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TiO_2 nanoparticles may alleviate cadmium toxicity in co-treatment experiments on the model hydrophyte *Azolla filiculoides*

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Abstract

Water ecosystems polluted by heavy metals, such as cadmium, may also be affected by the increasing presence of TiO_2 NPs. Several researchers have studied the effects of the two contaminants individually, however only a few studies on their joint action have been published for plants. Focusing on the aquatic environment, the hydrophyte *Azolla filiculoides* can be a useful model to assess if TiO_2 NPs may in some way alleviate the Cd injuries and improve the ability of the plant to cope with this metal. With this mechanistic hypothesis, after a pre-treatment with TiO_2 NPs, *A. filiculoides* plants were transferred to cadmium contaminated water with or without TiO_2 nanoparticles. After five days of treatment, cadmium uptake, morpho-anatomical and physiological aspects were studied in plants. The continuous presence of TiO_2 nanoparticles, though not increasing the uptake of cadmium in comparison with a priming treatment, induced a higher translocation of this heavy metal to the aerial portion. Despite the translocation factor was always well below 1, cadmium contents in the fronds, generally greater than 100 ppm, ranked *A. filiculoides* as a good cadmium accumulator. Higher cadmium contents in leaves did not induce damages to the photosynthetic machinery probably thanks to a compartmentalization strategy aimed at confining most of this pollutant to less metabolically active peripheral cells. The permanence of NPs in growth medium ensured a better efficiency of the antioxidant apparatus (proline and glutathione peroxidase and catalase activities), induced a decrease in H_2O_2 content, however was not able to lower the oxidative damage (in terms of TBARS).

Keywords: *Azolla filiculoides* · cadmium · histochemistry · oxidative stress · photosynthetic efficiency · TiO_2 nanoparticles

Introduction

Nanotechnologies are among the so-called “Key Enabling Technologies”, recognized by the EC to be essential for the sustainable innovation, scientific research, competitiveness and industry modernization and growth (European Commission 2019). Recognizing the opportunities offered by nanotechnologies, it is nonetheless central and urgent to produce scientific data in different disciplines to thoroughly understand the possible risks related to the release into the environment of nanoparticles (NPs), considered among the emerging contaminants. By material flow modelling advanced studies, predictions of the future environmental concentrations have been proposed for natural ecosystems, notwithstanding NPs chemical transformation, ageing processes, and their transfer to the food chain and in living organisms are not yet precisely known (Ibrahim et al. 2016, Bundschuh et al. 2018). TiO_2 NPs are among the most employed nanomaterials, with an annual production of about 5000 tons, estimated to increase annually up to 2025 (Weir et al. 2012). Because of the impressive increase in nanoparticles expected both in the soil and in marine and freshwater environments, it is necessary to characterize unambiguously TiO_2 NPs actions. Despite the substantial amount of data published on the subject, to date it is difficult to state with certainty whether TiO_2 NPs have toxic/harmful, neutral or positive effects on plant growth and development (Lyu et al. 2017). On the other hand, it is widely accepted that the effects of nanoparticles may depend on their characteristics (shape, size, charge, etc.), on the organism tested, and on the experimental conditions in which they are applied/studied

(Ruffini Castiglione et al. 2016). In view of this it is also very important to understand what effects they can exert when co-present with other pre-existing inorganic contaminants (Manesh et al. 2018). Deng and collaborators (2017) have performed a detailed bibliographic study on NPs and co-existing organic and inorganic contaminants and the combined effects/risks on biota. From the literature published so far the complexity of the problem emerges, related to reactive nanoparticles, that, in the presence of other contaminants, can increase or decrease the toxicity of conventional contaminants, being able to act at various levels, ranging from the bioavailability of the contaminant in the growth medium, to its ability to penetrate the organism, to its mobility and possible chemical and physical changes out or within the organism.

Heavy metals continue to cause great concern, even if a general decrease of the environmental emissions has been recorded in Europe (lead decreased by 93 %, mercury by 71 % and cadmium by 64 % between 1990 and 2016 (European Environmental Agency 2018). Cadmium has been ranked, among the heavy metals, as the seventh most toxic (ATSDR 2017). It is a by-product of zinc production, and it is mainly used in alkaline batteries, and to a lesser extent in pigments and platings, in coatings and as stabilizer for plastics. Cadmium possesses a specific toxicological profile (ATSDR 2012) describing its adverse effects for living organisms and human health. The harm induced by exposure will depend on many factors (e.g. dose, duration, type of contact) as well as on the bioavailability and/or the chemical interactions with other materials able to enhance or debase its activity. Though being a non-redox reactive metal, cadmium can indirectly cause oxidative stress (Wang et al. 2008), through the substitution of redox-active metals in proteins (Cuypers et al. 2011), and induction of NADPH oxidase activity, with the production of reactive oxygen species (ROS; Gallego et al. 2012).

Previous studies reported an induction of antioxidant response in seedlings of *Vicia* spp. treated with TiO_2 NPs (Ruffini Castiglione et al. 2014; Ruffini Castiglione et al. 2016). This response was able to maintain a low level of oxidative damage in roots from treated plants, suggesting a possible protective action of nanoparticles in plants exposed to abiotic stress (Ruffini Castiglione et al. 2016). On that basis, the hypothesis was made the TiO_2 NPs might have a protective action on plants subjected to cadmium stress.

As a significant portion of the nanomaterials of soil and air is expected to reach the aquatic environment and cadmium is characterized by high water solubility, a hydrophyte fern was used in the present experimentation. In particular, *Azolla filiculoides* has been chosen due to its high biomass production, metal accumulation ability (Valderrama et al. 2013; Naghipour et al. 2018) and free floating nature, all positive characteristics for phytoremediation purposes. In addition, the high water content typical of *A. filiculoides* fronds (Serag et al. 2000) drastically reduces the problems of disposal (Sood et al. 2012) and its ability to make symbiotic relationship with the heterocystous blue-green alga, *Anabaena azollae*, allows its growth also in contaminated low nitrogen environment. Despite the capacity of *Azolla* species to accumulate cadmium, it is known that this heavy metal is able to induce oxidative stress in ferns of this genus (Prasad and Singh 2011; Abhishek 2012). On the basis of what above, the clue that TiO_2 NPs may in some way alleviate the Cd injuries and improve the ability of the plant to cope with this metal represents our mechanistic insights.

In particular, the goals of the present work were: i) to assess the capacity and pattern of cadmium accumulation and translocation under priming and co-presence of TiO_2 NPs/Cd; ii) to highlight the eventual protective action of nanoparticles from cadmium induced stress and; iii) to characterize the pattern of activation of antioxidant response in the two different experimental conditions.

With these aims, plants of *Azolla filiculoides* were pre-treated with TiO_2 NPs and transferred to cadmium contaminated water with or without TiO_2 NPs. After five days of cadmium treatment, morpho-anatomical and physiological aspects of the response to the imposed culture conditions were explored.

Materials and methods

Plant material and treatments

Azolla filiculoides Lam. sporophytes were collected from a non-polluted pool of the 'Idrofitorio' at the Botanical Garden of Pisa, Italy (43°43'11"N, 10°23'46"E; the average year temperature and rainfall are 14.8 °C and 823 mm, respectively) in October 2017.

Next, the plants were transferred to plastic pots filled with deionized water and subjected to an acclimation period of 20 days in a growth chamber under controlled temperature and irradiance (25 ± 2 °C; 300 μmol m^{-2} s^{-1}), with a 16/8 h light/dark photoperiod.

Preliminary experiments with different Cd concentrations (from 2 mg L^{-1} to 20 mg L^{-1} of $CdCl_2$) indicated 5 and 10 mg L^{-1} as the highest concentration for which no damage to the plants was observed and the lowest concentration which induced visible damage (chlorosis symptoms) respectively.

Regarding TiO_2 NPs, a middle-low treatment concentration (50 mg L^{-1}) was chosen, possibly reproducing an actual environmental exposure, estimated by probabilistic material-flow modelling studies (Praetorius et al. 2012).

Commercial powder of TiO_2 was bought from US Research Nanomaterials Inc. (Houston, USA) as anatase or rutile NPs (nominal size of 30 nm) having at least 99.9% of purity (producers' information). Shape and size were previously characterized (Giorgetti et al. 2019): anatase NPs appeared prismatic or cylindrical with a size in the range 20-80 nm, rutile NPs were prismatic with cusp with size in the range 30-100nm. TiO_2 NPs (anatase + rutile, 1:1 ratio) were suspended in deionized water, sonicated (Sonifier 250, Branson) for 30 min at 80 W and lastly diluted at the selected concentration.

The experimental design foresaw a priming treatment with sonicated TiO_2 NPs for three days.

Healthy mature sporophytes were selected and transferred in plastic pots containing: deionized water (C), TiO_2 NPs (CNPs), $CdCl_2$ (5 and 10 mg L^{-1}; Cd5 and Cd10 respectively) and $CdCl_2$ (5 and 10 mg L^{-1}) plus TiO_2 NPs (Cd5+NPs and Cd10+NPs). Control and treated sporophytes were maintained for five days in the growth chamber under the same experimental conditions provided during the acclimation period.

At the end of the experiment, treated and control sporophytes were triple rinsed with deionized water and then processed for experimental determinations.

Scanning electron microscopy

For scanning electron microscopy (SEM) observations, the samples were fixed in 3% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.4) for 24 h and then dehydrated in a graded ethanol series. After critical-point drying, root and frond portions (three roots and frond portions of comparable developmental stage belonging to five different plants) were coated with gold, examined and captured with a scanning electron microscope (JSM-5410, Jeol, Tokyo, Japan).

Atomic absorption spectrometry analysis for cadmium content determination

Cadmium content in roots and fronds was determined according to Ciobanu et al. (2013) with minor modifications. In particular, the samples after drying to ash in muffle furnace at 525°C for 3 hours, were ground in porcelain mortar. The digested samples (65% HNO_3 and 1N HCl (1:1 v/v) and heating at 145°C till white fumes start appearing) were made at 25 mL with deionized water and filtered through filter paper. Heavy metal concentration was measured in a flame atomic absorption spectrometer (Thermo Scientific, ICE 3000 series).

Bioconcentration factor and translocation factor

The bioconcentration factor (BCF) was calculated according to Rahmani and Sternberg (1999) as follows:

$$BCF = \frac{metal\ concentration\ in\ plant\ \ dried\ biomass\ (mg\ kg^{-1})}{initial\ metal\ concentration\ in\ the\ external\ solution\ (mg\ L^{-1})}$$

The translocation factor (TF) was determined according to Luo et al. (2005) as follows:

$$TF = \frac{metal\ concentration\ in\ leaves\ (mg\ kg^{-1})}{metal\ concentration\ in\ roots\ (mg\ kg^{-1})}$$

Histochemical localization of Cd

In situ localization of cadmium in control and treated roots and fronds of *A. filiculoides*, was performed by dithizone (diphenylthiocarbazone) method, following the procedure described by Seregin and Ivanov (1997) with some modifications (Balestri et al. 2014b). Cadmium occurrence in plant cells/tissues was detectable as brown/reddish color precipitates. Five whole roots and leaves from five different plants were stained for 1.5 h with a dithizone solution (30 mg dissolved in 60 mL acetone and 20 mL distilled water), rinsed in water and immediately analyzed using a Leitz Diaplan light microscope (Wetzlar, Germany). Images were captured using a Leica DFC 420 (Leica Microsystems, Heerbrugg, Germany).

Water content, pigment determination and photosynthetic efficiency

The percentage of plant water content was estimated on the fresh weight basis with the formula:

$$water\ \% \ = \frac{FW - DW}{FW} x100$$

FW = Fresh weight, *DW* = Dry weight.

After recording fresh weight, roots or fronds were dried in an oven at 60° C to constant weight and reweighed to obtain the dry weight.

Frond chlorophylls (a, b and total) and carotenoids were extracted and determined as in Spanò and Bottega (2016), according to Hassanzadeh et al. (2009) and to Lichtenthaler (1987). Pigment contents were expressed as mg g^{-1}FW.

Photosynthetic efficiency was determined by analyzing chlorophyll a fluorescence by a portable fluorometer (MINI-PAM Walz, Effeltrich, Germany). Five records per pot were taken from 4 pots per treatment on light-exposed fronds, thus acquiring the operating Photosystem II (PSII) quantum yield (ΦPSII). The measurements were repeated following 30 min dark acclimation, to evaluate the maximum PSII quantum yield (Fv/Fm) (Genty et al. 1989). Consequently, the value of each thesis was the average of 20 measurements ± SE.

Oxidative stress and antioxidant response

Hydrogen peroxide content of plants was determined spectrophotometrically at 410 nm, using titanium chloride in H_2SO_4 for peroxide detection according to Jana and Choudhuri (1982). The amount of H_2O_2 in the extracts was expressed as μmol g^{-1}FW referring to a standard curve.

Lipid peroxidation in plants was estimated by determining the amount of thiobarbituric acid reactive substances (TBARS) according to Wang et al. (2013) with minor modifications as in Spanò et al. (2017). The concentration of TBARS, measured as specific absorbance at 532 nm by subtracting the non-specific absorbance at 600 nm, was expressed as nmol g^{-}FW.

Proline concentration was determined according to the method of Bates et al. (1973) with minor modifications, as in Spanò et al. (2013). Plant tissue was homogenized with 3% sulfosalicylic acid and the supernatant was incubated with glacial acetic acid and ninhydrin reagent (1:1:1) and boiled for 60 min. After cooling the reaction mixture and addition of toluene, the absorbance of toluene phase was read at 520 nm. Proline content was expressed as μmol g^{-1}FW referring to a standard curve.

For antioxidant enzymes, extraction was made as in Spanò et al. (2013) after grounding of plants in liquid nitrogen with a mortar and pestle at 4°C. The homogenate was then centrifuged at 15,000 g for 20 min. For ascorbate peroxidase, 2 mM ascorbate was added to the extraction medium and for glutathione reductase (GR, EC 1.6.4.2) the supernatant was desalted on a Sephadex G-25 column.

Supernatants were collected and stored at -80°C until their use for enzymatic assays. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) recording the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) as ascorbate was oxidised. Correction was made for the low, non-enzymatic oxidation of ascorbate by hydrogen peroxide (blank). Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to Navari-Izzo et al. (1997) following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mm^{-1} cm^{-1}). The activity of GR was determined as described by Rao et al. (1995) following the oxidation of NADPH at 340 nm. Catalase (EC 1.11.1.6) activity was determined as described by Aebi (1984) and calculated from the 39.4 mM^{-1} cm^{-1} extinction coefficient. A blank containing only the enzymatic solution was made. Guaiacol peroxidase (POX, EC 1.11.1.7) activity was determined as described by Arezky et al. (2001) using as substrate 1% guaiacol and measured determining guaiacol oxidation by H_2O_2 at 470 nm (extinction coefficient 26.6 mM^{-1} cm^{-1}), one unit oxidising 1.0 mmole guaiacol per min.

Histochemical localization of H_2O_2

In situ localization of hydrogen peroxide in control and treated roots and fronds of *A. filiculoides*, was performed by 3,3'-diaminobenzidine (DAB) staining (Daudi et al. 2012). This compound is oxidized by hydrogen peroxide occurring in plant cell/tissues to generate dark brown precipitates. Five whole roots and

leaves from five different plants were soaked in a freshly prepared incubation medium containing 1 mg mL^{-1} DAB for 4 hours at 25°C in complete darkness. To remove chlorophylls, leaves were fixed in ethanol:glycerol:acetic acid (3:1:1, bleaching solution) and placed in a water bath at 95°C for 15 min. After extensive rinsing the sample were immediately observed under light microscopy (Leitz Diaplan microscope; Wetzlar, Germany) and images were captured using a Leica DFC 420 (Leica Microsystems, Heerbrugg, Germany).

Statistical analysis

Data were expressed as mean of at least six replicates ± SE. Normality of distribution and homogeneity of variances were assessed by Shapiro-Wilk and Levene tests, respectively. The results were processed by one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons (Tukey test). The level of significance was $p < 0.05$

Results and discussion

Water ecosystems may be affected by the increasing presence of TiO_2 NPs as well as by Cd pollution following different contamination ways: both the contaminants can convey in freshwater basin ecosystems, via industrial plants waste, wastewater, polluted runoff, biosolid and soil, as well as via surface water in a total life cycle with a non-clear fate and prediction on how they may work together to exert interactive effects on the biota. Only a few studies on this joint action have been published for plants, taking into account NPs and heavy metals as occurring contaminants, co-present in the growth medium (Wang et al. 2015; Lopez-Luna et al. 2016; Ji et al. 2017; Rossi et al. 2018). Following a specific experimental design, *A. filiculoides* plants displayed interesting and peculiar responses depending on Cd concentration as well as on the simultaneous presence with TiO_2NPs in the growth medium.

Effects on plant morphology

To observe the effects of the imposed treatments on plant morphology, SEM analysis has been employed. Leaf morphology did not change considering the structure and organization in both the dorsal photosynthetic lobe and lower not photosynthetic thin-lobe leaf. Figure 1 shows the dorsal lobes of leaves, organized in two rows along the rhizome, with unicellular papilliform trichomes and stomata in the upper surface. On the contrary root morphology displayed noteworthy differences, depending on the treatments (Fig. 2). The simple priming treatment with TiO_2 NPs was not able to preserve root hair patterning when the plants were placed in contact with Cd (at both concentrations). Indeed, after 5 days of Cd treatments, root hairs were rarely or no detectable in the first millimeters (on average 4.5±0.3 mm) from the root apex (Fig. 2c, d). This result contrasts with what is reported in the literature on the *Arabidopsis thaliana* model system: Cd enhances the density and the length of root hairs, by the modulation of the expression of specific genes in a context in which auxin and auxin signalling pathway are involved (Bahmani et al. 2016). Given that recent studies suggest possible different roles of auxin (and cytokinins) in homorhizoic roots of ferns compared to allorhizoic root of *A. thaliana* (Augstein and Carlsbecker 2018), different root architecture induced by Cd may reasonably be explained. In addition, it is worth noting that in *Pteris vittata* treated with 100 μM Cd a drastic reduction of density and

length of root hairs was reported too (Balestri et al. 2014b) and a comparable root adaptive plasticity was shown in response to arsenic (Forino et al. 2012).

Interestingly, in the present experimentation, Cd in co-presence with TiO_2 NPs did not affect the root architecture that, in these experimental conditions, maintained a root hair *indumentum* set up comparable to the control, with a partial mitigation of the notorious Cd toxicity (Fig. 2). Indeed, the ability of nanoparticles to induce the proliferation of root hairs is known in the literature (Giordani et al. 2012) and could therefore compensate for the toxic action of cadmium on the root growth *habitus*.

Cadmium content

Cd was taken up from the growth medium by root hairs, as shown by root dithizone staining (Fig. 3) and particularly evident in Cd10 (Fig. 3g). According to Piñeros et al. (1998), the root apical region may be also involved in the uptake of cations, constituting an absorption site, reasonably active in Cd5 and Cd10 plants (Fig. 3e, 3g insert), that lacked root hairs in the first millimetres from the root apex (Fig. 2).

In accordance with most of data in literature (Dai et al. 2006; Tan et al. 2011; Valderrama et al. 2013; Balestri et al. 2014a, b; Valderrama et al. 2016) cadmium content progressively increased with its increasing concentration in the growth medium both in roots and in fronds (Table 1). In *Azolla* (Tan et al. 2011), just as in other plants (Ji et al. 2017), at a given Cd treatment, the content of this heavy metal was higher in roots, than in fronds (Table 1). The preferential accumulation in roots of *A. filiculoides*, though not surprising, as roots are in direct contact with the contaminant, could highlight a strategy of avoidance to protect the photosynthetic apparatus from possible negative effects. The continuous presence of NPs in the pots induced a slight decrease in root cadmium concentration in comparison with the simple priming with nanoparticles (Table 1). In addition, root bioconcentration factor was lower under the higher Cd concentration in growth medium, showing a limited capacity of accumulation of this heavy metal in this plant organ. A decrease in Cd concentration has been recorded in roots of rice co-treated with TiO_2 NPs and similar reduction were detected also in leaves (Ji et al. 2017). On the contrary, in fronds of *A. filiculoides,* the concentration of this heavy metal was significantly higher in plants grown in NPs, that, as a consequence, had a relatively higher TF and BCF. A similar positive effect on TF value was also recorded in *Bohemeria rigida* (Gong et al. 2017). Cd presence in leaves was marked as reddish aggregates mainly in membranous margin of the foliar lobes (Fig. 3f, h, j, l). These margins, formed by hetero-dimensional cells, contained chloroplasts, but at lower extent, compared to the assimilating cells below epidermis of the lobe central region, thicker and active for photosynthesis (Fig. 3b, d). In addition, in Cd5+NPs and Cd10+NPs treated plants, Cd was detected histochemically also in the unicellular papillae, characterizing superior epidermis. Foliar lobe Cd patterning suggests an endeavor to delocalize and sequestrate this toxic metal in less metabolically bioactive cells/tissues such the peripheral membranous margins of the foliar lobe, epidermis and papillae. This could be one of the possible mechanisms for heavy metal detoxification and defense strategies in tracheophytes (Küpper et al. 2000; Pielichowska and Wierzbicka 2004; Balestri et al. 2014b).

Pigment content and photosynthetic efficiency

Priming with NPs seemed to have a protective action on total chlorophyll content (Table 2), whose content generally declines under cadmium treatment (Dai et al. 2006; Prasad and Singh 2011). The continuous presence of TiO_2 NPs in the growth medium induced an increase, though not significant, in the concentration of this pigment despite the increase in cadmium frond content. There were not significant differences in carotenoids content among the different treatments, showing a protective action of TiO_2 NPs also on this pigment, as in literature a carotenoids cadmium-dependent decrease has been recorded (Dai et al. 2006). Operating PSII quantum yield (ΦPSII) and maximum PSII quantum yield (Fv/Fm) were not negatively affected, either by cadmium and NPs in the growth medium (Fig. 4), therefore photochemical efficiency might have benefited from the compartmentation of this heavy metal in the less metabolically active portions of the leaves, which seems to be a successful strategy for the protection of the photosynthetic apparatus.

Oxidative stress and antioxidant response

Cadmium treatment induced an increase in hydrogen peroxide content in comparison with control plants (Table 2).

The accumulation of this ROS, besides being a common trait in plants treated with this heavy metal (Lv et al. 2017; Leng et al. 2018), has also been recorded in plants amended with TiO_2 NPs (Giorgetti et al. 2019) and consistently, neither the priming nor the continuous treatment with TiO_2 NPs were able to reduce the increase in this signaling molecule (Table 2).

In situ determination of hydrogen peroxide by DAB staining (Fig. 5), performed on leaves and roots separately, showed a higher responsiveness of root compared to leaf compartment in Cd5 treatment (Fig. 5e, f), while, at higher concentration, the opposite occurrence was detected (Fig. 5g, h). It is worth noting that only the leaf central lobe and thicker region were stained (Fig. 5f, h). The co-presence of NPs induced a lower level of DAB staining but with a comparable differentiation between root and leaf (Fig. 5c, d, i, j, h, l). In addition, leaf staining in Cd10+NPs was partially extended to the membranous margin of the foliar lobe (Fig. 5l), in which the highest Cd accumulation was recorded by dithizone staining (Fig. 3l).

Although in most papers (Kapoor et al. 2016; Leng et al. 2018) a cadmium concentration-dependent increase in H_2O_2 content has been reported, in the present work the highest content of this ROS was detected under the lower concentration of this heavy metal, in accordance with histochemical data. A similar trend has sometimes been reported in literature (Chang et al. 2012) and is not surprising if the mechanism of induction of oxidative stress by cadmium is considered. In fact, this heavy metal produces ROS indirectly by different mechanisms among which the weakening of the antioxidant defence can play an important role (Srivastava et al. 2004; Gill and Tuteja 2010). In accordance CAT and GPX activities were particularly low just in Cd5 plants. On the other hand, the partial recovery in the activities of these antioxidant enzymes in Cd5+NPs plants could help to explain the lower contents of hydrogen peroxide recorded in particular just in these plants. Oxidative damage indicated as TBARS (Table 2), indirect indicator of lipid peroxidation and membrane injury, was always higher under cadmium treatment and despite the differences recorded in H_2O_2 concentration, the continuous presence of NPs was not able to reduce this damage. The partial lack of correlation between hydrogen peroxide content and oxidative damage in cadmium-treated plants has been already recorded in literature (Balestri et al. 2014a)

and further underlines the presence of an oxidative injury not completely ROS-mediated. In fact, the activation by cadmium of a lipoxygenase inducing lipid peroxidation has been already reported (Chaoui et al. 1997; Balestri et al. 2014a). In the complex antioxidant response, antioxidant molecules are involved. Among these, proline, compatible solute, besides its contribution to osmotic adjustment, protects macromolecules in stress conditions and can act as a radical scavenger (Hayat et al. 2012). Most of data in literature, report an accumulation of proline under heavy metal treatment (Alayat et al. 2014; Natarajan et al. 2018), while both decrease and increase in the content of this amino acid have been recorded in plants treated with TiO_2 nanoparticles (Ruffini Castiglione et al. 2014; Ruffini Castiglione et al. 2016). In *A. filiculoides* cadmium exposure induced a decrease in proline content in NPs primed plants (Table 2). Interestingly, under the simultaneous treatment with cadmium and NPs, a significant increase in this antioxidant molecule was recorded further showing that the continuous presence of nanoparticles helped to maintain a good antioxidant response. Besides molecules, such as proline, antioxidant enzymes play an important protective role in stress conditions. Both decrease and increase in the activity of these enzymes have been reported in the presence of cadmium (Li et al. 2013; Balestri et al. 2014a; Irfan et al. 2014) or TiO_2 NPs (Lei et al. 2008; Foltete et al. 2011; Ruffini Castiglione et al. 2014, 2016; Okupnik and Pflugmacher 2016).

The continuous presence of nanoparticles induced in itself a decrease in activity of APX and GPX and an increase in activity of POX and CAT in control plants (Table 2), confirming that the influence of NPs may be different on the different antioxidant enzymes as previously reported for other plant systems (Servin et al. 2013; Ruffini Castiglione et al. 2016). With the exception of GR, whose activity was not statistically different across the different treatments (Table 2), the presence of cadmium generally induced a decrease of enzymatic activity and for APX and POX this inhibition was concentration-dependent. The general impairment of antioxidant activity by cadmium is in accordance with its ability to indirectly induce oxidative stress by weakening the antioxidant defense (Srivastava et al. 2004; Gill and Tuteja 2010). A partial protection of the antioxidant enzymes GPX and CAT by nanoparticles was recorded only under Cd and NPs co-treatment, and limited to the treatment with the lower cadmium concentration.

Conclusions

The continuous presence of TiO_2 nanoparticles, though not increasing the uptake of cadmium in comparison with a priming treatment, induced a higher translocation of this heavy metal to the aerial portion of the plant. Although the translocation factor was always well below 1, cadmium contents in the fronds were generally greater than 100 ppm and therefore *A. filiculoides* can be considered a good cadmium accumulator, especially since the whole plant can be collected after the period of phytoremediation. Higher cadmium contents in leaves did not impair photochemistry, probably thanks to a compartmentalization strategy confining most of this pollutant to less metabolically active peripheral cells of the leaf. The permanence of NPs in growth medium despite ensuring a better efficiency of the antioxidant apparatus, in terms of proline and GPX and CAT activities, and inducing a decrease in H_2O_2 content, was not able to lower the oxidative damage (in terms of TBARS).

Acknowledgments

This work was supported by local funding of the University of Pisa (ex 60 %)

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Figure captions

Fig. 1. Portions of representative floating stems of *A. filiculoides*, as observed on scanning electron microscope, bearing two lateral rows of alternately arranged leaves on the dorsal surface. a) C plant sample. b) Cd10 plant sample. c-d) leaf epidermis with one-celled trichomes (papillae, Pp) and adjacent stomata (St) from Cd5+NPs. C=deionized water; Cd10=10 mg L^{-1} $CdCl_2$; Cd5+NPs=5 mg L^{-1} $CdCl_2$ plus TiO_2 NPs.

Fig. 2. Apical part of roots of *A. filiculoides* observed on scanning electron microscope. a) C root; b) CNPs root; c) Cd5 root; d) Cd10 root; e) Cd5+NPs root; f) Cd10+NPs root. C=deionized water; CNPs=TiO_2 NPs; Cd5=5 mg L^{-1} $CdCl_2$; Cd10=10 mg L^{-1} $CdCl_2$; Cd5+NPs=5 mg L^{-1} $CdCl_2$ plus TiO_2 NPs; Cd10+NPs=10 mg L^{-1} $CdCl_2$ plus TiO_2NPs.

Fig. 3. *In situ* Cd localization in *A. filiculoides* roots and mature leaves after treatment with dithizone. Root images correspond to a portion about 2 mm far from the apex except for g and for insert in e (about 5 mm from the apex). a) Detail of the root and root hairs of C plants. b) ventral side of the dorsal lobe of a C leaf: Mm: membranous margin of the foliar lobe; Cr: thick photosynthetic central region; Cv: pore of the leaf cavity. c) Detail of the root and root hairs of CNPs plants. d) feature of Cr and Mm of the leaf lobe in CNPs plants. e-f) Cd5 treatment: root and leaf representative images with brown/reddish Cd precipitates (arrows). g-h) Cd10 treatment: root and leaf representative images with brown/reddish Cd precipitates (arrows). i-j) Cd5+NPs treatment: root and leaf representative images with brown/reddish Cd precipitates (arrows); Cd positivity is also detectable in dorsal epidermis and papillae (Pp). k-l) Cd10+NPs treatment: root and leaf representative images with brown/reddish Cd precipitates (arrows); Cd positivity is also detectable in dorsal epidermis and papillae (Pp). C=deionized water; CNPs=TiO_2 NPs; Cd5=5 mg L^{-1} $CdCl_2$; Cd10=10 mg L^{-1} $CdCl_2$; Cd5+NPs=5 mg L^{-1} $CdCl_2$ plus TiO_2 NPs; Cd10+NPs=10 mg L^{-1} $CdCl_2$ plus TiO_2NPs.

Fig. 4. Operating (ΦPSII, left) and maximum potential (Fv/Fm, right) efficiency of photosynthesis of *A. filiculoides*, under different treatments. C=deionized water; CNPs=TiO_2 NPs; Cd5=5 mg L^{-1} $CdCl_2$; Cd10=10 mg L^{-1} $CdCl_2$; Cd5+NPs=5 mg L^{-1} $CdCl_2$ plus TiO_2 NPs; Cd10+NPs=10 mg L^{-1} $CdCl_2$ plus TiO_2NPs. Values are the results of 20 measures per treatment ± SE.

Fig. 5 Histochemical staining of roots and mature leaves for visual localization of hydrogen peroxide in *A. filiculoides*. Root images correspond to a portion about 2 mm far from the apex. a-b) Detail of the root and leaf dorsal lobe of C plants. c-d) Detail of the root and leaf dorsal lobe of CNPs plants. e-f) Detail of the root and leaf dorsal lobe of Cd5 plants. g-h) Detail of the root and leaf dorsal lobe of Cd10 plants. i-j) Detail of the root and leaf dorsal lobe of Cd5NPs plants. k-l) Detail of the root and leaf dorsal lobe of Cd10NPs plants.
Mm: membranous margin of the foliar lobe; Cr: thick photosynthetic central region; Vt: vascular trace which encircles almost the whole circumference of the leaf cavity (Lf).

C=deionized water; CNPs=TiO_2 NPs; Cd5=5 mg L^{-1} $CdCl_2$; Cd10=10 mg L^{-1} $CdCl_2$; Cd5+NPs=5 mg L^{-1} $CdCl_2$ plus TiO_2 NPs; Cd10+NPs=10 mg L^{-1} $CdCl_2$ plus TiO_2NPs.

Figure 1

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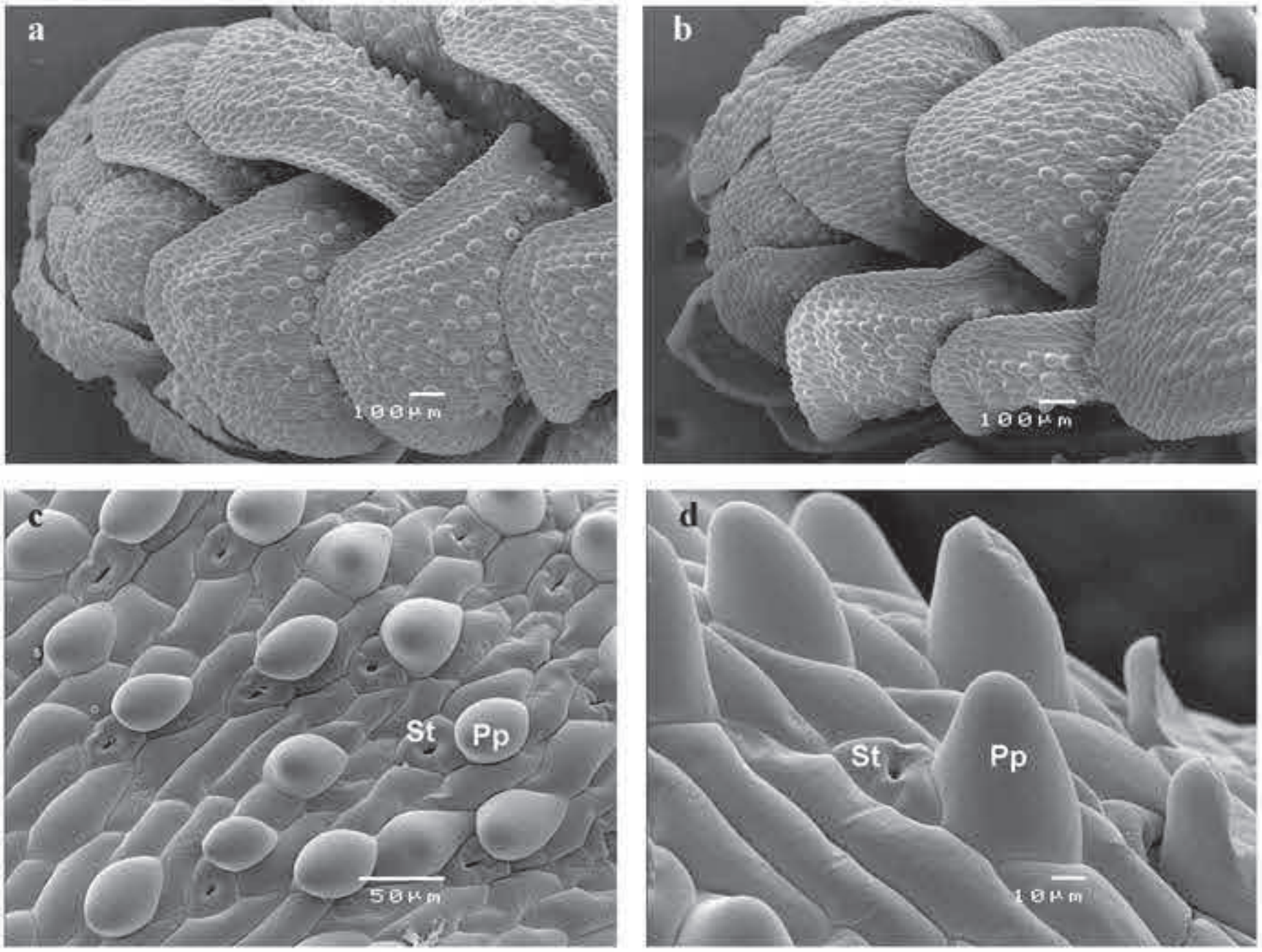

Figure 2

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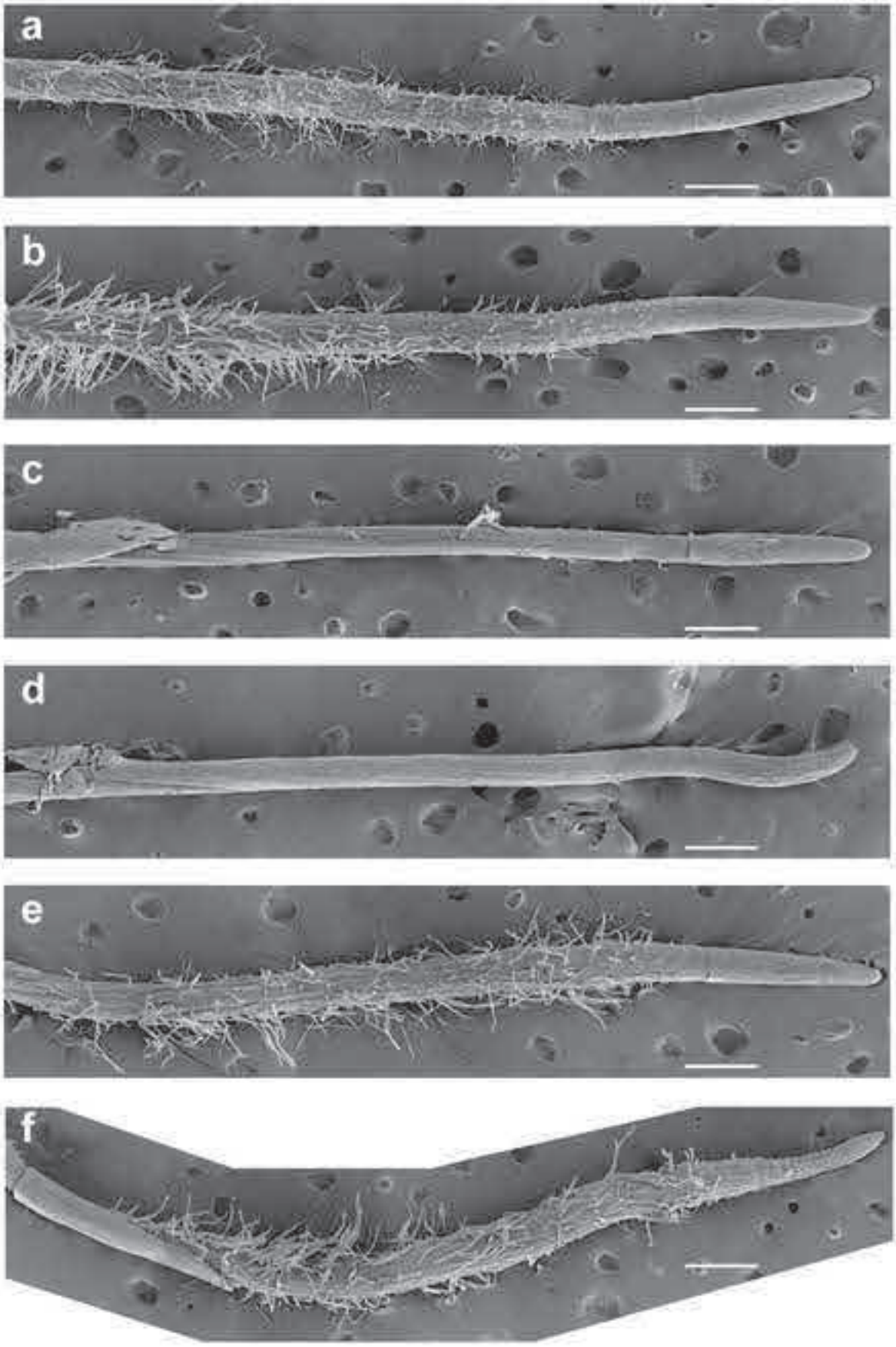

Figure 3

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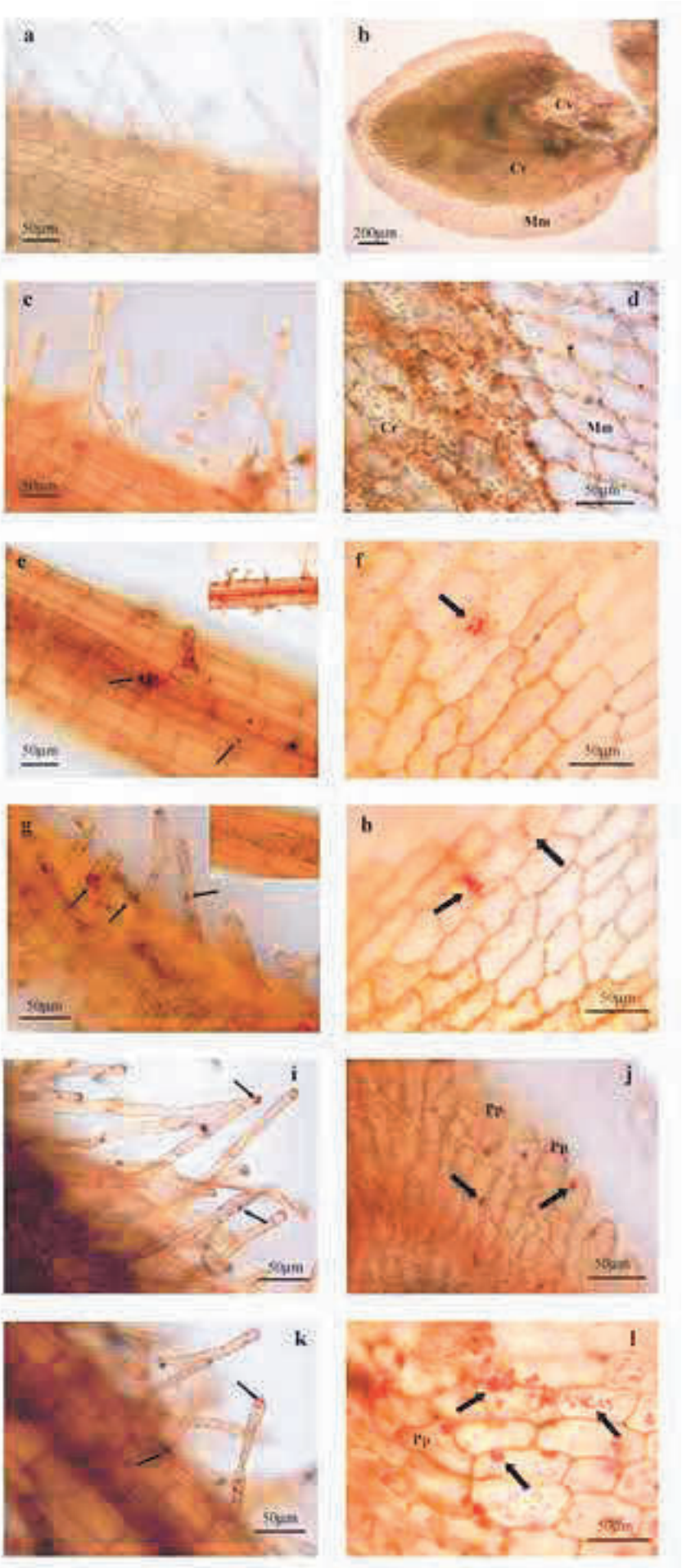

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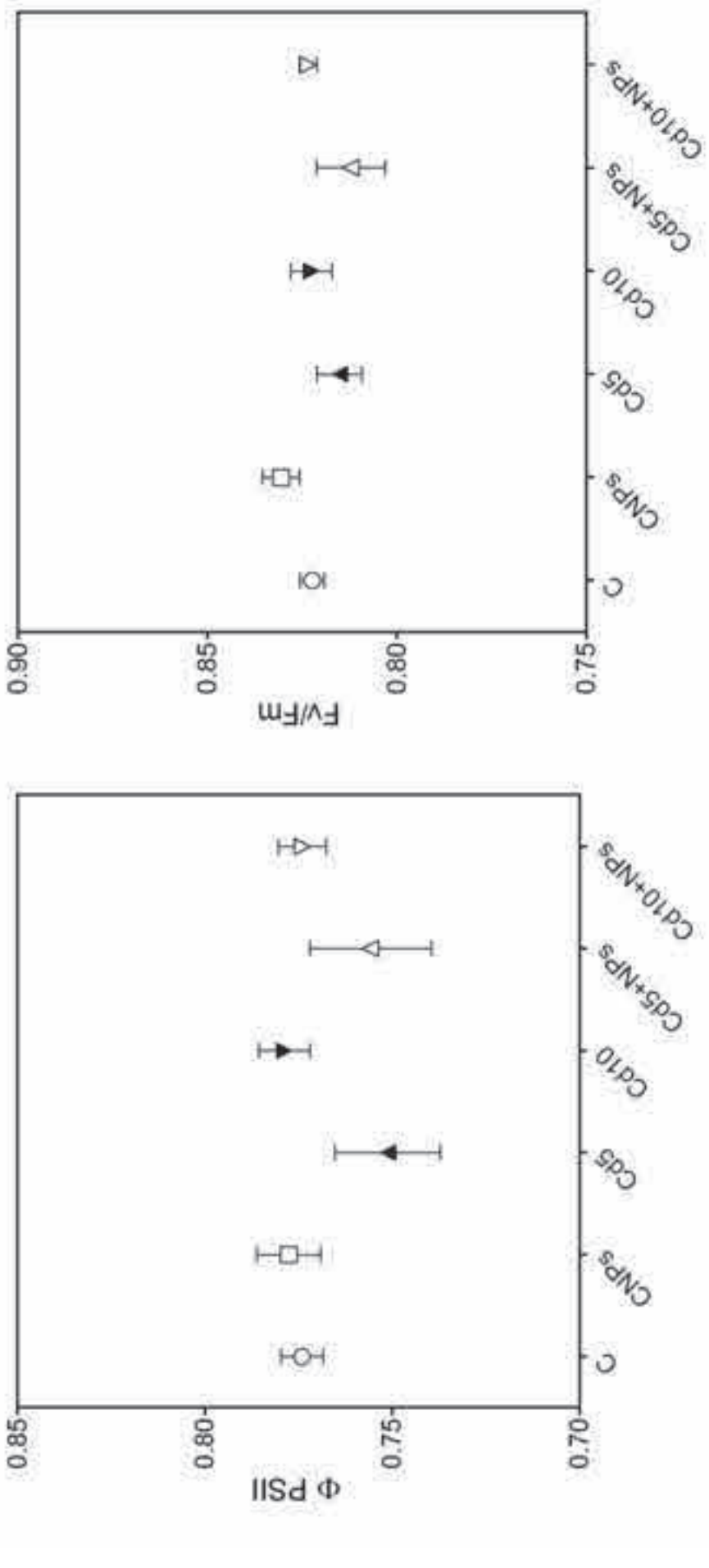

Figure 4

Figure 5

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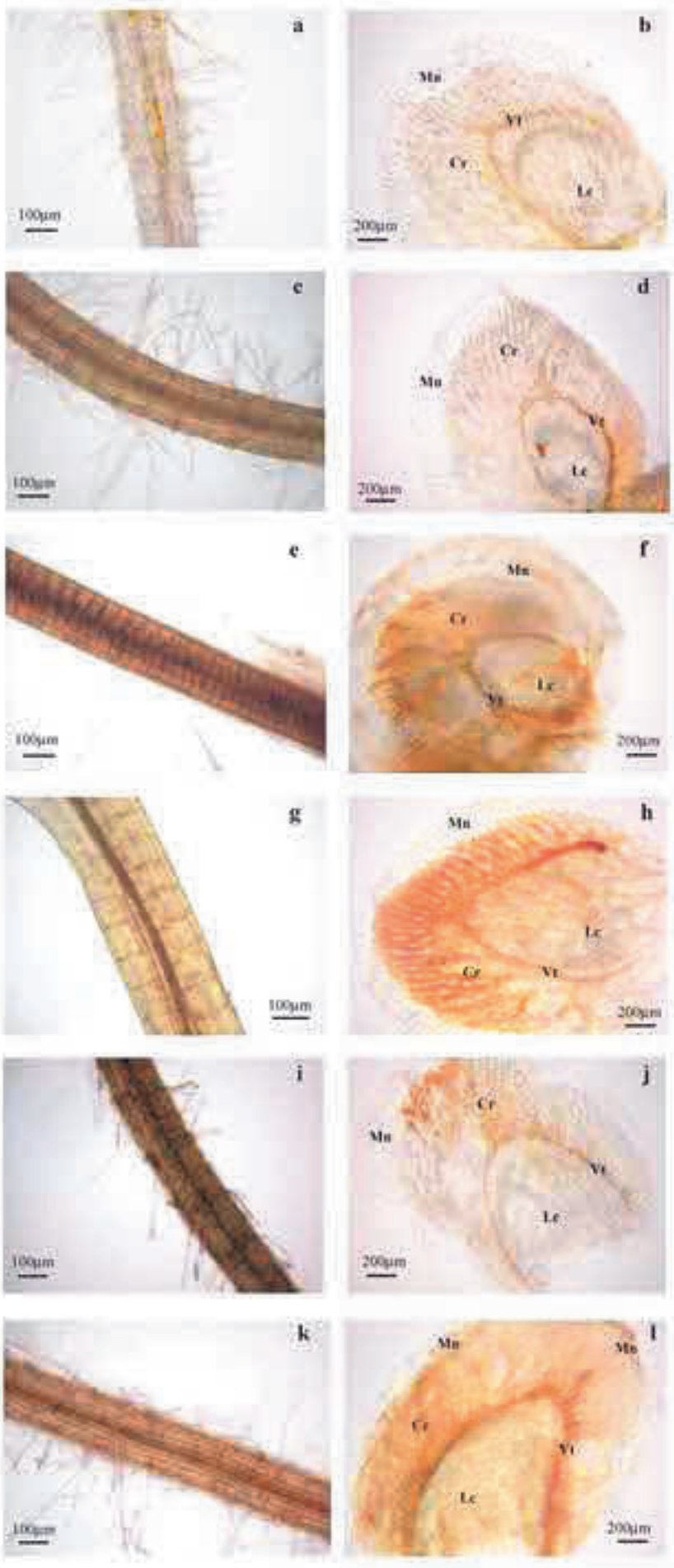

Table 1

Content of cadmium, bioconcentration factor (BCF) and translocation factor (TF) in roots and fronds of *Azolla caroliniana* under different treatments: C=deionized water; CNPs=TiO_2 NPs; Cd5=5 mg L^{-1} $CdCl_2$; Cd10=10 mg L^{-1} $CdCl_2$; Cd5+NPs=5 mg L^{-1} $CdCl_2$ plus TiO_2 NPs; Cd10+NPs=10 mg L^{-1} $CdCl_2$ plus TiO_2NPs. Values are means of at least six replicates ± SE. Different letters denote significant differences at $p<0.05$

	C	**Cd5**	**Cd10**	**CNPs**	**Cd5+NPs**	**Cd10+NPs**
Root Cd content (µg g^{-1} DW)	BDL	2299.43±31.20c	4203.95±67.26a	BDL	2176.63±20.45d	4049.30±50.23b
Frond Cd content (µg g^{-1} DW)	BDL	74.51±6.52d	649.95±13.09b	BDL	275.94±12.37c	933.22±26.89a
Root BCF	-	459.89	420.39	-	435.33	404.93
Frond BCF	-	14.90	64.99	-	55.19	93.22
TF	-	0.03	0.15	-	0.13	0.23

BDL: Below detection limit

Table 2

Contents of total chlorophylls (Tot chl) and carotenoids in fronds and contents of hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS) and proline and activities of ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX), guaiacol peroxidase (POX) and catalase (CAT) in plants of *Azolla caroliniana* under different treatments: C=deionized water; CNPs=TiO_2 NPs; Cd5=5 mg L^{-1} $CdCl_2$; Cd10=10 mg L^{-1} $CdCl_2$; Cd5+NPs=5 mg L^{-1} $CdCl_2$ plus TiO_2 NPs; Cd10+NPs=10 mg L^{-1} $CdCl_2$ plus TiO_2NPs.

Values are means of t least six replicates ± SE. Different letters denote significant differences at $p<0.05$

	C	Cd5	Cd10	CNPs	Cd5+NPs	Cd10+NPs
Tot chl (mg g^{-1} FW)	0.65±0.05a	0.62±0.02a	0.61±0.03a	0.70±0.08a	0.73±0.02a	0.85±0.10a
Carotenoids (mg g^{-1} FW)	0.11±0.01a	0.10±0.00a	0.10±0.00a	0.12±0.01a	0.12±0.00a	0.12±0.01a
H_2O_2 content (µmol g^{-1} FW)	2.45±0.14d	5.41±0.15a	3.82±0.17bc	2.78±0.04d	4.31±0.13b	3.42±0.06c
TBARS content (nmol g^{-1} FW)	11.58±0.21b	15.94±0.83a	16.62±0.24a	11.06±0.20b	15.63±0.54a	14.54±0.56a
Proline content (µmol g^{-1} FW)	0.99±0.02b	0.47±0.01c	0.31±0.01c	0.92±0.21b	1.35±0.09a	1.22±0.16ab
APX (U mg^{-1} protein)	1.07±0.03a	0.62±0.04c	0.42±0.03d	0.85±0.05b	0.64±0.03c	0.49±0.02d
GR (U mg^{-1} protein)	0.12±0.02a	0.10±0.02a	0.10±0.02a	0.10±0.02a	0.09±0.00a	0.09±0.00a
GPX (U mg^{-1} protein)	2.31±0.01a	1.16±0.01d	1.83±0.04b	1.16±0.10d	1.39±0.02c	1.47±0.02c
POX (U mg^{-1} protein)	0.052±0.003b	0.028±0.000c	0.022±0.000f	0.056±0.001a	0.027±0.000d	0.026±0.000e
CAT (U mg^{-1} protein)	4.39±0.40c	1.75±0.11d	2.56±0.19d	6.55±0.53a	5.49±0.41b	2.21±0.12d