plants of *A. blanchetiana* treated with 0.145 µM Cu and 275 µM Zn (Table 1). The content of AA was significantly increased in the plants treated with 14.5 µM Cu (Table 1). The activity of APX remained unchanged in all Cu and Zn treatments (Table 1).

Table 1. Effect of Cu and Zn at different concentrations on endogenous contents of metals (n = 3), SOD and APX activities (n = 5), ascorbic acid and polyamine contents (n = 3) and shoots and roots growth parameters (n = 10) of *Aechmea blanchetiana*. Significant differences in relation to the control (0.145 and 2.75 µM) (P < 0.05, Tukey test) are indicated by different letters.

Parameters	Cu [µM]				Zn [µM]			
	0.0	0.145	1.45	14.5	0.0	2.75	27.5	275
Cu [mg kg^{-1}(dm)]	23.0b	11.0b	24.0b	72.0a	20.0a	16.0a	20.0a	18.3a
Zn [mg kg^{-1}(dm)]	20.0a	16.0a	20.0a	18.33a	26.33c	41.0c	159.33b	233.33a
SOD [U mg^{-1}(dm)]	4.36ab	5.49a	3.19b	3.62ab	3.90b	2.80bc	1.44c	4.58a
APX [ΔA g^{-1}(dm) min^{-1}]	12.38a	10.57a	11.07a	9.60a	8.16a	6.96a	9.16a	9.48a
AA [mg g^{-1}(dm)]	3.38ab	4.23ab	2.86b	4.51a	4.72a	4.95a	6.83a	6.64a
Spm [mg g^{-1}(dm)]	1.23a	1.08a	0.78a	0.65a	0.62a	0.51a	0.81a	0.86a
Spd [mg g^{-1}(dm)]	0.26a	0.30a	0.30a	0.17a	0.14a	0.12a	0.22a	0.17a
Put [mg g^{-1}(dm)]	0.61a	0.47a	0.40a	0.42a	0.38a	0.49a	0.55a	0.47a
Number of leaves [plant^{-1}]	17.0a	19.0a	19.0a	19.0a	18.0b	22.0a	19.0b	18.0b
Shoot dry mass [mg plant^{-1}]	86.0a	94.0a	76.0a	59.0a	72.0b	102.0a	87.0ab	80.0ab
Root dry mass [mg plant^{-1}]	13.7a	15.1a	10.9a	14.9a	10.0b	17.0a	13.0ab	13.0ab
Number of roots [plant^{-1}]	8.0a	8.0a	10.0a	10.0a	8.0b	11.0ab	12.0a	10.0ab
Root fresh mass [g plant^{-1}]	0.09a	0.07ab	0.05b	0.05b	0.05a	0.06a	0.06a	0.05a
Root length [cm]	6.19ab	7.27a	4.09b	5.18ab	5.95a	7.23a	5.18a	6.29a

There were no statistically significant changes in the average values of polyamines between treatments, although the content of Spm tended to decrease (Table 1) with the accumulation of Cu in the leaf tissues, following a linear model (Spm = -205.2 × [Cu] + 1449.4, r^2 = 0.98) and increased when the content of Zn in the leaf tissue increased (Spm = 102.8 × [Zn] + 445.4, r^2 = 0.66) (Table 1). The highest values of Spm were observed in tissues treated with 27.5 and 275 µM Zn. The levels of Spd remained constant for all treatments and showed values lower than those of Put and Spm.

Histochemical analyses showed that 1.45 and 14.5 µM Cu and 27.5 and 275 µM Zn induced the accumulation of H_2O_2 in leaf tissues (Fig. 1). There was no DAB staining of H_2O_2 deposits on the adaxial leaf surface following treatments with 0.145 µM Cu (Fig. 1*B*) or 2.75 µM Zn (Fig. 1*C*) and in the negative control (Fig. 1*A*). After exposure to 14.5 µM Cu, H_2O_2 was observed in the apoplast of many epidermal cells and around the stomata (Fig. 1*D*) and in the wall and cytoplasm of mesophyll cells (Fig. 1*E*). Plants treated with 27.5 µM Zn showed H_2O_2 deposits in the guard cells and in nuclei of mesophyll cells (Fig. 1*F*), whereas plants treated with 275 µM Zn had deposits of H_2O_2 in the stomata and inside cell walls on the of epidermal cells (Fig. 1*G*).

Cu did not significantly affect shoot lengths, number of leaves or shoot fresh mass (Table 1). In contrast, the root fresh root mass of plants treated with higher concentrations of Cu was significantly lower in comparison to that of plant roots exposed to lower concentrations. The root length was highest in plants exposed to 0.145 µM Cu. Plants growing at 2.75 µM Zn showed significantly higher dry masses of shoots and roots than plants under other treatments (Table 1).

Discussion

A. blanchetiana effectively absorbed Cu and Zn in this study as indicated by their increased concentrations in plant tissues. However, the occurrence of increased contents of H_2O_2 in leaf tissues indicated that those higher contents were potentially toxic to the plants. In contrast, the plants grew in all treatments for 130 d without any visible symptoms, such as chlorosis or necrosis. They showed only few disturbances in the

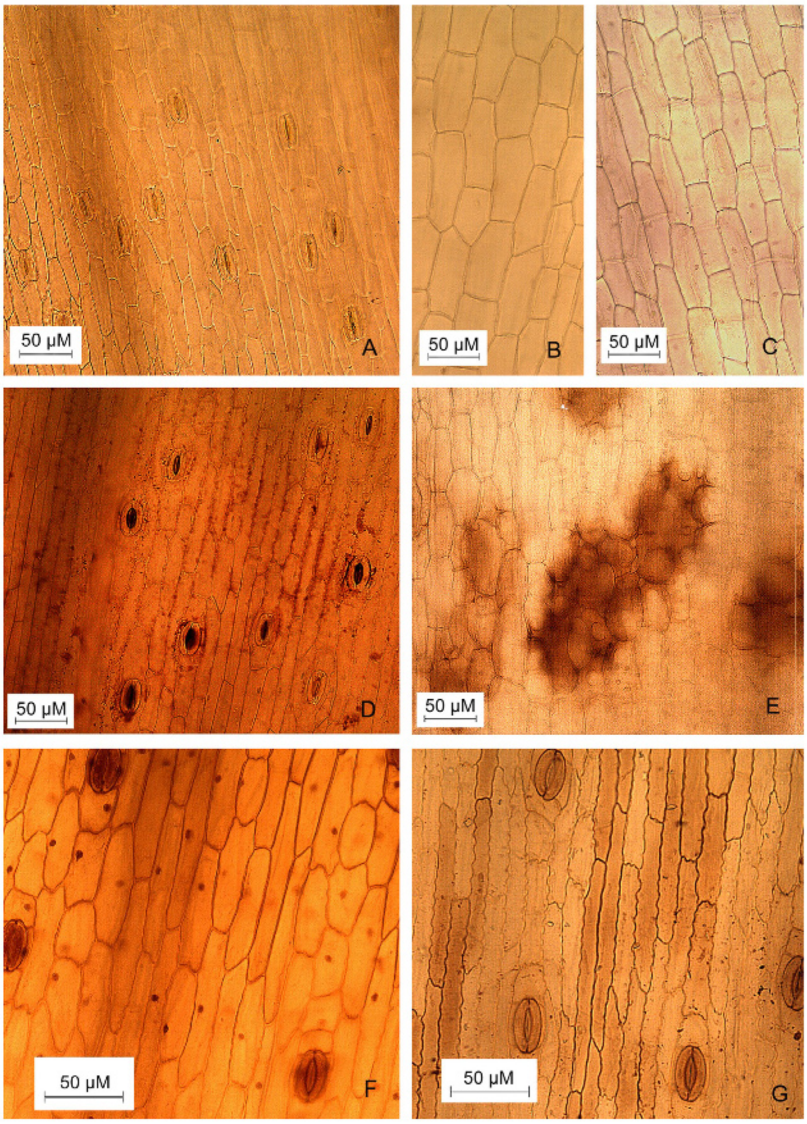

Fig. 1. Localization of H_2O_2 (dark brown precipitates of polymerized DAB) in leaves of *A. blanchetiana* treated with Cu with the DAB uptake method: *A* - negative control with no DAB precipitates; *B* - adaxial epidermal cells of Cu treated plants; *C* - adaxial epidermal cells of Zn treated plants; *D* - abaxial epidermal cells of plants treated with 1.45 µM Cu; *E* - mesophyll cells of plants treated with 14.5 µM Cu (H_2O_2 accumulation is intense and diffused in adjacent cells); *F* - abaxial epidermal cell of plants treated with 27.5 µM Zn; *G* - abaxial epidermal cell of plants treated with 275 µM Zn (accumulation in the walls and cytoplasm of cells).

metabolites and compounds analyzed and in growth and biomass production.

Populus tremula and *Populus alba* are able to uptake Zn from contaminated soil and retain levels that are four times higher than their normal Zn content in roots and 10 times higher in leaves (Durand *et al.* 2010). The bromeliad *Tillandsia tricholepis* can accumulate more than 127 mg kg^{-1} of Zn (Bermudez *et al*. 2009). Brazilian bromeliads *Canistropsis bilbergioides* and *Vriesea carinata* can accumulate 40 and 58 mg(Zn) kg^{-1}(f.m.), respectively (Elias *et al.* 2006, 2008). *A. blanchetiana* was able to accumulate more Zn than the bromeliads *T. tricholepis*, *Canistropis billbergioides* and *Vriesea carinata* but less than both species of *Populus*.

H_2O_2 accumulation in the leaves of *A. blanchetiana* in response to exposure to the highest concentrations of Cu and Zn may indicate that the antioxidant pool is operating inefficiently and further physiological disturbances may occur (Pellinen *et al.* 1999, Langebartels *et al.* 2002). H_2O_2 may also function as a signaling molecule for further antioxidant responses, as recognized by many authors (Bors *et al.* 1989, Iriti and Faoro 2003, Iriti *et al.* 2006, Groppa and Benavides 2008).

The higher contents of AA in the Cu and Zn treated plants are probably related to the increasing tolerance of *A. blanchetiana* to these elements. The effects of Cu and

Zn on total SOD activity appears to be related to the presence of Cu/Zn-SOD isoform in the cytosol and chloroplasts (Alscher *et al.* 2002, Resende *et al.* 2003). A decrease in SOD activity in plants of *Lupinus luteus* grown in hydroponic solution contaminated with different concentrations of Cu was observed after 7 d, suggesting that SOD was directly affected by the Cu toxicity (Mourato *et al.* 2009). In contrast, Panda and Khan (2004) observed a constant elevation of SOD activity in plants of *Hydrilla verticillata* grown in increasing concentrations of Cu and Cd and a reduction in SOD activity in plants exposed to higher concentrations of Zn and Cr. In fact, other factors, such as subcellular distribution, could affect the enzymatic performances of the plants exposed to heavy metals (Cuypers *et al.* 2002).

High contents of polyamines in cell, especially of Spm, are correlated with plant tolerance to environmental stresses (Alcázar *et al.* 2006). The comparatively high content of Spm with respect to Put and Spd in the *A. blanchetiana* plants may contribute to the characterization of the species as tolerant to Cu and Zn. However, the trend of decreasing content of Spm in *A. blanchetiana* plants grown in higher concentrations of Cu seems to be associated with its degradation (Alcázar *et al.* 2006) and a possible delay of polyamines biosynthesis.

The reduction in growth and fresh masses of roots was the only macroscopic symptom of Cu toxicity observed in the *A. blanchetiana* plants. Previous studies have shown that excess Cu can inhibit plant growth, increasing the leakage of root cell membranes, leading to loss of vigor (Shen *et al.* 1998, Murphy *et al.* 1999, Fernandes 2006). On the other hand, the treatment with Zn did not cause typical symptoms of toxicity in the plants, and Zn free medium was more deleterious to the plants than the presence of the metal. Similarly, plants of *Populus alba* cultivated *in vitro* started to show toxicity only when exposed to 500 μM Zn (Castiglione *et al.* 2007).

In conclusion, *A. blanchetiana* tolerated Cu and Zn showing few indications of phytotoxicity. The H_2O_2 accumulation observed in the leaf tissues of plants submitted to the highest concentrations of Zn and Cu may either represent a loss of the antioxidative capacity or a signal for further stimulation of the antioxidative system. Zn and Cu are essential to the development and growth of plants and are probably better tolerated than other non-essential elements, requiring prolonged exposure to these metals to produce symptoms of toxicity. Longer exposure of the plants to high contents of both elements will elucidate all of these aspects. Considering these results and the young age of the experimental plants, we conclude that *A. blanchetiana* possesses bioaccumulator characteristics and is indicated for active biomonitoring use, specially when compared with others bromeliad species as *Tillandsia*.

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