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Awaiting better times: A quiescence response and adventitious root primordia formation prolong survival under cadmium stress in *Tetradenia riparia* (Hochst.) *Codd*

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ABSTRACT

Plants survive stressful conditions by employing three main strategies: escape, tolerance, and survival. Experiments were conducted to pinpoint the response strategy adopted by *Tetradenia riparia* to cadmium (Cd) stress by monitoring several physiological parameters and plants' ability to recover once the stress receded. Cadmium (30 and 150 μM) strongly affected shoot and root growth in a dose dependent manner, with almost complete inhibition of shoot growth, stomatal opening and CO_2 assimilation in plants exposed to 150 μM $CdSO_4$. Independently of the $CdSO_4$ concentrations, plants excluded Cd from the aboveground tissues, with concentrations in shoots remaining around 0.1 μmol g^{-1} dry mass. Furthermore, Cd stress was associated with a decline in methylation of Lys4 of histone H3, likely associated with the transition from an active to a quiescent state. Surprisingly, Cd also induced the initiation of root primordia in *T. riparia* stems, which, once placed in a Cd-free media, quickly (24 h) developed into adventitious roots, which were likely the driving factor of the rapid resumption of leaf elongation and photosynthetic activity, which increased almost 20-fold over the 3 weeks of recovery. Therefore *T. riparia* ability to survive Cd stress was mediated by quiescence, which associated with an excluder strategy and stem root primordia formation, enabled rapid resumption of growth under Cd-free conditions.

1. Introduction

Cadmium (Cd) is a highly toxic trace pollutant for humans, animals and plants, and its presence in the environment has been widely recognized as a serious concern since the 1960s (Pan et al., 2010). Cadmium is naturally present in soils, however recent advances in industry and agriculture stimulate increased deposition of metals in the environment and more than 90% of Cd in soils is currently from anthropogenic sources (Pan et al., 2010). Particularly, high levels can be found when agricultural expedients are used, such as contaminated irrigation water (Caldas and Machado, 2004). It has now been demonstrated that Cd has cytotoxic, mutagenic and/or carcinogenic effects in animal and has been classified as a human carcinogen (Waalkes, 2003). Given that plants can easily absorb Cd, they are an important source of Cd exposure for humans through the food chain (Ueno et al., 2009; Lux et al., 2011a,b; Gallego et al., 2012; Uraguchi and Fujiwara, 2012, 2013; Clemens et al., 2013). Therefore intensive researches have been performed to understand the levels of Cd accumulation in the different plant organs and the effects of this heavy metal on plant physiology (Lux et al., 2011a,b; Clemens et al., 2013; Uraguchi and Fujiwara, 2013). In plants, Cd is a non-essential element and has been found to induce complex changes in plants at genetic, biochemical and physiological levels that ultimately result to substantial growth reductions (Sandalio et al., 2001; Cosio et al.,

Abbreviations: C_i, intercellular CO_2 concentration; ETR, photosynthetic electron transport rate; F_m, maximum fluorescence yield; F_s, steady state fluorescence yield; F_v/F_m, maximum quantum yield; g_s, stomatal conductance; H3-K4, lysine 4 of histone H3; NPQ, non-photochemical quenching; PFDa, absorbed photon flux density; P_n, net photosynthetic rate; PSII, photosystem II; Φ_{PSII}, actual quantum yield; RWC, relative water content.

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2006; Lopez-Millan et al., 2009; Gallego et al., 2012). Cadmium alters a wide array of processes in growing plants, amongst which plant water relations (Haag-Kerwer et al., 1999; Perfus-Barbeoch et al., 2002; Lefevre et al., 2014), leaf gas exchanges (Baryla et al., 2001; Küpper et al., 2007; He et al., 2011; Kieffer et al., 2009; Wang et al., 2009; Parmar et al., 2013), carbohydrate and ion homeostasis (Besson-Bard et al., 2009; Wu et al., 2012; He et al., 2015), and oxidative status (He et al., 2011, 2013a,b).

Plants survive stressful conditions by employing three main strategies: escape (*i.e.* escaping the stress, *e.g.*, short life cycle limited to the wet season in Mediterranean environments), tolerance and survival. The tolerance strategy involves different mechanisms that enable the plant to tolerate and grow in the presence of the stress (Lawlor, 2013). By contrast, the survival strategy is a form of stress resistance by which cells, tissues, and organs can cease growing in presence of the stress, while maintaining key cellular functions, which enable them to recover rapidly, with minimal damage, once the stress has receded (*cf.* Lawlor, 2013). This kind of survival has also been termed quiescence, and examples can be found in plants exposed to long periods of drought and salinity (Granot et al., 2009; Julkowska and Testerink, 2015) or in flooded plants (Bailey-Serres and Voesenek, 2008; Colmer et al., 2009; Luo et al., 2011; Bailey-Serres et al., 2012; Sasidharan and Voesenek, 2013). This inherent flexibility of plant in response to a changing environment has been related to the ability of the epigenetic status to alter rapidly and reversibly (Luo et al., 2012). In response to external signals (*e.g.*, stress), plants activate specific signaling pathways that alter physiological reactions and transcription rates of responding genes. Chromatin status, which is governed by pattern and quantity of histone modifications that alter DNA–histone interaction and accessibility of transcription factors, has been implicated in the regulation of eukaryotic gene activity (Chinnusamy and Zhu, 2009; Kim et al., 2008). In particular the primary level controlling gene expression is the structure of chromatin, and modifications of the histone H3 at lysine 4 (H3-K4) have been associated with the transition from quiescent into active state as they dictate either gene silencing or gene activation (Tsuji et al., 2006; Granot et al., 2009).

Plants have formed the basis of traditional medicine for thousands of years and, while in developing countries a large proportion of the population relies heavily on medicinal plants to meet primary health care needs, increased scientific interest and consumer demand have boosted their use also in developed countries in the past decades (Gurib-Fakim, 2006; Annan et al., 2013; Barthwal et al., 2008). Therefore, in parallel to the increased use of medicinal plants, concerns regarding the safety of their use have risen as they have been recognized as an important source of heavy metal toxicity to both humans and animals and a prolonged consumption of such medicinal plants may be detrimental to health (Dwivedi and Dey, 2002; Haider et al., 2004; Lux et al., 2011a,b). Indeed, despite plants do not require Cd for growth or reproduction, the bioaccumulation index of Cd in plants exceeds that of all other trace elements (Grant et al., 1998), and, due to its high toxicity, the World Health Organization has set the maximum permissible limit of Cd in medicinal plants to 0.3 $\mu g\, g^{-1}$ dry mass (World Health Organization, 1998).

Tetradenia riparia (Hochst.) Codd belongs to the Lamiaceae family and is a widespread aromatic shrub with a height of approximately 1–3 m occurring throughout eastern and tropical Africa (Gazim et al., 2014). *T. riparia* occupies a wide range of ecologically contrasting habitats but is often found on hillsides and river banks (Gairola et al., 2009). The family Lamiaceae is rich in aromatic species, which are used as culinary herbs, folk medicines and fragrant scents (Gairola et al., 2009), and in particular *T. riparia* is a well-known herbal medicine and has traditionally been used in the treatment of various illnesses including coughs, dropsy, fever, and malaria (Gairola et al., 2009; Gazim et al., 2011). Despite being widely used, to date virtually nothing is known about its response to abiotic stress and its potential and safe use in marginal soils (*i.e.* in areas with a possible pollution of soils by heavy metals) has yet to be evaluated. Henceforth, with the present study we aimed to evaluate the physiological responses of *T. riparia* to the addition of Cd in the growing media. Therefore, following the observation that *T. riparia* exhibited the survival strategy response, we tested the following hypotheses: (i) the quiescent response and the prolonged survival of *T. riparia* to Cd stress is associated with low Cd concentrations in shoot tissues; (ii) the Cd-induced quiescence enables plants to recover rapidly, with minimal damage, once the stress has receded.

2. Materials and methods

2.1. Plant material

Rooted plants were established from cuttings (3–4 internodes) taken from *T. riparia* plants grown in a naturally lit glasshouse for 1 year in standard potting mix. The cuttings were propagated by inserting them in aerated water for 3–4 weeks in the same glasshouse (with average day/night temperatures during the experimental period of 35/25 °C and an average humidity of 76% in the two experiments). Rooted cuttings were then transferred to an aerated nutrient solution, which contained: 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.50 mM $MgSO_4$, 40 μM Fe(Na)-EDTA, 1 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.1 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$, and buffered with 1 mM MES. The pH of the solution was adjusted to 5.8 using KOH. Solutions were changed weekly. Two months after transferring the cuttings to the aerated nutrient solution, plants were selected for shoot and root uniformity, and $CdSO_4$ was added to the nutrient solutions to obtain the required final concentrations.

2.2. Physiological responses of T. riparia to different concentrations of $CdSO_4$ (Expt 1)

Experiment 1 (Expt 1) consisted of three treatments with five replicates in a completely randomized block design, where plants were exposed to increasing Cd concentrations: 0 μM $CdSO_4$, considered as the control treatment, and 30 and 150 μM $CdSO_4$. Plants were then harvested 5 weeks after imposing the treatments.

2.3. Recovery of T. riparia following Cd stress (Expt 2)

In the second experiment (Expt 2) plants were grown for 5 weeks with a solution containing 150 μM $CdSO_4$ and subsequently were transferred in aerated water to assess recovery in the following 3 weeks (*i.e.*, only the root system and the basal portion of the stem bathing in the Cd-free media). In addition, as in the first experiment we observed that Cd strongly induced the formation of root primordia in the top internodes of the stems, cuttings (with all leaves removed except the pair of leaves on the top internode) from the top 3–4 internodes of the stems of control and treated plants were inserted in aerated water to assess leaf and root formation for the following 3 weeks.

2.4. Leaf and root elongation and leaf sampling (Expt 1 and 2)

In Expt 1, leaf length was measured every 2–4 days for the duration of the experiment to determine its extension during the treatment period. At different time points (1, 7, 14 and 35 days), young fully expanded leaves (one for each plant in all treatments) were collected between 11.00 h and 11.30 h for subsequent analyses of ions, total soluble sugars and leaf pigments. These

leaf tissues were snap-frozen in liquid N_2, stored at −80 °C, freeze-dried, and then stored at −20 °C. In addition, the relative water content (RWC) of the leaves was measured using the following formula RWC = (FM − DM)/(TM − DM) × 100, where FM is leaf fresh mass, DM is leaf dry mass after the leaves were dried at 65 °C for 3 d, and TM is the turgid mass after leaves were soaked in water for 4 h at room temperature.

In Expt 2, root number, root and leaf length were measured every 2–4 days for the entire duration of the experiment to determine their extension during the treatment period.

2.5. Leaf gas exchange parameters (Expt 1 and 2)

At different time points in both experiments, net photosynthetic rate and stomatal conductance were calculated on young fully expanded leaves in each treatment, using the open gas exchange system Li-6400 (LiCor Inc., Lincoln, NE, USA) as described in Bazihizina et al., 2015. Briefly leaf gas exchange measurements were taken on four plants from each treatment at ambient relative humidity (50–60%), ambient CO_2, flow rate of 400 μmol s^{-1}, leaf chamber temperature at 30 °C and photosynthetically active radiation of 300 μmol m^{-2} s^{-1}.

In Expt 1, using the integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc.) of the open gas exchange system Li-6400 (LiCor Inc., Lincoln, NE, USA) chlorophyll fluorescence was measured on the same leaves used for gas exchange measurements. Maximum quantum yield (F_v/F_m) was determined after a 30 min dark acclimation of selected leaves using a dark leaf clip. Actual quantum yield (Φ_{PSII}) was measured on light adapted-leaves and was calculated as $\Phi_{PSII} = (F_{m'} - F_s)/F_{m'}$, which measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry (Maxwell and Johnson 2000). Fluorescence parameters were also used to calculate the non-photochemical quenching (NPQ = $(F_m - F_{m'})/F_{m'}$; Redondo-Gomez et al., 2006), which is linearly related to heat dissipation (Maxwell and Johnson 2000). ETR was calculated as ETR = $\Phi_{PSII} \times PFDa \times 0.5$, where PFDa is absorbed light in μmol photon m^{-2} s^{-1} and 0.5 is a factor that accounts for the partitioning of energy between PSII and PSI (Maxwell and Johnson 2000).

2.6. Plant harvest (Expt 1)

In Expt 1, plants were sampled at the beginning of the experiment and 5 weeks after applying the treatments for the determination of shoot and root fresh and dry mass. Roots were carefully washed three times and each plant was separated into roots, leaves and stems and their fresh mass was recorded. Tissue samples were then oven-dried at 60 °C for 1 week to determine their dry mass.

2.7. Leaf pigment analyses (Expt 1)

Cold 100% methanol was added to 10–20 mg of the ground freeze-dried tissues and the samples were incubated in darkness, on ice. After 30 min, samples were centrifuged at 9300*g* for 10 min at 4 °C, the supernatants were removed and their absorbance was determined at 470, 665.2 and 652.4 nm, using a Tecan Infinite 200 Spectrophotometer (Männedorf, Switzerland). Total chlorophyll and carotenoid concentrations were calculated using the equations from Wellburn (1994).

2.8. Determination of K and Cd concentrations in plants (Expt 1)

Potassium concentrations in dry plant tissues were obtained after digesting of ground tissues in 0.5 M HNO_3 by shaking vials for 48 h in dark at 25 °C. Diluted extracts were analysed for K concentrations (Digiflame DV710, NT Laboratory). Cd concentrations in dry plant tissues were obtained following a digestion in a mixture of concentrated HNO_3 and $HClO_4$ (2:1 v.v., Sigma-Aldrich, Italy) using a digester (VELP Scientifica, Italy). Cd concentrations were then determined with an inductively coupled plasma-optical emission spectrometer (ICP-OES, OPTIMA 2000 DV, PerkinElmer, USA). The ICP analytical standard (AA/ICP calibration/check standards for environmental analyses, 1 g L^{-1}) for Cd was supplied by Sigma-Aldrich.

2.9. Acid extraction of proteins and immunoblotting (Expt 1)

Leaves of 5 weeks treated plants of *T. riparia* were ground using a T25 Ultra-Turrax (Ika Labortechnik) in an extraction buffer (100 mM Tris–HCl pH 7,5, 2 mM EDTA 5 mM 2-Mercaptoethanol 2 mM Phenylmethylsulfonyl fluoride, HCl 0.25 M) supplemented with a protease inhibitor cocktail (Sigma-Aldrich St. Louis, MO product number P9599). The acid soluble proteins were TCA-Aceton precipitated and protein concentration estimated with the Bradford Assay (Bio-Rad Hercules CA product number 500-0006). Acid soluble proteins (20 μg) were separated in 15% SDS-PAGE and stained with Comassie brilliant blue R (Merck Darmstadt) or blotted on nitrocellulose paper (Bio-rad Hercules CA product number 162-0115). The following two different antibodies were probed to evaluated the dimethylation of H3: abH3 (Sigma-Aldrich St. Louis, MO product number H0164) for immunodetection of total histon3 fraction and anti-dimethylated H3K4 (Sigma-Aldrich St. Louis, MO product number D5692). Immunodetection was performed using secondary antibody of goat anti-rabbit horseradish peroxidase conjugate (Sigma-Aldrich St. Louis, MO product number A0545). To strip the nitrocellulose a stripping buffer composed by 62.5 mM Tris–HCl pH 6.7, 100 mM 2-Mercaptoethanol 2% SDS was used, and after the stripping, the nitrocellulose paper was incubated with secondary antibody of goat anti-rabbit horseradish peroxidase conjugate to evaluate background signal.

2.10. Statistical analyses

Statistical analyses were conducted using GraphPad for Mac 6th Edition, and analysis of variance (ANOVA) was used to identify overall significant differences between treatments. Tukey *post-hoc* test was used for a posteriori comparison of individual means (with at least $P < 0.05$ as significant level).

3. Results

3.1. Plant growth (Expt 1)

Increasing concentration of Cd in the nutrient solutions reduced plant growth, with substantial declines in plant dry mass increments and leaf elongation (Table 1 and Fig. 1). For example, with 150 μM $CdSO_4$ there was no net dry mass increment for both shoots and roots. The used Cd concentrations resulted toxic for root

Table 1
Net dry mass increments in *Tetradenia riparia* in response to different concentration of $CdSO_4$ in the culture media (Expt 1). Values are mean ± S.E. (n = 5). Different lower case letters in each column indicate significant differences between control and treated plants. Initial leaf, stem and root dry mass were respectively (g): 7.1 ± 0.6; 20.5 ± 3.1; 4.8 ± 0.4.

$CdSO_4$ treatment	Net dry mass increment (g plant^{-1})		
	Leaves	Stems	Roots
0	26.8 ± 3.3a	21.1 ± 7.2a	13.4 ± 4.2a
30	5.3 ± 0.5b	3.0 ± 2.2b	3.0 ± 0.8b
150	2.3 ± 0.5c	1.6 ± 1.6b	0.0 ± 0.9b

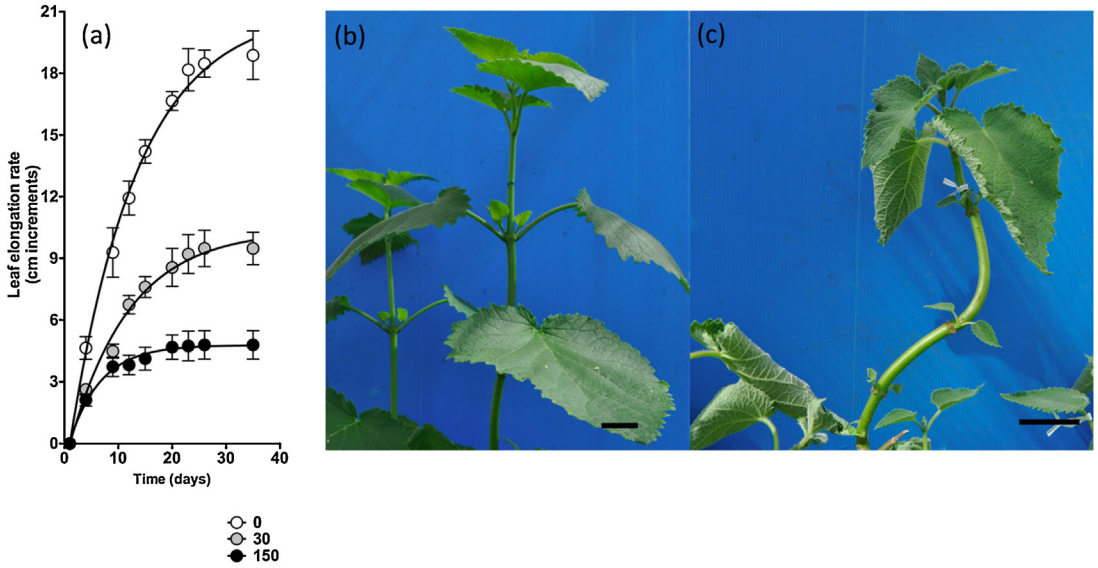

Fig. 1. Response of *Tetradenia riparia* to increasing concentration of $CdSO_4$ in the culture media (Expt 1): (a) leaf elongation; (b) example of a stem of a plant grown under control conditions (scale bar, 5 cm); (c) example of a stem of a plant exposed to 150 μM CdSO4 for 5 weeks (scale bar, 5 cm). In (a) values are mean ± S.E. (n = 5).

growth (Table 1), and with 150 μM $CdSO_4$ the entire plant root system was completely necrotic (Fig. S1). As for plant dry mass, leaf elongation was strongly inhibited in time (more than 60% declines in both Cd treatments, Table S1) by the stress in all Cd treatments, although the reductions were accentuated with 150 μM $CdSO_4$ (Fig. 1a). Cadmium immediately affected plant water relations, and one day after applying the treatments, independently from the Cd concentrations, relative water content (RWC) of the youngest expanded leaves declined to almost 50% compared to that in control plants (Fig. S2a). However, with time, in plants treated with 30 μM $CdSO_4$, RWC recovered to values similar to those of control plants, meanwhile in plants exposed to 150 μM $CdSO_4$ RWC was at most times lower than that in control plants.

3.2. Gas exchange and chlorophyll fluorescence (Expt 1)

In all times considered, net CO_2 assimilation and stomatal conductance were reduced by Cd treatments (Fig. 2). One day after the treatments stomatal conductance decreased by 60 and 80% in plants exposed to 30 and 150 μM $CdSO_4$, respectively, and in both cases values never returned to those measured in control plants. Leaf photosynthetic activity had a similar trend to the one observed for stomatal conductance.

Chlorophyll fluorescence results showed that the maximum quantum efficiency of PSII was not affected by Cd treatments throughout the entire experimental period (Table 2, Fig. S3). However, although after one day of treatment, for all Cd concentrations considered there were no significant changes in

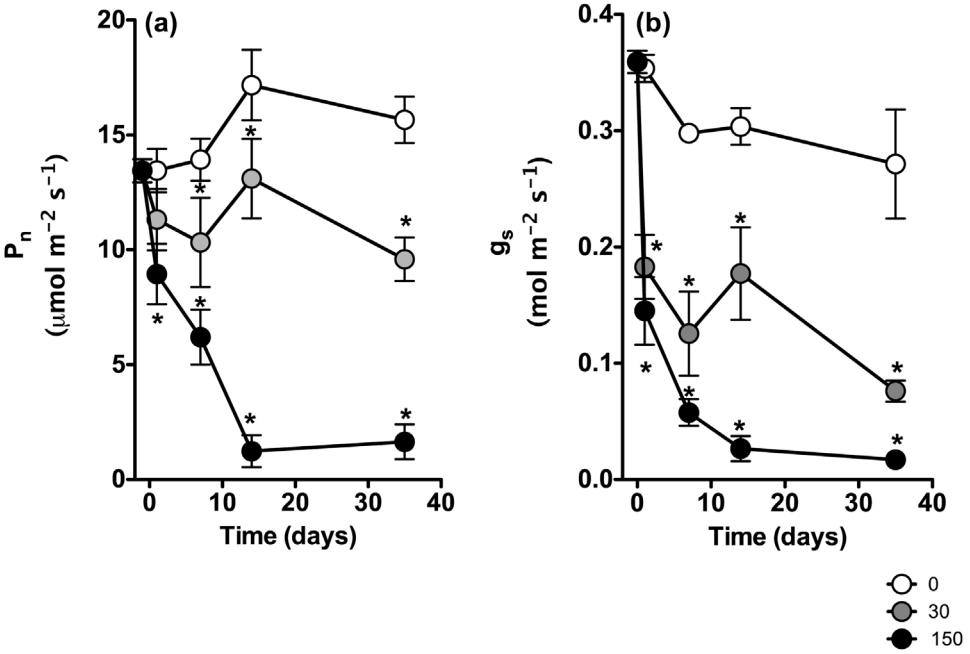

Fig. 2. Net CO_2 assimilation (a) and stomatal conductance (b) in time in plants grown with control solution or 30 and 150 μM $CdSO_4$ (Expt 1). Values are mean ± S.E. (n = 5). Asterisks indicate significant differences between control and Cd treated plants. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2
Chlorophyll fluorescence parameters and total chlorophyll and carotenoid concentrations in young leaves of *Tetradenia riparia* exposed to increasing concentration of CdSO4 in the culture media (Expt 1). Values are mean ± S.E. (n = 5). Different lower case letters in each row indicate significant ($P < 0.05$) differences between control and treated plants. Fv/Fm, maximum potential photosystem II efficiency; ETR, electron transport rate; ΦPSII, actual photosystem II efficiency; NPQ, non-photochemical quenching.

Parameter	$CdSO_4$ treatment (μM)		
	0	30	150
F_v/F_m	0.93 ± 0.00a	0.91 ± 0.02a	0.90 ± 0.02a
ETR (μmol $e^- m^{-2} s^{-1}$)	93.48 ± 3.74a	75.13 ± 4.33b	35.59 ± 8.79c
Φ_{PSII}	0.18 ± 0.01a	0.14 ± 0.01b	0.11 ± 0.01c
NPQ	1.59 ± 0.04a	2.04 ± 0.20b	3.16 ± 0.36c
Total chlorophyll (μg mg^{-1} dry mass)	8.51 ± 0.62a	8.68 ± 0.57a	6.10 ± 0.51b
Carotenoid (μg mg^{-1} dry mass)	1.52 ± 0.10a	1.71 ± 0.11a	0.96 ± 0.07b

Table 3
Cd and K concentrations in leaves, stems and roots of *Tetradenia riparia* exposed to increasing concentration of $CdSO_4$ in the culture media (Expt 1). Values are mean ± S.E. (n = 5). Different lower case letters in each row indicate significant differences between control and treated plants. nd, not detectable.

Parameter	$CdSO_4$ treatment (μM)								
	Leaves			Stems			Roots		
	0	30	150	0	30	150	0	30	150
Cd (μmol g^{-1} dry mass)	nd	0.03 ± 0.01a	0.07 ± 0.01c	nd	0.06 ± 0.01a	0.12 ± 0.01b	nd	8.55 ± 2.62a	23.94 ± 3.47b
K (μmol g^{-1} dry mass)	1120 ± 25a	1177 ± 35a	994 ± 71a	1093 ± 132a	1009 ± 99a,b	902 ± 38b	854 ± 32a	498 ± 53b	533 ± 76b

all measured chlorophyll fluorescence parameters, in time there was a progressive decline in the actual quantum yield of PSII and in the electron transport rate, associated with a progressive increase in the non-photochemical quenching (Fig. S3). For example, with 150 μM $CdSO_4$, at the end of the 5 weeks of treatments, Cd stress resulted in a 60% and 40% declines in ETR and Φ_{PSII}, respectively, and a 2-fold increase in NPQ compared with control plants (Table 2).

3.3. Total chlorophyll and carotenoid concentrations in youngest expanded leaves (Expt 1)

For the first 14 days after treatment, for all Cd concentrations considered there were no significant declines in both total chlorophyll and carotenoid concentrations in the youngest expanded leaves (Fig. S4). However, 5 weeks of treatment with 150 μM $CdSO_4$ resulted in 27–37% declines in total chlorophyll and carotenoid concentrations. By contrast, 30 μM $CdSO_4$ did not affect the concentration of these two leaf photosynthetic pigments (Table 2).

3.4. Cd and K concentrations (Expt 1)

In all Cd treatments, Cd accumulation was limited in stems and especially in leaves in comparison to roots (Table 3). Independently from the Cd concentration in the culture media, Cd concentrations in both leaves and stems remained within or close 0.1 μmol Cd g^{-1} dry mass. At the end of the experiment, for both Cd treatments, root K concentration decreased as compared with control plants. In both leaves and stems, K concentrations did not vary between

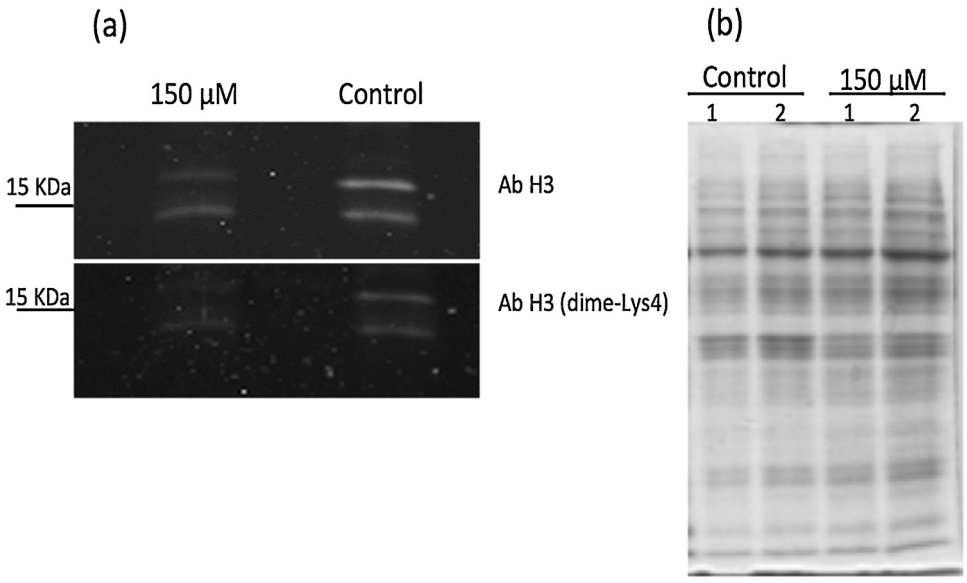

Fig. 3. Loss of histone H3 de-dimethylation at lysine 4 in *Tetradenia riparia* after exposure for 5 weeks to 150 μM $CdSO_4$. (a) Wstern Blot analysis on 20 μg of acidic soluble extracts from treated and control leaves, shows that Histone H3 lacks dimethylation in lysine 4 under Cadmium treatment. (b) Gel loading control: two different amount of protein (10 and 20 μg) were loaded in the gel and then stained with Coomassie brilliant blue.

Table 4
Number of stems with preformed root primordia, recovery rate, time required for the emergence of root primordia and new adventitious roots formation (scored with a visual evaluation) in Cd-treated plants transferred into a Cd-free media and in cuttings taken from control and Cd-treated plants and placed into a Cd-free media for 3 weeks. Prior to the recovery treatment, plants were either grown in a control solution or in a solution with 150 μM $CdSO_4$ for 5 weeks. Values are mean ± S.E. (n = 5). Different lower case letters in each column indicate significant differences between treatments. nd, not detected as root primordia were visible only on the soft-wood stems. * root primordia were already visible prior to the recovery treatment.

		Stems with visible root primordia (%)	Recovery rate (%)	Root primordia visible (days from recovery)	Emergence new roots (days from recovery)
Control plants	Cuttings	9 ± 6a	100	6.5 ± 0.2a	9.7 ± 0.4a
Cd-treated plants	Entire plants	nd	80	5.0 ± 1.7a	10.2 ± 3.6a
	Cuttings	88 ± 10b	100	0b*	1.2 ± 0.2b

control plants and plants treated with 30 μM $CdSO_4$ (Table 3), whereas at 150 μM $CdSO_4$, leaf and stem K concentrations decreased by approximately 10–15% as compared with concentrations in control plants.

3.5. Histone post translational modifications following Cd stress (Expt 1)

The dynamics of histone modifications after 5 weeks of $CdSO_4$ treatments were analyzed, focusing on the demethylation of lysine 4, as this modification has previously been associated in plants with decondensed, transcriptionally active chromatin (Pfluger and Wagner 2007; Granot et al., 2009). Compared with the control plants, exposure for 5 weeks to 150 μM $CdSO_4$ led to the loss of the demethylation in lysine 4 (Fig. 3) meanwhile this drastic change did not occur in plants treated with 30 μM $CdSO_4$ (Fig. S5). Moreover, we loaded on the SDS-PAGE 10 and 20 μg of acidic extracts and then performed a Comassie Brillant Blue staining (Fig. 3b), in order to confirm the estimation of proteins through Bradford Assay.

3.6. Growth recovery and adventitious root formation following Cd stress (Expt 2)

Cd stress strongly induced the initiation of root primordia on the stem of treated plants, resulting in the formation, after 2–3 weeks of treatment, of several primordia, mostly in the first few internodes of the stems of Cd-treated plants (Table 4 and Fig. 4). Therefore, once cuttings taken from these top internodes from Cd-treated plants were transferred in a Cd-free media, these new root primordia quickly developed into adventitious roots, despite their activation and development in adventitious roots was strictly local, and primordia that remained dry never got activated. Indeed, in Cd-treated cuttings, adventitious root growth occurred very quickly, with visible roots already after 24 h of recovery treatment meanwhile in control plants root primordia initiation and subsequent adventitious root formation occurred on average only after 6–10 days (Table 4 and Figs. 4 and 5).

In the recovery experiment, most plants previously exposed to Cd (80% of all plants in the recovery experiment and 100% of all plants for the cutting experiment, see Table 4) were able to form

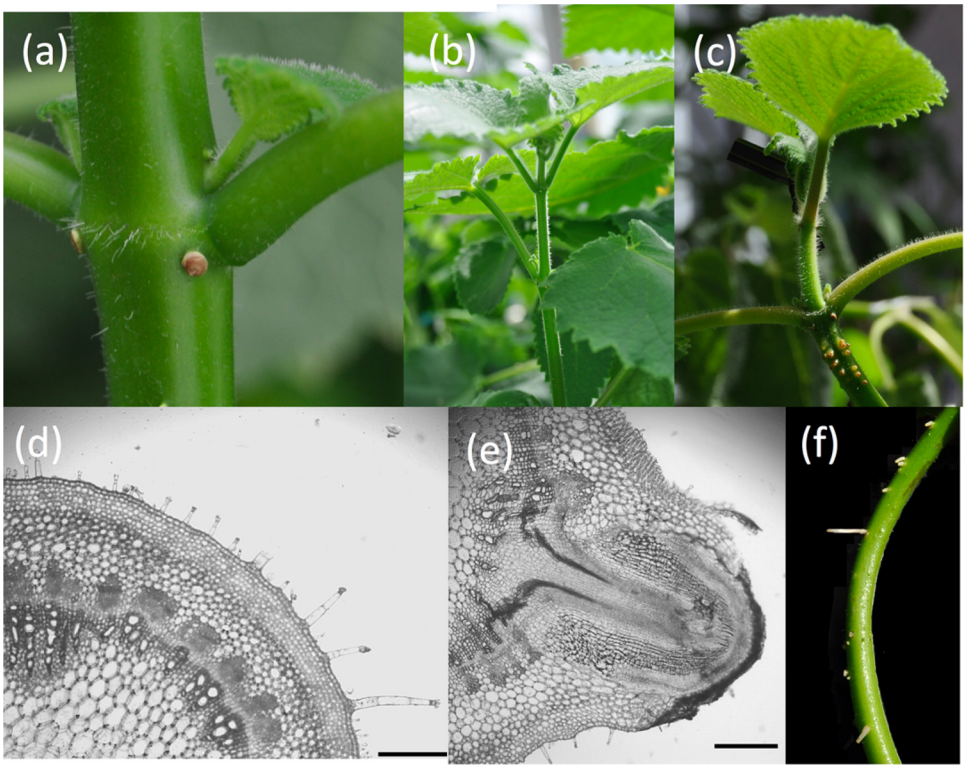

Fig. 4. Root primordia and adventitious roots in stems of *Tetradenia riparia* plants (Expt 2). (a) Constitutively preformed root primordia present on all internodes in all plants, which however did not always hasten into adventitious root formation; (b) typical stem of a control plant; (c) Cd induced initiation of new root primordia on the stem of a plant grown under Cd stress; (d) section of a stem of *T. riparia* without root primordia (scale bar, 200 μm); (e) section of a root primordia on a stem of a Cd-treated plant (scale bar, 200 μm); (f) the Cd-induced root primordia developed in 24–48 h into adventitious roots when in a Cd-free media.

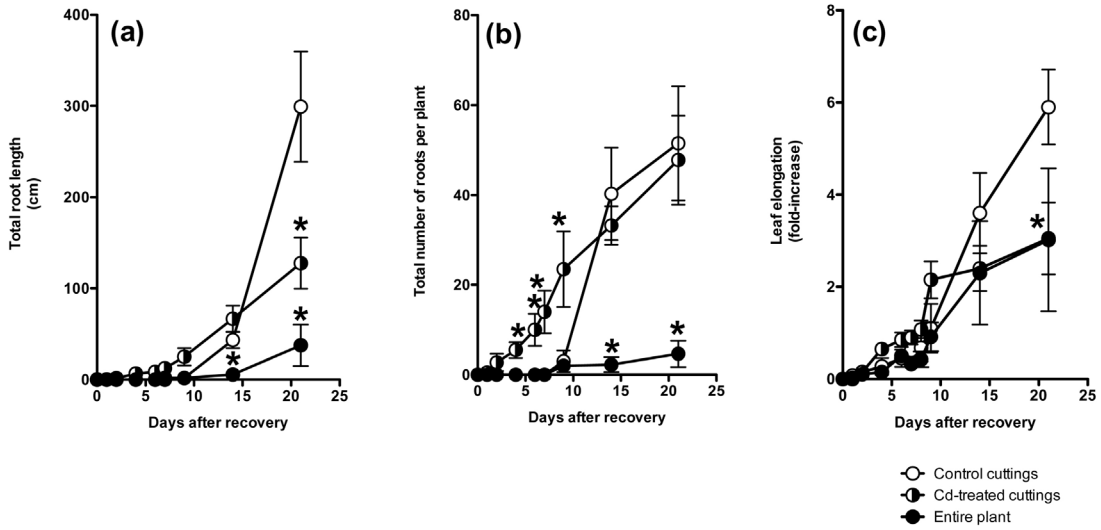

Fig. 5. Recovery of plants previously exposed to 150 μM $CdSO_4$ and of cuttings (first 3–4 internodes of the stems) of *Tetradenia riparia* plants previously exposed to a control solution or 150 μM $CdSO_4$ and then transferred to a Cd-free media: (a) total root length; (b) number of main adventitious roots; (c) leaf elongation rate. Cuttings from control plants were taken to evaluate their recovery speed and rate. Values are mean ± S.E. (n = 5). Asterisks indicate significant differences between cuttings taken from control plants and cuttings taken from Cd treated plants. $^*P < 0.05$.

new roots and leaves once placed in a Cd-free media, as shown by the progressive increase in leaf elongation, leaf photosynthetic activity and stomatal conductance (Figs. 5 and 6, Table S2). In particular, when cuttings were taken from plants previously treated with 150 μM $CdSO_4$, leaf photosynthetic activity increased from an average value of 0.3 to an average value of 5.6 μM CO_2 $m^{-2}\,s^{-1}$ during the 3-week recovery period (Fig. 6a). Despite the observed increases in leaf elongation and photosynthetic activity in cuttings previously treated with Cd, 3 weeks after the recovery treatment, leaf and root lengths of cuttings from control plants were approximately double than those of cuttings from treated plants (Fig. 5). When considering the recovery of the entire plants (*i.e.*, with only the root system and the basal portion of the stem bathing in the Cd-free media), recovery was significantly slower, as the initiation of root primordial in Cd-treated plants occurred mainly in the first few internodes of the stems, and adventitious root formation was seen on average only 10 days after the recovery treatment (Table 4, Fig. 5).

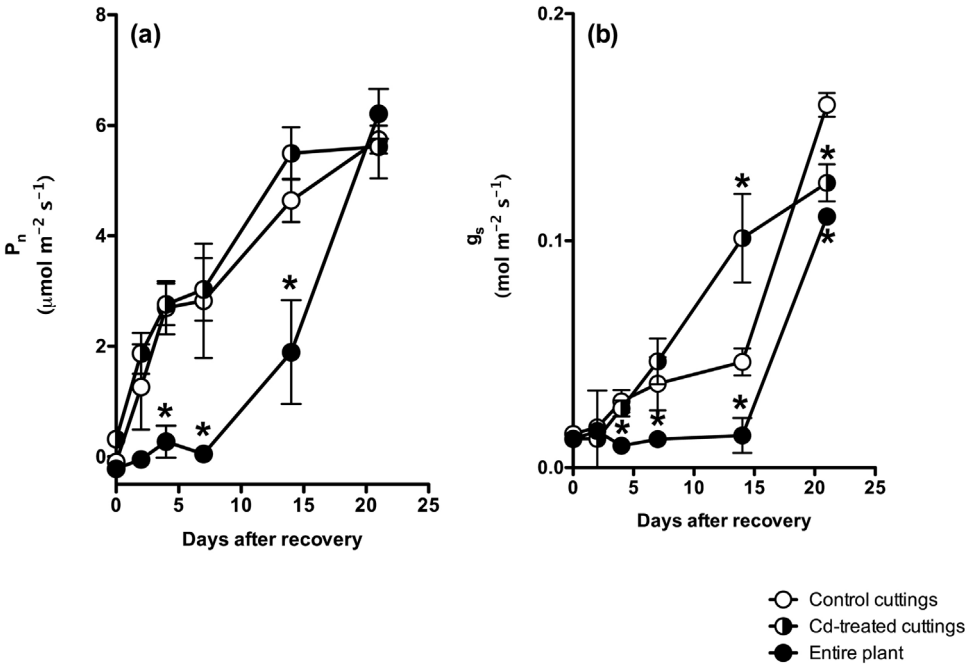

Fig. 6. Net CO_2 assimilation (a) and stomatal conductance (b) in time measured in the leaves of plants previously exposed to 150 μM $CdSO_4$ and in leaves of cuttings (first 3–4 internodes of the stems) of plants previously exposed to control or 150 μM $CdSO_4$ and subsequently transferred into a Cd-free media. Cuttings from control plants were taken to evaluate their recovery speed. Values are mean ± S.E. (n = 5). Asterisks indicate significant differences between cuttings taken from control plants and cuttings taken from Cd treated plants. $^*P < 0.05$. Net CO_2 assimilation and stomatal conductance in control and Cd-treated plants in the considered period were, respectively: 9.97 ± 0.21 and −0.33 ± 0.11 (μM CO_2 $m^{-2}\,s^{-1}$); 0.336 ± 0.003 and 0.004 ± 0.000 (mol H_2O $m^{-2}\,s^{-1}$).

4. Discussion

The quiescence strategy has been considered pivotal in prolonging plant survival under stressful conditions and to improve survival rate and generation of new tissues after the stress has receded (Greenway and Gibbs 2003; Fukao and Bailey-Serres, 2004; Bailey-Serres and Voesenek, 2008; Colmer et al., 2009; Luo et al., 2011). We observed that, following Cd stress, *T. riparia* adopted a quiescence-like response and there was an enhanced formation of adventitious root primordia in the stems. We therefore subsequently tested and confirmed the following hypotheses: (i) the quiescent response and the prolonged survival of *T. riparia* to Cd stress is associated with low Cd concentrations in shoot tissues; (ii) the Cd-induced quiescence enables plants to recover rapidly, with minimal damage, once the stress has receded; and (iii) root primordia will quickly develop into adventitious roots in more favourable conditions, therefore enabling the plants to rapidly exploit new environmental opportunities.

Cadmium affected *T. riparia* growth in a dose dependent manner, and 150 μM $CdSO_4$ quickly arrested leaf elongation rates and resulted in widespread necrosis and eventually death of the entire root system after 5 weeks of treatment (Fig. S1). This strong reduction in the root growth, and subsequent root death, with 150 μM $CdSO_4$ could be directly linked with the large accumulation of Cd in root tissues, as already observed for other species (*e.g.*, Perfus-Barbeoch et al., 2002). Cadmium concentrations were found to be several folds higher in roots than in shoots, with concentrations up to 241 μmol g^{-1} dry mass. By contrast, Cd concentrations in shoots remained within or close to 0.1 μmol g^{-1} dry mass, a clear indication of restricted accumulation of Cd in shoots. However, with both 30 and 150 μM $CdSO_4$, Cd concentrations in leaves and stems exceeded by far the limit for maximum Cd levels for medicinal plants (0.3 μg g^{-1} dry mass, *i.e.* 0.003 μmol g^{-1} dry mass), indicating that this species is not suitable as a medicinal plant when cultivated in substrates with even moderate Cd concentrations. Nevertheless, this exclusion of Cd from the above-ground tissues was sufficient to avoid the irreversible damage of these tissues, and shoots of treated plant remained alive, as clearly shown by the fast resumption of photosynthesis and leaf elongation once the stress receded.

Cadmium stress quickly affected shoot water relations, as shown by the substantial declines in leaf relative water content and stomatal conductance in the first 24 h of treatments. Indeed, as declines in leaf photosynthetic rate were paralleled by declines in stomatal conductance and C_i (Fig. S2b), these results support the view that photosynthesis was initially limited by CO_2 diffusion as a consequence of low leaf stomatal conductance (Medrano et al., 2002). This result is in accordance with the increasing evidence suggesting that exposure to toxic metal concentrations initially affects plant water relations (Barcelo and Poschenrieder, 1990; Sagardoy et al., 2010; Bazihizina et al., 2014). For instance, in *Arabidopsis thaliana*, exposure to 50–100 μM Cd resulted in a progressive decline in leaf stomatal conductance, with a complete stomatal closure 4–6 days after the treatments (Perfus-Barbeoch et al., 2002). Several factors could explain these initial declines in stomatal opening following Cd stress. Cadmium has been found to cause strong depolarization of root cortical cells, down to values within the range of the diffusion potential (Sanz et al., 2009). This altered root membrane functionality could explain the rapid declines in stomatal conductance as it is now well established that changes at the root level can quickly, through rapid systemic long-distance signals, regulate stomatal behaviour (Comstock 2002; Christmann et al., 2007; Choi et al., 2014). In alternative, it has been suggested that Cd can enter the guard cells through calcium channels thus affecting guard cell regulation and stomatal opening (Perfus-Barbeoch et al., 2002). However, given the observed responses in *T. riparia*, this second hypothesis seems less likely as Cd concentrations measured in shoots was low, even after 5 weeks of treatments. Nevertheless, from the seventh day on, prolonged Cd stress led substantial declines in the actual efficiency of PSII and ETR and increases in non-photochemical quenching, which coincided with great reductions in CO_2 assimilation rates. This would indicate that, although initially decreased photosynthesis was associated with declines in stomatal conductance, subsequently Cd impaired leaf photosynthetic machinery. Indeed, although the type of damage varies based on the species used and the environmental contingencies (*e.g.*, Krupa et al., 1993; Küpper et al., 2007), photosynthetic reactions belong to the most important sites affected by Cd, with PSII often identified as one of the main target (Küpper et al., 2007).

The hypothesis that Cd induced a quiescent-like response in *T. riparia* was further confirmed by the histone H3 posttranslational modifications, which resulted in the loss of the methylation of Lys4 of histone H3 (H3-K4) after Cd stress. Indeed, histone modifications, such as methylation and acetylation, in the chromatin surrounding genes are thought to regulate transcriptional activity and gene expression, with the methylation of H3-K4 generally associated with transcriptionally active chromatin (Pfluger and Wagner 2007; Granot et al., 2009). As plants are sessile organisms it has been shown that gene expression is dynamically controlled in response to the appearance or disappearance of an environmental stress through reversible and dynamic changes in histone modification (Tsuji et al., 2006), and declines in dimethylation of H3-K4 have been linked with the entry of the plants into a quiescent state during stressful periods (Granot et al., 2009).

Adventitious roots develop ectopically from aboveground organs and form naturally from stem tissue and/or under stressful environmental conditions (*e.g.* flooding, drought or salt stress, Wang et al., 2009; Liao et al., 2012; Steffens and Rasmussen, 2016) to replace the existing root system that has either been killed or whose function is impaired by the stress (Pezeshki 2001; Dawood et al., 2014). In particular, despite being phytotoxic, heavy metal stress has been found to induce specific 'stress-induced morphogenic responses' (cf. Potters, 2007; Xu et al., 2011), including the enhanced formation of adventitious roots (Gad and Atta-Aly, 2006). In agreement with these findings, our data show that high Cd stress leads to characteristic stress-induced morphogenic responses: a reduction in primary root growth and, at 150 μM $CdSO_4$, a concomitant induction of root primordia formation in most stems (Fig. 4). As reported for *Solanum dulcamara* under flooded conditions (Dawood et al., 2014), root primordia activation and development into adventitious roots was local, as primordia that remained dry never developed into a root (data not shown). These Cd-induced root primordia enabled a much faster resumption of plant growth (root and leaf elongation and leaf photosynthetic activity) compared with control cuttings and treated plants that had only the primordia-free portion of the stem exposed to the Cd-free, where *de novo* initiation of root primordia was required (Table 4). This result can be explained by considering that in cuttings previously treated with Cd, during the first week of recovery, leaf photosynthetic activity was correlated with root elongation rather than leaf growth (Table S3). It would therefore be reasonable to expect that carbohydrate retained or newly synthesized in leaves after recovery were initially used for a quick adventitious root growth. Subsequently, from the second week of recovery, there was a change in the trend, and leaf photosynthetic activity was correlated mainly with the relative leaf elongation, which would suggest a shift in photoassimilate partitioning.

5. Conclusion

Based on the remarkable survival capacity of *T. riparia* under Cd stress and the growth resumption under optimal conditions, we bring forward the hypothesis that this species adopted the quiescence strategy in response to Cd stress. Indeed the response of *T. riparia* to Cd stress was associated with: (a) a 'quiescence response', *i.e.*, no biomass accumulation, associated with histone H3 modification; (b) a limited Cd accumulation in shoot tissues; and (c) the induction of root primordia. What might be the physiological and ecological significance of the observed responses in *T. riparia* in a Cd-rich environment? It has been suggested that both quiescence and stress induced morphogenic responses in general are a mechanism for stress evasion (Potters, 2007). Given that *T. riparia* is not a metallophyte, and by definition adaptive responses to Cd could not have evolved under natural selection, the response described in the present study are likely to represent an inherent general purpose response of *T. riparia* to transient adverse growth conditions, which enables plants to quickly resume normal growth in more favourable conditions, increasing plants' ability to rapidly exploit new environmental opportunities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.05.006.

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