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Zinc and cadmium as modulating factors of the morphophysiological responses of *Alternanthera tenella* Colla (Amaranthaceae) under *in vitro* conditions

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Abstract

Zinc (Zn) plays an important role in the physiological processes in plants and may mitigate trace element stress. The aim was to evaluate the morphophysiological responses of *Alternanthera tenella* plants exposed to cadmium (Cd) and Zn under *in vitro* conditions. Segments of *A. tenella* were transferred to flasks containing medium supplemented with different combinations of Cd (0, 75, or 150 µM) and Zn (0, 750, or 1,500 µM) concentrations, totalizing nine treatments. We assessed the growth traits, anatomy, chlorophyll *a* fluorescence by OJIPs, and tolerance index (TI). With exposure only to Cd, the plants showed physiological disorders. Zn supplementation in the medium had a positive effect on the physiological performance of plants. At concentrations ≤ 750 µM, it can partially mitigate the deleterious effects of Cd. Plants grown with Cd and Zn showed intermediate TI. The results proved the potential of Zn as a mitigator of Cd-induced stress in plants.

Keywords: chlorophyll *a* fluorescence; electron transport flux; phytoremediation; plant tissue culture; trace element.

Introduction

Contamination and accumulation of industrial pollutants in the environment due to human activities can lead to potential damage to human health resulting from continuous exposure to their components (Jeong *et al.* 2020). The trace elements are among the elements that are pollutant and harmful to the ecosystem; they can

Highlights

- Both Cd and Zn alone and co-exposure can affect the morphophysiological traits of *Alternanthera tenella*
- Cd alone can induce physiological disorders in *A. tenella*
- Zn is a mitigator of Cd-induced stress in *A. tenella* plants

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Abbreviations: DI_0/CSm – dissipated energy flux per cross-section; F_0 – initial fluorescence; F_I – fluorescence intensity at 30 ms; F_J – fluorescence intensity at 2 ms; F_K – fluorescence intensity at 0.3 ms; F_m – maximal fluorescence intensity; F_P – fluorescence peak (= F_{300ms}); F_t – fluorescence at time t after start of actinic illumination; F_V/F_0 – ratio of the de-excitation rate constants for photochemical and nonphotochemical events; K_P – photochemical de-excitation rate constant; PI_{total} – total performance index, which measures the performance up until the final electron acceptors of PSI; RC/CSm – total number of active reaction centers; $SFI_{(ABS)}$ – PSII structure and functioning index; V_I – relative variable fluorescence at 30 ms (step I); V_J – relative variable fluorescence at 2 ms (step J); V_K – relative variable fluorescence at 0.3 ms (step K); W_K – represents the damage to oxygen-evolving complex; W_L – indicates disturbance in the thylakoid membranes, reducing the energetic connectivity between the PSII units; ΔV_{IP} – relative variable fluorescence amplitude of the increase from I to P = relative contribution of the increase from I to P to the increase in OJIP; φD_0 – quantum yield of energy dissipation (at t = 0); φE_0 – quantum yield of electron transport (at t = 0); φP_0 – maximum quantum yield of primary photochemistry (at t = 0); φR_0 – quantum yield of reduction of end electron acceptors at the PSI acceptor side; ψR_0 – efficiency/probability by which electrons move from PSII to PSI acceptor side.

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accumulate in the air, water, and soil, especially in mining areas, where their bioavailability can be high (Alloway 2013, Wang *et al.* 2017a, Logiewa *et al.* 2020).

Trace elements can have distinct effects on plants (Shahid *et al.* 2016, Xu *et al.* 2020). Some metals such as copper (Cu), zinc (Zn), iron (Fe), cobalt (Co), and manganese (Mn) are considered essential and have a known function in the cellular metabolism of plants (Kirkby 2012, Lange *et al.* 2017). In contrast, others such as cadmium (Cd), lead (Pb), silver (Ag), and mercury (Hg) have no metabolic role in plants and can induce toxicity symptoms (Amari *et al.* 2017, Karri *et al.* 2018).

The presence of trace elements such as Cd at high concentrations in plants can limit growth, decrease pigment content, induce photosynthetic performance and anatomy disorders, cell damage is caused by increased reactive oxygen species (ROS). In addition, the plants may present visual symptoms of stress such as chlorosis or necrosis and even plant death (Ahmad *et al.* 2015, Amari *et al.* 2017). For some plant species, Cd can be toxic even in minimal amounts. It is often considered a contaminant affecting numerous physiological and biochemical processes (Amari *et al.* 2017, Samiei *et al.* 2020).

Zn is an important element for plants, as it regulates physiological and metabolic processes (cofactor of peroxidases, regulator of cell multiplication) (Kirkby 2012, Verma *et al.* 2016, Sturikova *et al.* 2018). Moreover, the Zn concentration influences the growth, development, and performance of the PSI and/or PSII in plants (Mazaheri-Tirani and Dayani 2020). Zn can compete with other metals at absorption sites at high concentrations, reducing leaf area, inducing oxidative stress, chlorosis, or necrosis in plants, among other deleterious effects (Adhikari *et al.* 2016). Thus, the toxicity of metals in plants depends on their concentration.

Co-exposure to Cd and Zn in plants may enable Cd-induced detoxification and proper functioning of cellular functions. Previous studies on co-exposure of these two metals (Cd and Zn) in *Alternanthera tenella* and *Cosmos bipinnatus* plants have shown that Zn can suppress the Cd uptake, protecting plants against phytotoxic effects or contributing to detoxification of this metal by increasing the antioxidant system activity (Rodrigues *et al.* 2017, Du *et al.* 2020). From a more advanced perspective, this present study examined the level of Cd-induced disorders in the photosynthetic apparatus (by chlorophyll *a* fluorescence) in *A. tenella* plants and the role of Zn in mitigating the stress induced by this nonessential trace element.

Plants can develop morphophysiological mechanisms to survive and reproduce even with high concentrations of trace elements in their environment. The capacity of plants to uptake or accumulate pollutants in their biomass may allow their use for phytoremediation proposals (Carolin *et al.* 2017, Rodrigues *et al.* 2017). Some species of the Amaranthaceae family, such as *A. tenella*, have been identified for their ability to accumulate and stabilize contaminants present in soils degraded by mining or human activities (Pereira *et al.* 2016, Ayangbenro and Babalola 2017, Men *et al.* 2018).

The assessment of morphophysiological responses of plants under the effect of trace elements during their development and growth can be carried out under *in vitro* conditions. *In vitro* culture enables a careful study of physiological and anatomical processes of plants since it can isolate the effects of a trace element from the effects of other stresses (Martins *et al.* 2016, 2020, 2021; Rodrigues *et al.* 2017). Chlorophyll (Chl) *a* fluorescence analysis in plants grown under stress may also be advantageous to evaluate photochemical changes in PSII and or PSI caused by trace elements in plants (Martins *et al.* 2020).

Considering the potential for bioaccumulation of the species *A. tenella* pointed out by Rodrigues *et al.* (2017), further analysis of its physiological mechanisms can help understand Cd-tolerance strategies, including co-exposure to Zn. Thus, the objective of this work was to evaluate the morphophysiological responses of *A. tenella* plants exposed to different concentrations of Cd and Zn under *in vitro* conditions. In addition, we assessed the potential of Zn for the mitigation of Cd-induced stress.

Materials and methods

Plant material and exposure to Cd and Zn: Nodal segments (2.5 ± 0.5 cm) obtained from *A. tenella* plants previously cultured in the MS medium (Murashige and Skoog 1962) without any plant growth regulator were used as explants (Rodrigues *et al.* 2017). This step was carried out with MS medium at full-strength (440.0 mg L^{-1} $CaCl_2.H_2O$; 1,900.0 mg L^{-1} KNO_3, 370.0 mg L^{-1} $MgSO_4.7H_2O$, 170.0 mg L^{-1} $KH_2.PO_4$, 1,650.0 mg L^{-1} NH_4NO_3, 22.3 mg L^{-1} $MnSO_4.4H_2O$, 6.2 mg L^{-1} H_3BO_3, 0.83 mg L^{-1} KI, 0.25 mg L^{-1} $Na_2MoO_4.2H_2O$, 8.6 mg L^{-1} $ZnSO_4.7H_2O$, 0.025 mg L^{-1} $CoCl_2.6H_2O$, 0.025 mg L^{-1} $CuSO_4.5H_2O$, 27.85 mg L^{-1} $FeSO_4.7H_2O$, 37.25 mg L^{-1} Na_2EDTA, 0.5 mg L^{-1} pyridoxine.HCl, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} thiamin.HCl, 2.0 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol). The plant material (nodal segments) was subcultured for 45 d in 500-mL glass flasks with 30 mL of modified (absence of Zn = 0 µM or Zn + 0 µM Cd) MS medium. After 45 d, new nodal segments were collected from these plants and transferred to 500-mL glass flasks containing 30 mL of modified MS medium, supplemented with Zn (0, 750, or 1,500 µM). Zn treatments were combined with three concentrations of Cd (0, 75, or 150 µM), nine treatments in total. Cadmium nitrate [$Cd(NO_3)_2.4H_2O$] and zinc sulfate [$ZnSO_4.7H_2O$] were used as sources of Cd and Zn, respectively. The concentrations of Cd and Zn were chosen according to Rodrigues *et al.* (2017).

At all stages, the media were supplemented with 20 g L^{-1} sucrose and solidified with 6 g L^{-1} agar. The pH of all culture media was adjusted to 5.8 and autoclaved at 120°C for 20 min. The plant material was kept in a growth room for 45 d (modified MS medium – absence of Zn), then for 30 d (co-exposure to Zn-Cd) at 26 ± 2°C, with a 16-h photoperiod (08:00 to 00:00 h) under slim LED lamps (*Blumenau*® 36W/6500K), emitting 70 µmol(photon) m^{-2} s^{-1} of PAR.

Plant growth and tolerance index (TI): After 30 d of culture (co-exposure to Zn-Cd), the *A. tenella* plants were washed in running water. The growth was analyzed in 25 plants per treatment, which were randomly divided into five different repetitions (replications). The quantification of total dry mass (DM) [mg per plant] (aerial part + roots) was performed with the aid of an analytical balance. The dry mass of *A. tenella* plants (aerial part and root) in each treatment was also used to calculate the TI according to the methodology proposed by Wilkins (1957) with modifications. The TI was determined as follows: TI = $[(DM_{treatment})/(DM_{control})] \times 100$, with varying TI values that can range from 0 [maximum sensitivity = 0%] to 100 [maximum tolerance = 100%]. $DM_{treatment}$ = dry mass of plants grown in medium containing trace element(s). $DM_{control}$ = overall mean of dry mass of plants in the control treatment (0 μM Cd + 0 μM Zn).

Anatomical analyses: At the end of the experiment, the anatomy of the *A. tenella* plants was also analyzed. Five plants were randomly collected from each treatment and fixed in a FAA solution (formaldehyde, acetic acid, and 50% ethanol at a ratio of 0.5:0.5:9) (Johansen 1940). Cross- and paradermal sections of *A. tenella* were obtained as described by Martins *et al.* (2020). Anatomical analyses were performed on five different samples (repeats) per treatment. After mounting the slides, photomicrographs of the cross- and paradermal sections were obtained using an optical microscope (*Bioval*, *L-2000A-Flur*) coupled to a *Leica EC3* digital camera (*Wetzlar*, Germany). Measurements of anatomical characteristics were performed using *UTHSCSA-Imagetool*® software calibrated with a microscopic ruler. The photomicrographs of the stem and leaves were obtained from two different cross-sections for each organ and the paradermal ones from four fields per sample (repeat). In stem sections, the number of vascular bundles and cross-area [μm^2] was measured. In leaf sections, the number of vessel elements, stomatal density [mm^{-2}], and stomata size [μm^2] was analyzed.

Assessment of Chl *a* fluorescence transient OJIP and JIP test: The assessments of Chl *a* fluorescence transients of *A. tenella* plants exposed to different concentrations of Cd and Zn were carried out after 30 d of culture in 20 plants per treatment, between 8:00 and 10:00 h with the aid of a *HandyPEA* portable fluorometer (*Hanstech*, King's Lynn, Norfolk, UK). The measurements were performed on the second leaf fully expanded from the apex. The analyzed leaf area was previously dark-adapted using leaf clips (*Hansatech*®) for 30 min. Based on the fluorescence intensities, we obtained the JIP test parameters, as well as the OJIP transients. The treatment without the addition of Cd and Zn (0 μM Cd + 0 μM Zn) was considered the control. The interpretation and normalization of the JIP test parameters were done according to Strasser *et al.* (2004) and Wang *et al.* (2016).

Statistical analysis: The experimental design was completely randomized and in a factorial scheme (3 × 3), with three concentrations of Cd (0, 75, and 150 μM) and three concentrations of Zn (0, 750, and 1,500 μM). The obtained data were subjected to analysis of variance (*ANOVA*), and the means were compared by the *Scott-Knott* cluster test ($p<0.05$). The analyses were performed using the *Sisvar*® program (Ferreira 2011).

Results

***In vitro* plant growth and tolerance index**: After 30 d of exposure of *A. tenella* plants to different concentrations of Cd and/or Zn, we observed differences in morphology, growth, and the tolerance index (TI) (Fig. 1). Plants grown without Zn and exposed to 75 or 150 μM Cd showed necrosis and a mortality rate of approximately 45%. Visual symptoms that indicated toxicity, such as leaf necrosis and chlorosis, were also observed in plants cultured under co-exposure to high concentrations of metals (75 or 150 μM Cd + 1,500 μM Zn). Regarding the growth, the total dry mass of the plants showed a decrease as a function of Cd concentrations when the plants were exposed to 750 and 1,500 μM Zn. Plants grown without Cd had a higher total dry mass (Fig. 1*A*). Cd concentrations also influenced the TI of plants, with a linear reduction as a function of Cd concentrations being observed. The TI was higher in plants grown without Cd and in those grown with Zn addition (Fig. 1*B*).

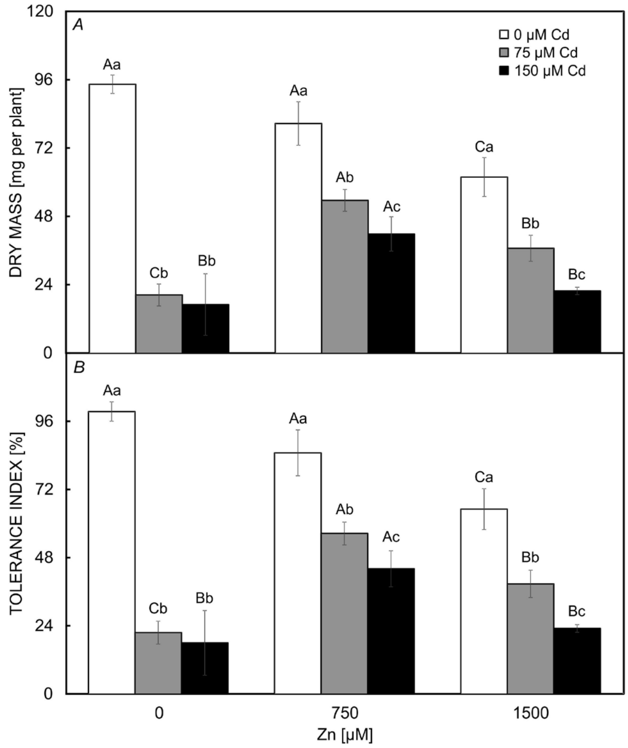

Fig. 1. Dry mass and tolerance index of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of Cd (0, 75, and 150 μM) and Zn (0, 750, and 1,500 μM) concentrations. Means ± SE (n = 5) followed by *the same letter* (*uppercase letters* comparing Zn concentrations at each Cd concentration and *lowercase letters* comparing Cd concentrations at each Zn concentration) do not differ significantly according to *Scott-Knott* test ($p<0.05$).

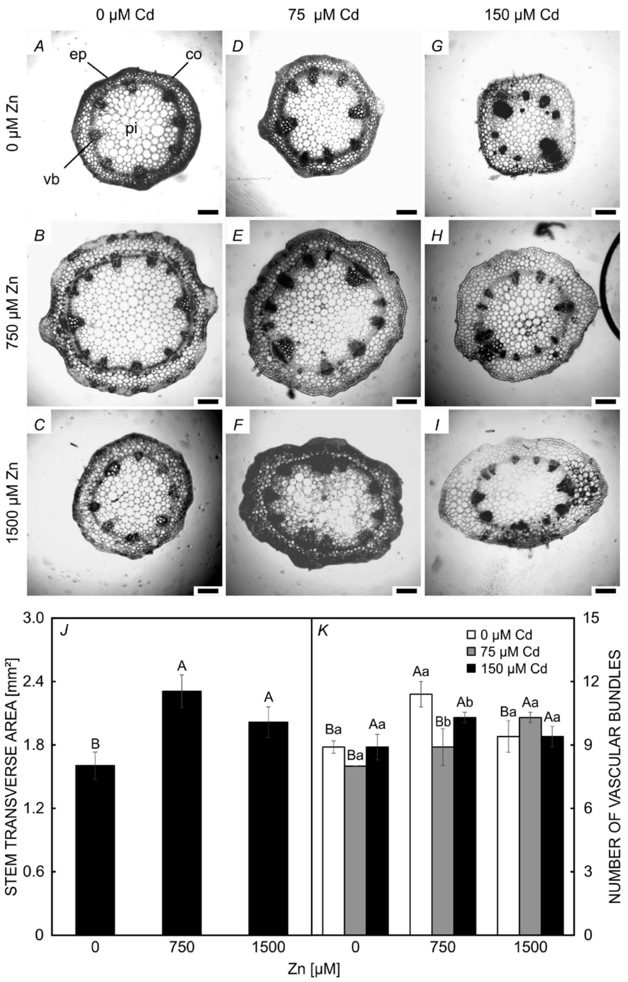

Fig. 2. Cross-sections of the stem of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of the concentrations of Cd (0, 75, and 150 μM) and Zn (0, 750, and 1,500 μM). For each anatomical characteristic, the means ± SE ($n = 5$) followed by *the same letter* (*uppercase letters* comparing Zn concentrations and *lowercase letters* comparing Cd concentrations at each Zn concentration), do not differ significantly according to *Scott-Knott* test ($p<0.05$). co – collenchyma; ep – epidermis; vb – vascular bundle; pi – pith. Bar = 200 μm.

Anatomical analyses (stem/leaf): Among the stem anatomy characteristics, the cross-section of the stem area was affected only by increasing Zn concentrations, while the number of vascular bundles was influenced by both Cd and Zn (interaction between factors). Plants grown without Zn (0 μM) had the lowest values for the cross-sectional area compared to plants grown with Zn (Fig. 2*A–J*). Plants grown with 0 and 1,500 μM Zn showed similar values for the number of vascular bundles. Among plants grown with 75 μM Cd, the highest values were observed in those exposed to 1,500 μM Zn (Fig. 2*A–I*,*K*).

In leaves, stomata size was influenced only by Cd concentrations, and stomatal density was affected by both metals but independently (without interaction). Plants grown with 75 μM Cd had the largest stomata (Figs. 3, 4*A*). Stomatal density increased as a function of Zn concentrations. When comparing stomatal density between treatments with Cd, a marked decrease was observed in plants exposed to this metal (Figs. 3, 4*B*).

The number of vessel elements, in turn, was influenced in conjunction by Cd and Zn. In leaves grown in a medium without Zn, the number of vessel elements decreased as a function of Cd concentrations. Furthermore, the number of vessel elements was higher in leaves grown in medium with 750 μM Zn and 75 μM Cd. At the highest concentration of Cd (150 μM), an enhanced Zn concentration induced a greater number of vessel elements (Fig. 3, 4*C*).

Chl *a* fluorescence transients of plants *in vitro*: Cd and Zn impacted the photosynthetic apparatus of *A. tenella* plants cultured *in vitro*. Changes were found in all JIP test parameters and there was an interaction between Cd and Zn concentrations on all parameters related to fluorescence. Plants not exposed to Cd showed a reduction in V_K and V_J values when cultured with Zn; however, V_I values were similar regardless of Zn concentrations (Fig. 5). In contrast, plants exposed to Cd exhibited an increase in V_K and V_J with an increase in Zn concentration (Fig. 5*D*,*E*). V_I values were higher in plants grown with 150 μM Cd and/or 1,500 μM Zn (Fig. 5*F*).

Changes in the functionality and integrity of the thylakoid membrane [$W_L = (F_L - F_0)/(F_K - F_0)$] and the oxygen-evolving complex (OEC) [$W_K = (F_K - F_0)/$

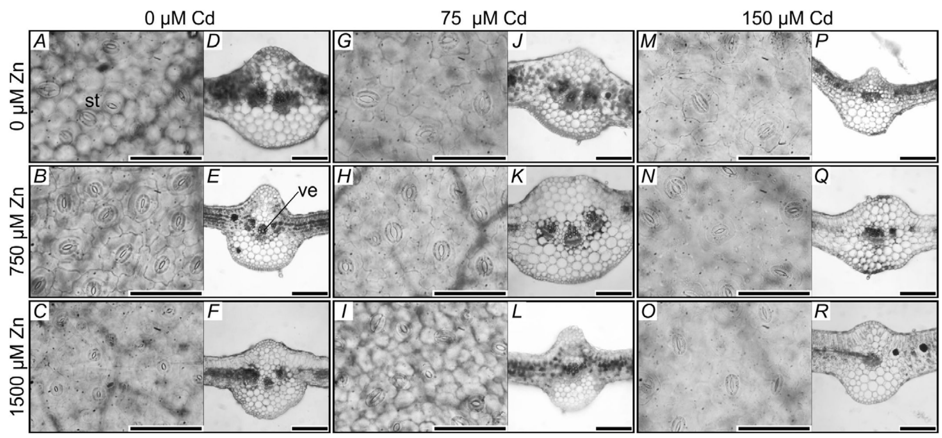

Fig. 3. Paradermal and cross-sections of leaves of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of concentrations of Cd (0, 75, and 150 μM) and Zn (0, 750, and 1,500 μM). st – stomata; ve – vessel element. Bar = 100 μm.

$(F_J - F_0)$] were influenced by both factors. Pronounced increases in W_L and W_K were mainly observed in plants co-exposed to 150 µM Cd and 750 or 1,500 µM Zn. Similarly, increases in W_L and W_K values were also observed in plants grown with 75 µM Cd + 1,500 µM Zn. These responses were confirmed by forming positive amplitudes of L-bands and K-bands in these treatments (Fig. 6).

Changes in the O–I phase ($V_{OI} \geq 1.0$) were also verified as a function of treatments. Plants exposed to 150 µM Cd had a smaller amplitude compared to those grown without Cd. Plants co-exposed to Cd and 1,500 µM Zn also showed a reduction in the amplitude of the $V_{OI} \geq 1.0$ curve (Fig. 7).

Reductions in ΔV_{IP} [= $(F_P - F_I)/(F_P - F_0)$] values were observed in 150 µM Cd treatments, as well as in the treatment with 75 µM Cd + 1,500 µM Zn (Fig. 8*A*). *A. tenella* plants grown in medium supplemented with 75 and 150 µM Cd showed increased initial fluorescence (F_0) and DI_0/CSm. In addition, under 150 µM Cd, there was a linear increase in F_0 and DI_0/CSm as a function of increasing Zn concentrations (Fig. 8*B*,*H*). The values of F_P were the highest in plants exposed to 150 µM Cd (Fig. 8*C*).

The F_V/F_0 and K_P values were higher in plants grown with Zn (750 µM and 1,500 µM) when the media did not have Cd supplementation. On the other hand, in the presence of 150 µM Cd, the plants showed a reduction in F_V/F_0 and K_P with an increasing concentration of Zn added to the culture medium (Fig. 8*D*,*E*). Regarding the quantum yield parameters, there was a significant decrease in φP_0 and φE_0 in plants grown in medium containing 75 µM Cd + 750 µM Zn, 75 or 150 µM Cd + 1,500 µM Zn (Fig. 8*F*,*G*). In these treatments, an increase in φD_0 values was also observed (Fig. 8*I*). The values of ψR_0 and φR_0 decreased in plants grown in medium supplemented with 150 µM Cd, regardless of the Zn concentration. Similarly, there was a decrease in ψR_0 and φR_0 in plants grown with 75 µM Cd + 1,500 µM Zn (Fig. 8*J*,*K*). In the Cd-free culture media, Zn supplementation increased the values of RC/CSm, $SFI_{(ABS)}$, and PI_{total} (Fig. 8*L–N*). However, when Cd and Zn were added, there was a reduction in RC/CSm and $SFI_{(ABS)}$. Exposure to 150 µM Cd induced a decrease in PI_{total} values, with a reduction also observed in plants grown with 75 µM Cd + 1,500 µM Zn (Fig. 8*N*).

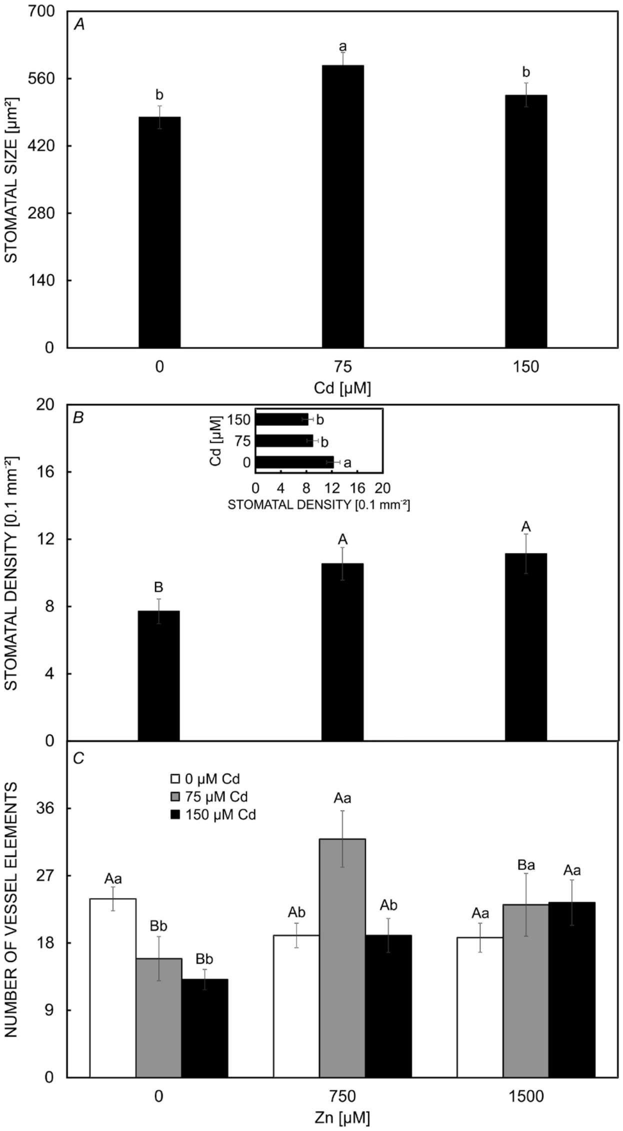

Fig. 4. Anatomical characteristics of the stem of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of the concentrations of Cd (0, 75, and 150 µM) and Zn (0, 750, and 1,500 µM). (*A*,*B*) Size and density of *A. tenella* leaf stomata after 30 d of *in vitro* culture as a function of concentrations of Cd (0, 75, and 150 µM) or Zn (0, 750, and 1,500 µM) (without interaction). (*C*) The number of vessel elements of *A. tenella* leaves after 30 d of *in vitro* culture as a function of concentrations of Cd (0, 75, and 150 µM) and Zn (0, 750, and 1,500 µM) (with interaction). For each anatomical characteristic, the means ± SE ($n = 5$) followed by *the same letter* (*uppercase letters* comparing Zn concentrations and *lowercase letters* comparing Cd concentrations), do not differ significantly according to *Scott-Knott* test ($p<0.05$).

Discussion

The growth, anatomical characteristics (stem/leaves), and Chl *a* fluorescence in *A. tenella* plants cultured *in vitro* treated with different concentrations of Cd and/or Zn were described in this study.

The decrease in total dry mass and the presence of visual symptoms such as chlorosis and necrosis in the leaves of *A. tenella* plants due to the presence of Cd confirmed its toxic effect. Zn did not have a pronounced negative impact on the growth and performance of the photosynthetic apparatus of the plants. The tolerance index (TI) reflected the sensitivity of *A. tenella* plants to trace elements (Cd and Zn). The TI was low (TI < 35) when the plants were grown in medium containing only Cd, intermediate (35 < TI < 60) when they were exposed to Cd and Zn, and high (TI > 60) when they were grown with Zn, according to the TI described by Lux *et al.* (2004). The significant reduction in mass accumulation as a function of Cd concentrations shows the low tolerance of plants of this species to this trace element under conditions of Zn deficiency. In contrast, at the tested Zn concentrations, we

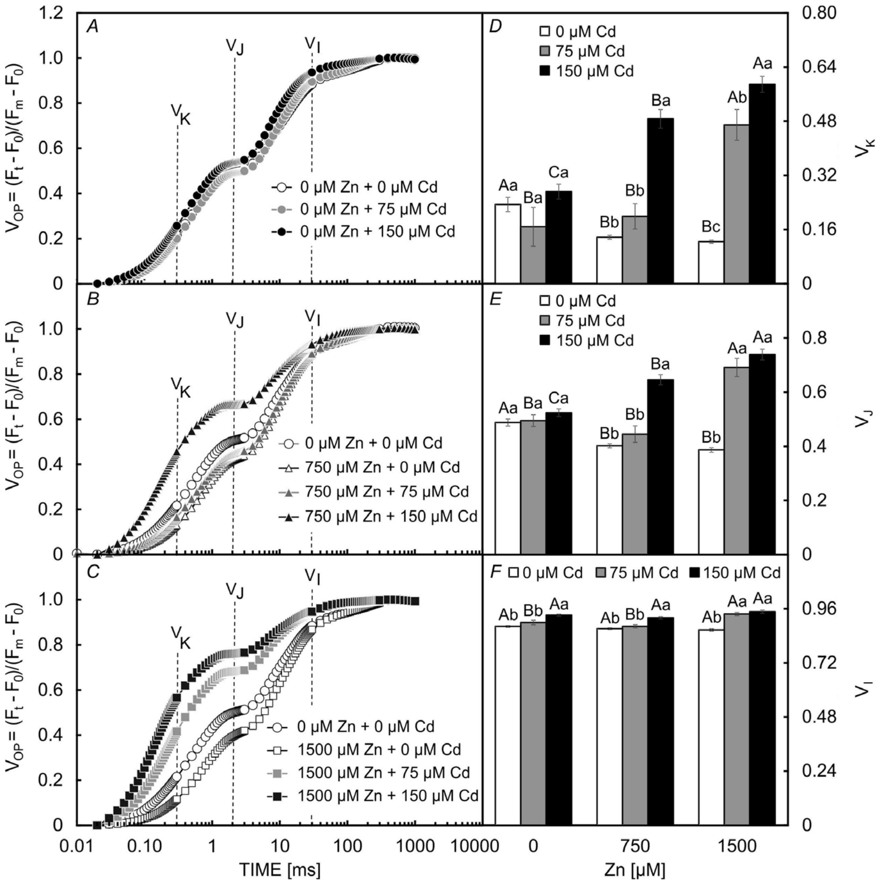

Fig. 5. Relative variable fluorescence of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of concentrations of Cd (0, 75, and 150 µM) and Zn (0, 750, and 1,500 µM). (*A–C*) Relative variable fluorescence between F_0 and F_m (V_{OP}); (*D*) relative variable fluorescence at step K (0.3 ms); (*E*) variable relative fluorescence at step J (2 ms); (*F*) relative variable fluorescence at step I (30 ms). Means ± SE (*n* = 16) followed by *the same letter* (*uppercase letters* comparing Zn concentrations at each Cd concentration and *lowercase letters* comparing Cd concentrations at each Zn concentration) do not differ significantly according to *Scott-Knott* test ($p<0.05$). V_I – relative variable fluorescence at 30 ms (step I); V_J – relative variable fluorescence at 2 ms (step J); V_K – relative variable fluorescence at 0.3 ms (step K).

can suggest that Zn (at concentrations up to 750 µM) was partially effective to mitigate Cd stress. Similar effects on *in vitro* culture growth were reported by Pérez-Romero *et al.* (2016) and Wiszniewska *et al.* (2017), showing that the toxicity of Cd usually results in growth disorders due to its easy accumulation in plant tissues and, therefore, it harms essential physiological processes. On the other hand, Zn is a micronutrient and can play an important role in plants, increasing the biomass of aerial parts and roots and facilitating the photolysis of water during photosynthesis (Abbas *et al.* 2017, Wu *et al.* 2020).

The anatomical characteristics of *A. tenella* plants are consistent with the literature. The morphological responses of *A. tenella* plants were also described under exposure to other trace elements under *in vitro* conditions (Rodrigues *et al.* 2017, Martins *et al.* 2020).

Exposure to Zn stimulated the increase in the cross-sectional area of the stem; however, this element did not have a major impact on the formation of vascular bundles in this organ. The increase in the cross-sectional area appears to be related to the role of Zn in cell division of the cortex and pith. This element is involved in the synthesis of tryptophan (auxin precursor), cell division, and maintenance of the membrane structure (Lacerda *et al.* 2018). However, at high concentrations, Zn can induce changes in the process of cell division and inhibition of cell elongation (Somavilla *et al.* 2018, Alam *et al.* 2020). This statement makes sense since plants grown with 1,500 µM Zn showed a decreasing trend in cross-sectional area. Thus, at higher concentrations than those tested, the plants would possibly show a more significant reduction in the cross-sectional area of the stem.

In leaves, stomatal morphology and density changes are typical responses of plants to stress conditions, such as water deficit or exposure to trace elements (Andrade *et al.* 2019, Caine *et al.* 2019, Pires-Lira *et al.* 2020). Cd induced an antagonistic response to that of Zn. Exposure to 75 µM Cd led to the formation of larger stomata, which can influence CO_2 absorption and transpiration. Smaller stomata tend to be more functional, but this can also interfere with mass flux by modulating transpiration (Pereira *et al.* 2016, Martins *et al.* 2019). The reduction in stomata density and a decrease in the number of vessel elements may indicate a response to Cd that led to a reduction in mass flux. It can influence the uptake and translocation of Cd (among other nutrients) from the culture medium to the aerial part of the plants. This may be related to a strategy of plants to resist or adapt to excess trace elements (Wafee *et al.* 2018).

The number of vessel elements in leaves can control the translocation of elements absorbed by the roots to the aerial part (Martins *et al.* 2016, Rodrigues *et al.* 2017). A reduction in nutrient translocation from the medium may have impaired mass accumulation, the amount of water needed for the photosynthetic reaction, and reduced performance of the photosynthetic apparatus.

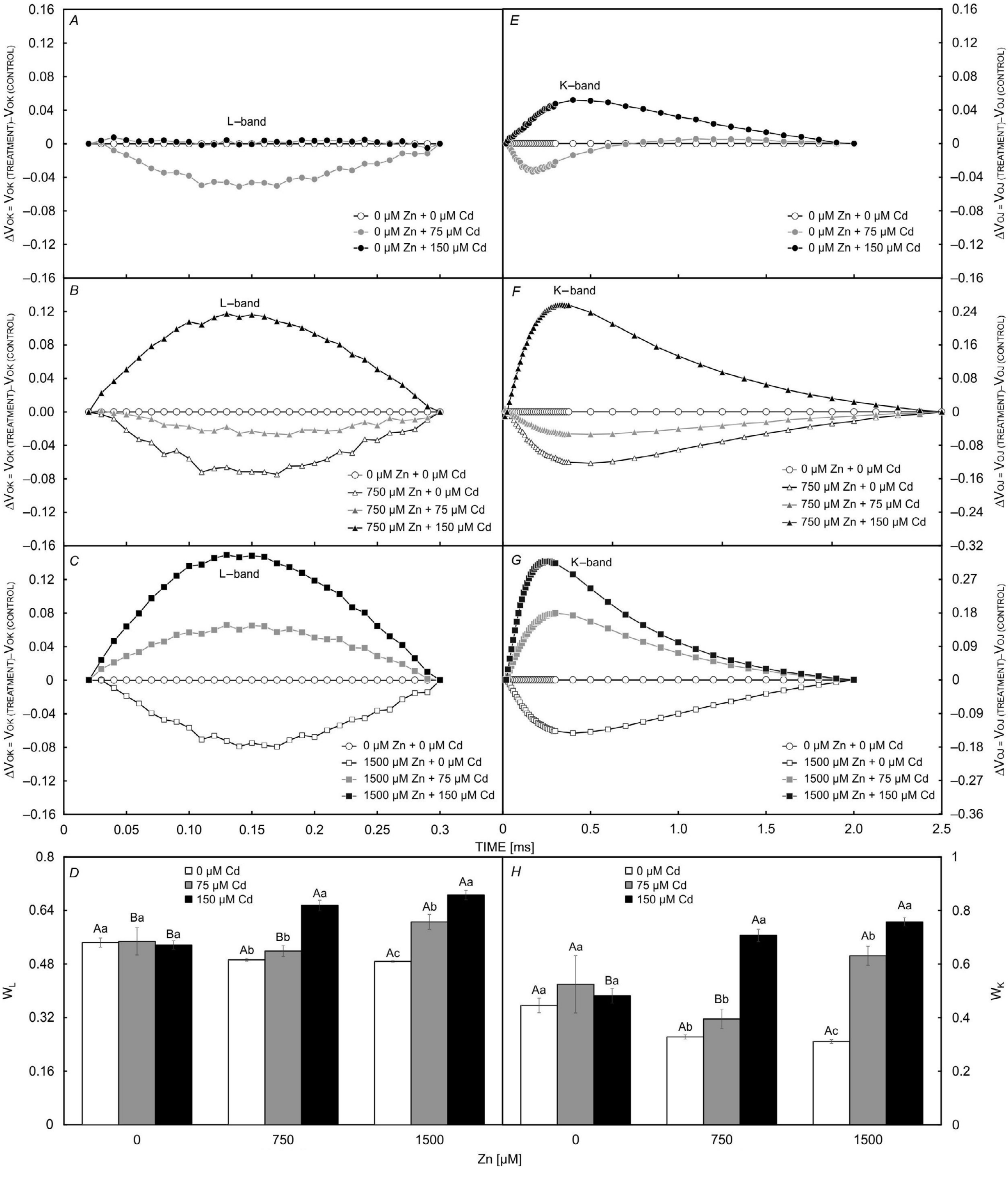

Fig. 6. Transient chlorophyll *a* fluorescence between points O–K and O–J of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of concentrations of Cd (0, 75, and 150 µM) and Zn (0, 750, and 1,500 µM). (*A–C*) Kinetic differences between points O and K, showing the L-band; (*D*) W_L values (indicates a disturbance in the thylakoid membranes, reducing the energetic connectivity between the PSII units). (*E–G*) Kinetic differences between points O and J, showing the K-band; (*H*) W_K values (represents the damage to oxygen-evolving complex). Means ± SE (n = 16) followed by *the same letter* (*uppercase letters* comparing Zn concentrations at each Cd concentration and *lowercase letters* comparing Cd concentrations at each Zn concentration) do not differ significantly according to *Scott-Knott* test (p<0.05).

In plants grown without Zn, we observed a reduction in the number of vessel elements as a function of Cd concentrations. The effects of Cd toxicity on *A. tenella* leaf tissues have also been shown by Rodrigues *et al.* (2017). Cd can inhibit cell division as well as alter the cell cycle in leaf tissues (Hendrix *et al.* 2018).

The damage observed in the anatomy and morphology of the plants was reflected in the plant physiology. An increase in V_K and V_J can indicate damage to the system involved in the oxidation of water molecules of PSII and partial blockage of electron transfer from Q_A to Q_B in the electron transport chain at the acceptor side of PSII, respectively (Kalaji *et al.* 2016, Zhang *et al.* 2016). In this context, the decrease in V_K and V_J values observed in plants grown only with Zn may indicate an improvement in the activities of the oxygen-evolving complex (OEC) on the electron donor side of PSII and the essentiality of this element for good performance of primary photochemical activities. In contrast, the increase in V_K and V_J values observed as a function of Cd concentrations in conjunction with 1,500 µM Zn indicates the occurrence of disorders in OEC activity or a reduction in Q_A^- accompanied by a weak transfer of electrons to Q_B, resulting in higher fluorescence emission (Martins *et al.* 2020). Concerning this fact, the effects of Cd are manifested both on the donor and on the acceptor side of PSII and affect the activities of the photosynthetic apparatus. Thus, although toxic at high concentrations, Zn is presented in this study as an element mitigating Cd-induced stress in *A. tenella* plants and plays a key role in photosynthesis and electron transport.

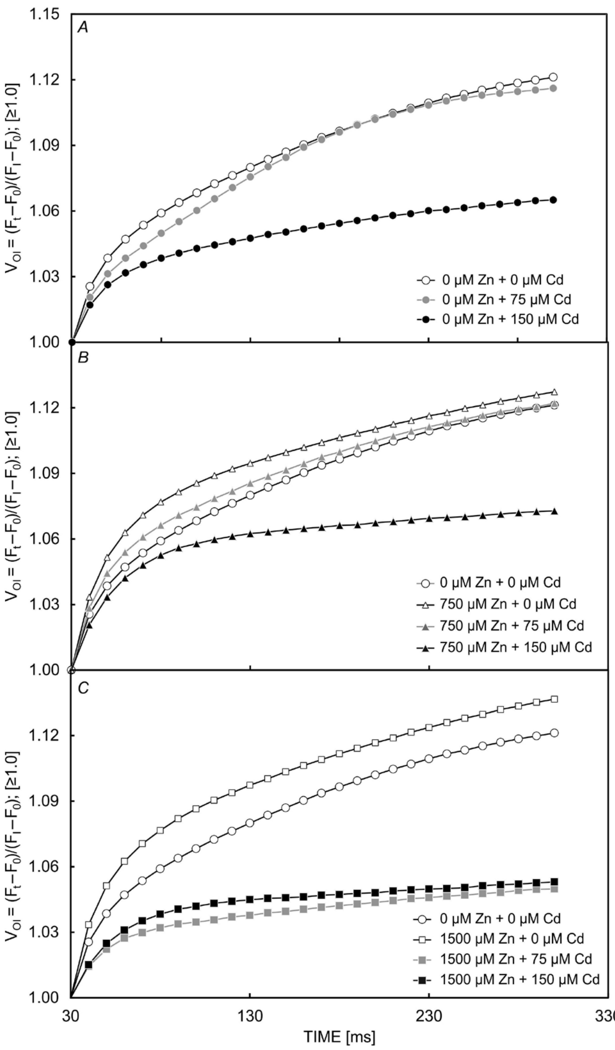

Fig. 7. Normalization of transient chlorophyll *a* fluorescence between F_0 and F_I ($V_{OI} \geq 1.0$) of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of concentrations of Cd (0, 75, and 150 µM) and Zn (0, 750, and 1,500 µM).

The formation of a positive L-band can signal disorders and weak connectivity between PSII subunits or even damage to thylakoid membranes (Paunov *et al.* 2018, Zhang *et al.* 2018). The increase in W_L values of plants grown with high concentrations of Zn (1,500 µM) and Cd confirmed this result. The presence of both trace elements in excess caused harmful effects to electron transport linked to poor performance of the photosynthetic apparatus. Thus, our results suggest that plants co-exposed to high concentrations of both trace elements can show damage to their thylakoid membranes and consequent impairment of the electron transport chain. It could be related to Chl content, as reported for *A. tenella* plants under concentrations higher than 50 µM Cd and excess Zn (Rodrigues *et al.* 2017). At high concentrations, these trace elements can disrupt the chloroplast ultrastructure, dismantle the thylakoid, reduce the Chl biosynthesis, and impair the electron transport and the connectivity between the PSII subunits (Amari *et al.* 2017, Adamakis *et al.* 2021, Janeeshma *et al.* 2021, Rajput *et al.* 2021).

The presence of positive K-bands can also indicate physiological disorders in PSII related to damage of the OEC (Kalaji *et al.* 2016). Wang *et al.* (2017b) and Zhang *et al.* (2018) reported an increase in W_K, representing damage in the transport of electrons from OEC to P_{680}^+. It represents an important target for metallic contaminants because the inhibition of the biochemical and biophysical processes of photosynthesis affects, in particular, the physiology of the whole plant. In the present study, the presence of Zn in plants grown with 75 µM Cd showed better performance of the thylakoid membranes and the OEC at the donor side of PSII, evidenced by the presence of negative L-band and K-band. It also indicates improved energy absorption, activities, and connectivity between PSII units.

The O–I and I–P phases are associated with electron transfer dynamics from the intersystem to PSI (Souza *et al.* 2019). Reduction of amplitudes in the O–I interval ($V_{OI} \geq 1.0$) was observed when plants were co-exposed to high concentrations of the two trace elements. This decrease in the values of $V_{OI} \geq 1$ may indicate a reduction in electron flux and pool size of the final electron acceptors of PSI (Yusuf *et al.* 2010, Souza *et al.* 2019). The damages induced by excess Cd led to a reduction in ΔV_{IP} values, suggesting a smaller contribution of emission of Chl *a* fluorescence and reduction in PSI units (Paunov *et al.* 2018). Thus, excess Cd and Zn in plants can lead to disorders in the photosynthetic apparatus.

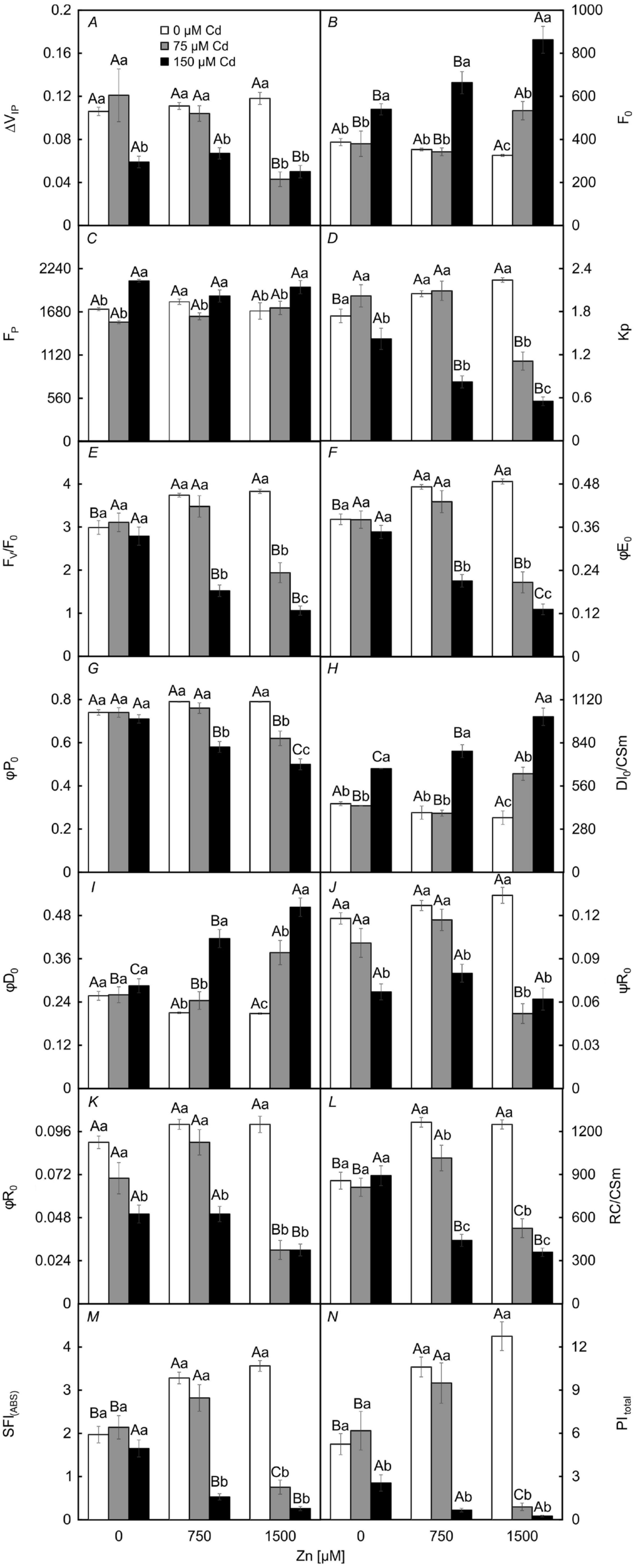

Fig. 8. Amplitude of the relative variable fluorescence of the increase from I to P (ΔV_{IP}) and JIP test parameters of *Alternanthera tenella* plants after 30 d of *in vitro* culture as a function of concentrations of Cd (0, 75, and 150 µM) and Zn (0, 750, and 1,500 µM). Means ± SE (n = 16) followed by *the same letter* (*uppercase letters* comparing Zn concentrations at each Cd concentration and *lowercase letters* comparing Cd concentrations at each Zn concentration) do not differ significantly according to *Scott-Knott* test ($p<0.05$). DI_0/CSm – dissipated energy flux per cross-section; F_0 – initial fluorescence; F_P – fluorescence peak; F_V/F_0 – ratio of the de-excitation rate constants for photochemical and nonphotochemical events; K_P – photochemical de-excitation rate constant; PI_{total} – total performance index, which measures the performance up until the final electron acceptors of PSI; RC/CSm – total number of active reaction centers; $SFI_{(ABS)}$ – PSII structure and functioning index; ΔV_{IP} – relative variable fluorescence amplitude of the increase from I to P = relative contribution of the increase from I to P to the increase in OJIP; φD_0 – quantum yield of energy dissipation (at t = 0); φE_0 – quantum yield of electron transport (at t = 0); φP_0 – maximum quantum yield of primary photochemistry (at t = 0); φR_0 – quantum yield of reduction of end electron acceptors at the PSI acceptor side (RE); ψR_0 – efficiency/probability by which electrons move from PSII to PSI acceptor side.

The PSII, intersystem, and PSI activities, as a function of the treatments, were also expressed through the JIP test parameters. An increase in F_0 can signal a reduction in a number of active reaction centers (RCs), reflecting a decrease in the constant rate of trapped energy (Lotfi *et al.* 2018). In this work, plants exposed to high concentrations of Cd and Zn presented increased F_0 values and reduced RC/CSm, followed by a high energy dissipation as shown by DI_0/CSm values (low rate of trapped energy). Furthermore, with the increase in F_0 and the decrease in the F_V/F_0 ratio values, it was possible to verify that plants exposed to high concentrations of Cd presented a reduction in the constant rate of trapped energy in PSII centers. A decrease in K_P values may reflect abundant energy loss in plants under trace element stress or signal damage to PSII centers (Kumar *et al.* 2020). Reduced F_V/F_0 values may reflect the decline in electron transport resulting from low OEC activity for the donor side of PSII or a decrease in trapped energy in the RCs of PSII (Ghassemi-Golezani and Lotfi 2015, Pontes *et al.* 2020), which corroborates the increased values of W_K. The higher values of F_V/F_0 and K_P, in addition to the decreased values of W_K, observed in plants grown with Zn without Cd supplementation, proved the positive effects of this trace element during the *in vitro* culture of *A. tenella*.

Under co-exposure to both trace elements at high concentrations, the plants also presented decreased quantum yield (φP_0 and φE_0), thus showing a state of stress in the plants. Meng *et al.* (2016) and Kalisz *et al.* (2016) reported that a decrease in quantum yield values might be the result of photoinhibition due to photochemical damage in PSII. In addition, a reduction of φE_0 values indicates a lower efficiency in electron transport, especially from Q_A to Q_B (Kalaji *et al.* 2016). In contrast, an increase in φP_0 and φE_0 may reflect better electron transfer between Q_A and Q_B, with a high connection between the PSII antennas (Lotfi *et al.* 2018, Singh *et al.* 2018).

In this study, *A. tenella* plants grown in media with co-exposure to both trace elements at high concentrations presented physiological disorders resulting in the inactivation of RCs (as evidenced by RC/CSm values), which led to a greater dissipation of energy (φD_0). The inactivation of RCs is a negative response induced by stress caused by trace elements (Meng *et al.* 2016, Zhang *et al.* 2017). In this situation, Paunov *et al.* (2018) stated that a smaller number of active RCs would indicate that less energy is used in the electron transport system, thus, the unused energy must be dissipated. A decrease in RC/CSm values is usually accompanied by increased values of F_0 and φD_0. Increased F_0 and φD_0 values reflect a reduction in the dynamics of transport and use of excitation energy, which may reflect a greater dissipation of energy in the form of heat (Meng *et al.* 2016). This response was even higher in plants exposed to 150 µM Cd, which presented high values of F_0 and simultaneously increased F_P, which can indicate low energy trapping efficiency in the RCs (Martins *et al.* 2015). It led to an increase in DI_0/CSm and reduced PI_{total}. Higher energy dissipation (DI_0/CSm) can reduce the energy necessary for photochemical transformations (Pastuszak *et al.* 2020).

The ψR_0 parameter is sensitive to the effect of trace elements and is associated with a significant decrease in PSI activity. A decrease in ψR_0 under exposure to high concentrations of trace elements may indicate a decrease in efficiency or probability of transfer of trapped electrons from PSII to PSI (Rastogi *et al.* 2019, Faseela *et al.* 2020). This response occurs by the increase in V_I. Other parameters also confirmed that the negative effects of excess trace elements went beyond PSII in *A. tenella* plants. This lower efficiency of the photosynthetic apparatus beyond the intersystem was confirmed by the reduced values of φR_0. The decrease in the values of this parameter may indicate photoinhibition with lower efficiency of PSII electrons to reach the final electron acceptors of PSI (Wang *et al.* 2017b, Zhuo *et al.* 2017, Chattopadhyay *et al.* 2020).

The mitigating effect of Zn and its essentiality as a micronutrient was evidenced by the PI_{total} and $SFI_{(ABS)}$ parameters. The increased values of these parameters in *A. tenella* plants grown with Zn, even when co-exposed to 75 µM Cd, demonstrated how this micronutrient acts positively on the dynamics of electron transport from PSII to PSI. However, it should be noted that Zn at high concentrations can increase the Cd-induced stress in plants. The PI_{total} and $SFI_{(ABS)}$ parameters are good indicators of photosynthetic performance. They specify how stressors acted on the efficiency and functionality of the photosynthetic apparatus of plants (Yusuf *et al.* 2010, Kalaji *et al.* 2016). The decrease in $SFI_{(ABS)}$ observed in plants without Zn or with the highest concentration of Cd demonstrated instability and difficulty for the plants to conserve energy and promote a decreased PSII performance (Stirbet *et al.* 2018). This may lead to an interruption in the electron transport rate and the general photosynthetic activity of several plants (Gupta 2020). Thus, a decrease in the overall performance of the photosynthetic apparatus is expected, as seen through the reduced values of PI_{total}. Furthermore, lower values of PI_{total} indicated that the damage went beyond the intersystem and negatively affected the overall performance of the photosynthetic apparatus.

Conclusion: Cd induced an antagonistic response to that of Zn. With exposure only to Cd, plants showed physiological disorders and reduced plant growth. Zn increased the cross-sectional area of the stem and had positive effects on the physiological performance of plants, such as stability in the structure and functionality of the photosynthetic apparatus. The co-exposure to both trace elements at high concentrations resulted in the inactivation of RCs (RC/CSm) and greater dissipation of energy (φD_0 and DI_0/CSm). Exposure to Zn at concentrations ≤ 750 µM may partially mitigate the deleterious effects of the Cd concentrations evaluated. Therefore, from an eco-toxicological point of view, the excess of both trace elements (Cd and Zn) represents toxicity to the species under study.

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