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ORIGINAL PAPER

Seedlings of medicinal plants treated with either a cytokinin antagonist (PI-55) or an inhibitor of cytokinin degradation (INCYDE) are protected against the negative effects of cadmium

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Abstract Cytokinins are plant hormones that regulate diverse processes during plant growth and development. Targeted manipulation of their metabolism or perception has already shown benefits in agriculture. Recently, we described substances that can affect plants' endogenous cytokinin status: a cytokinin antagonist, PI-55, and an inhibitor of cytokinin degradation, INCYDE (2-chloro-6-(3-methoxyphenyl)aminopurine). The effects of these substances on seedling growth in the medicinal plant *Bulbine natalensis* Baker (Asphodelaceae) and the metal tolerant *Rumex crispus* L. (Polygonaceae) under abiotic stress caused by cadmium (Cd) were tested. Cd is known for its negative effects on plant growth and its toxicity to humans. Treatment with either PI-55 or INCYDE had positive effects on seedling shoot and root growth and the fresh weight of treated seedlings grown in the presence of Cd. Even a single application of either compound at submicromolar concentrations was sufficient to reverse the inhibitory effects of Cd. Our results demonstrate that modulating cytokinin status with inhibitors of cytokinin perception and/or degradation may be useful in protecting plants against the adverse effects of high Cd levels.

Keywords Cytokinin · Heavy metals · Cadmium · Cytokinin antagonist · Cytokinin degradation

Abbreviations

ABA	Abscisic acid
AHK	*Arabidopsis thaliana* histidine kinase
BA	N^6-benzyladenine
CKX	Cytokinin oxidase/dehydrogenase
DMSO	Dimethylsulfoxide
INCYDE	Inhibitor of cytokinin degradation
JA	Jasmonic acid

Introduction

Heavy metals such as cadmium (Cd) are important abiotic stress factors that limit plant performance and yield. Cd is one of the most toxic non-essential elements, having high mobility and the ability to inhibit the main physiological processes in plants. It is phytotoxic and interacts with photosynthesis, respiration and nitrogen metabolism in plants. This causes poor growth and low biomass due to oxidative stress caused by the generation of free radicals and active oxygen species (Hendry et al. 1992; Sanitá di Toppi and Gabbrielli 1999).

Cytokinins are a class of plant hormones that regulate the cell cycle and diverse developmental and physiological processes, including responses to biotic and abiotic stress. Unlike abscisic acid (ABA), ethylene and jasmonic acid (JA), they are not fundamental plant stress hormones.

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Nevertheless, they affect stress processes in a number of ways (Ha et al. 2012). It has been suggested that in longer term responses to abiotic stress, hormones such as ABA and cytokinins may regulate the production, metabolism and distribution of metabolites essential for stress survival and recovery (Pospíšilová and Dodd 2005). Cytokinin treatment reduces the negative effects of water deficits on chlorophyll and carotenoid content, which are important protective factors against oxidative stress caused by heavy metal uptake, and on photosynthetic activity (Metwally et al. 1997). Moreover, the transcription of many stress-induced genes is stimulated by cytokinins (Hare and Van Staden 1997). In tobacco and maize, endogenous cytokinin levels fall during water stress, primarily due to the activity of cytokinin oxidase/dehydrogenase (CKX), a key enzyme in cytokinin degradation (Alvarez et al. 2008; Havlová et al. 2008). In maize, CKX is induced by ABA and abiotic stress, so there is a direct connection between cytokinin down-regulation and abiotic stress tolerance. Another example is the expression of *SAG12-IPT* in cassava leaves, which delays leaf senescence and increases drought resistence (Zhang et al. 2010). Conversely, *Arabidopsis* lines that have reduced cytokinin sensitivity due to mutations in the cytokinin receptors AHK2, AHK3, or AHK4 are more resistant to drought, osmotic, and cold stress (Jeon et al. 2010; Tran et al. 2007). This suggests that both increases in cytokinin levels and decreases in the intensity of cytokinin signalling could potentially be exploited to increase stress tolerance and promote acclimatization in plants.

We have recently described multiple new synthetic cytokinin derivatives with specific biological effects. Systematic testing led to the identification of two very potent inhibitors of AtCKX2: 2-chloro-6-(3-methoxyphenyl)aminopurine and 2-fluoro-6-(3-methoxyphenyl)aminopurine (Zatloukal et al. 2008). The first of these compounds was named INCYDE (inhibitor of cytokinin degradation). It modulates endogenous cytokinin levels *in planta* and is a suitable tool for in vivo studies of cytokinin action (Aremu et al. 2012). A second screen of the cytokinin derivative library resulted in the identification of PI-55, the first known molecular antagonist of cytokinin activity at the receptor level. PI-55 accelerated the germination of *Arabidopsis* seeds and promoted root growth and formation of lateral roots, thus phenocopying the known consequences of a reduced cytokinin status. This clearly demonstrated its ability to inhibit cytokinin perception *in planta* (Spíchal et al. 2009). Both INCYDE and PI-55 were used in this work to assess the effect of modulating cytokinin levels and responses, respectively, on Cd stress.

The effects of treatment with PI-55 and INCYDE on germination and seedling growth in two plant species (*Bulbine natalensis* and *Rumex crispus*) grown under abiotic stress conditions caused by Cd exposure were investigated. *B. natalensis* is extensively used in traditional medicine in South Africa; the widespread *R. crispus* is a metal-tolerant plant with a relatively high biomass that is also used as a medicinal plant (Zhuang et al. 2007).

Materials and methods

Chemicals

Dimethylsulfoxide (DMSO) and mercuric chloride were obtained from Sigma (St., Luis, MO). N^6-benzyladenine was obtained from OlChemIm (Olomouc, Czech Republic). 2-chloro-6-(3-methoxyphenyl)aminopurine (INCYDE) and 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55) were synthesized in by the Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany AS CR (Olomouc, Czech Republic) as previously described (Zatloukal et al. 2008; Spíchal et al. 2009).

Plant material

Seeds of *B. natalensis* Baker and *R. crispus* L. were obtained from the Univerzity of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa. The seeds were stored in brown paper bags at 10 °C in the dark for 6 months before the experiments.

Determination of the optimal PI-55 and INCYDE concentration for germination and seedling establishment

All seeds were surface decontaminated with 0.1 % mercuric chloride for 2 min and rinsed thoroughly with distilled water prior to germination. The first experiment to determine the optimal concentrations of PI-55 and INCYDE for germination and seedling establishment was performed as described by Street et al. (2007). Disposable Petri dishes containing two Whatman No. 1 filter paper discs were used for germination. Three replicates of 15 seeds each were used. Test solutions of either PI-55 or INCYDE in distilled water at concentrations ranging from 0.001–10 μM were added at the start of the experiment. Additional control experiments were performed involving treatment with distilled water, 0.1 % DMSO solution and 10 μM N^6-benzyladenine (BA). The treated Petri dishes were placed in plant growth chambers at 25 ± 0.5 °C under 16:8 h light: dark conditions, with a photosynthetic photon flux density of 80.4 ± 3.5 μmol M^{-2} s^{-1}. The Petri dishes were checked daily and distilled water was added periodically to maintain moisture levels. Germination (scored as the emergence of a radicle with a length of at least 2 mm) was recorded every day. The experiment was terminated 2 weeks after the first observed germination. The number of lateral roots produced by the *Rumex* seedlings and adventitious roots produced by the *Bulbine* seedlings were counted, and the lengths of the roots and shoots and the fresh masses of the seedlings were recorded.

Testing of plant response to PI-55 and INCYDE in the presence of Cd

The second experiment was done in Petri dishes as described above but in the presence of 5 mg L^{-1} Cd ($CdNO_3$). Three replicates of 25 seeds each were used and samples of the two plant species were treated with 10 nM PI-55, 10 nM INCYDE, and 10 μM BA (*B. natalensis*); or 10 nM PI-55, 100 nM INCYDE, 10 μM BA (*R. crispus*). These concentrations were selected based on the results of the initial experiment to determine the optimal concentration of PI-55 and INCYDE for germination and seedling establishment for the two species under investigation. Each treatment was applied in two different ways: (1) compounds diluted in distilled water were added once at the start of the experiment, after which only water or water containing Cd (5 mg L^{-1}) and DMSO (0.1 %) was added every second day to maintain an appropriate moisture level, or (2) the tested compounds were added at the indicated concentrations every second day during watering. Germination (2 mm radicle emergence) was recorded every second day. The experiment was terminated after 21 days when the seedlings were developed, and morphological parameters (root and shoot length, fresh seedling mass and number of lateral or adventitious roots) were measured for each seedling.

Statistical analyses

All arcsine-transformed germination and seedling growth data were analyzed using one-way analysis of variance, and Duncan's Multiple Range test at the 5 % level of significance was performed to separate the means of the treatments. All analyses were performed using release 12 of the GenStat® statistics package.

Results

Effect of PI-55 and INCYDE on germination and seedling development

In the initial experiments, plants were treated with PI-55 and INCYDE at concentrations of 1 nM–10 μM to establish the optimal concentrations for germination and seedling development. Neither compound alone had any significant effect on the germination of *B. natalensis* and *R. crispus* (Table 1). Characterisation of seedling growth revealed that plant organs responded differently to the PI-55 and INCYDE treatments. In *R. crispus*, PI-55 had no effect on shoot and root growth at any of the tested concentrations (Fig. 1a–c). However, INCYDE caused dose-dependent inhibition of both root and shoot growth (Fig. 1d, e). As was observed with the cytokinin control (10 μM BA), INCYDE at 10 μM strongly reduced the lengths of the shoots and roots (Fig. 1d, e), and to lesser extent, overall seedling weight (Fig. 1f). In the case of *B. natalensis*, treatment with PI-55 at concentrations of up to 1 μM did not significantly affect seedling growth. However, growth was reduced by approximately 30 % at the 10 μM concentration (Fig. 2). Treatment with INCYDE had no effect on shoot length (Fig. 2d) but higher concentrations (10 μM) significantly inhibited root growth (Fig. 2e).

Table 1 The effects of a cytokinin antagonist (PI-55) and an inhibitor of cytokinin degradation (INCYDE) on *R. crispus* and *B. natalensis* germination, along with control (Ctr), N^6-benzyladenine (BA) and solvent control (DMSO) treatments

Treatment	*B. natalensis*		*R. crispus*	
	Number of seeds	Germination (%)	Number of seeds	Germination (%)
Ctr	7.7 ± 1.15	76.7	7.7 ± 0.58	76.7
DMSO	7.0 ± 1.73	70	7.3 ± 0.58	73.3
BA	8.7 ± 1.15	86.7	7.7 ± 1.53	76.7
PI-55				
10 μM	5.0 ± 1.73	50	8.3 ± 0.58	83.3
1 μM	6.7 ± 1.15	66.7	7.0 ± 1	70
0.1 μM	6.0 ± 1.73	60	7.7 ± 0.58	76.7
10 nM	7.0 ± 1	70	7.0 ± 2.65	70
1 nM	7.0 ± 1	70	7.7 ± 2.08	76.7
INCYDE				
10 μM	6.3 ± 1.15	63.3	7.7 ± 2.0	76.7
1 μM	6.7 ± 0.6	66.6	6.3 ± 3.7	63.3
10 nM	8.7 ± 1.52	86.7	6.0 ± 2	60
1 nM	7.0 ± 2.64	70	6.3 ± 0.5	63.3

Values show mean ± SD of three replicates. The total number of seeds per replicate was ten. The differences between the treatments are not statistically significant

Effect of PI-55 and INCYDE on growth of *B. natalensis* and *R. crispus* in the presence of Cd

The effects of PI-55 and INCYDE on growth under Cd stress were investigated using concentrations that had no adverse effects on seedling development in the first set of experiments. For *B. natalensis*, both compounds were applied at concentrations of 10 nM, while *R. crispus* was treated with PI-55 at 10 nM and INCYDE at 100 nM. A Cd

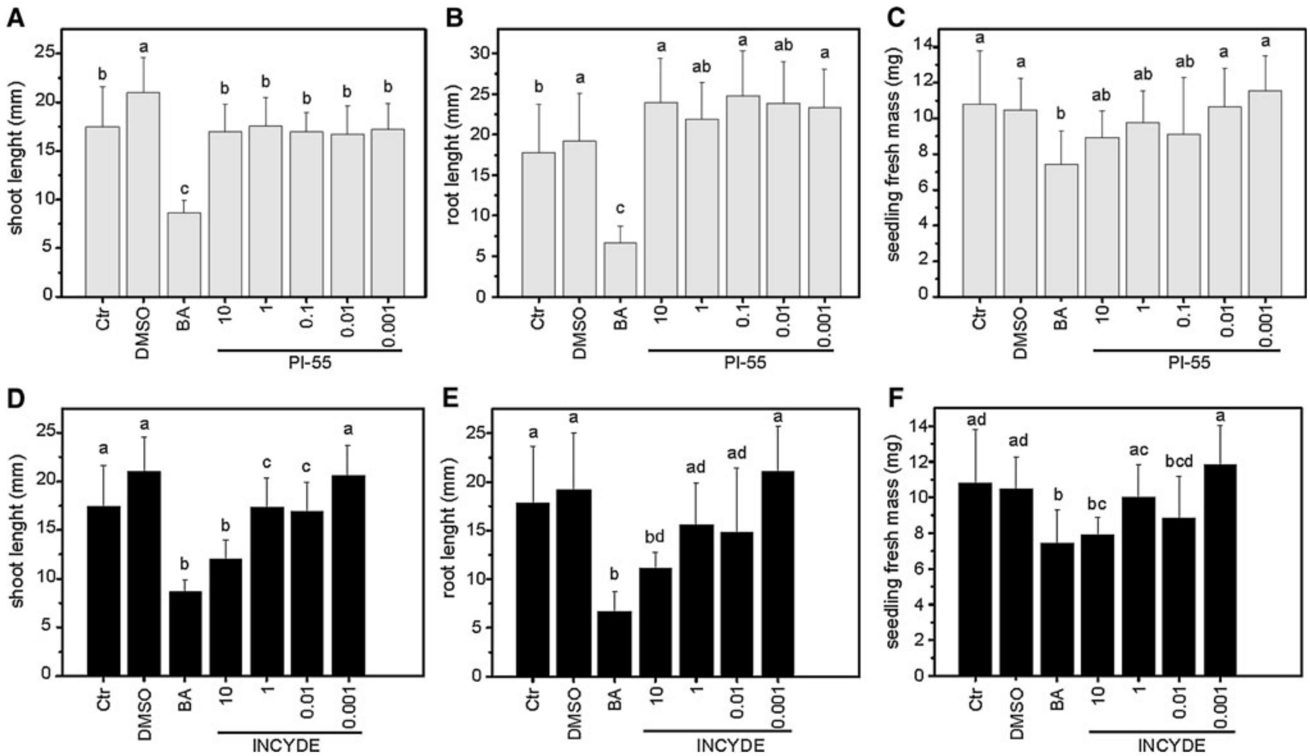

Fig. 1 The effects of the cytokinin antagonist PI-55 (**a-c**) and the inhibitor of cytokinin degradation INCYDE (**d-f**) on *Rumex crispus* growth. Seeds were germinated in water containing 0.001–10 µM of one of the two compounds, and the effects of the treatment on the seedlings' shoot length (**a**, **d**), root length (**b**, **e**), and overall weight (**c**, **f**) were estimated. Results for control (Ctr), N^6-benzyladenine (BA) and solvent control (DMSO) treatments are also shown. *Bars* show mean values and SD (n = 45); standard error bars with *different letter(s)* denote results that differ significantly ($p < 0.05$)

concentration of 5 mg L^{-1} was used to induce abiotic stress; this is a typical concentration found in heavily Cd-contaminated soil solutions (Alloway 1990).

Cd treatment caused significant inhibition of *R. crispus* growth (Fig. 3), reducing seedling weight and shoot length by 30 % relative to the untreated control (Fig. 3a, d). Cd treatment also inhibited root growth by 40 % (Fig. 3c) and partially reduced the number of lateral roots (Fig. 3b). These negative effects on the root system were more pronounced in the presence of 10 µM BA (Fig. 3b, c). This is consistent with the known function of cytokinins as negative regulators of the root system (Cary et al. 1995; Skoog and Miller 1957). In contrast, BA had positive effects on shoot length and seedling weight in Cd-treated seedlings (Fig. 3a, d).

The effect of PI-55 and INCYDE were tested in two ways: the compounds were either applied once at the start of the experiment or continuously throughout the whole experiment. Both compounds had positive effects on *R. crispus* seedlings grown in the presence of Cd (Fig. 3). PI-55 treatment overcame the negative effects of Cd, increasing the fresh weight of the seedlings, their root and shoot lengths (Fig. 3a, c, d), and the number of lateral roots produced (Fig. 3b). Even with only a single application at the beginning of the experiment, PI-55 protected root development in Cd-treated plants, doubling the number of lateral roots produced relative to the untreated control (Fig. 3b).

Treatment with INCYDE had positive effects on shoot length and seedling fresh weight similar to those observed with BA (Fig. 3a, d). However, the typical cytokinin-like negative effect on the root system was not observed even under long-term INCYDE treatment (Fig. 3b, c): the roots of seedlings treated with both INCYDE and Cd were almost as long as those of untreated control seedlings (Fig. 3c). Seedlings treated with INCYDE also produced around 30 % more lateral roots than controls treated with Cd alone (Fig. 3b).

In the monocotyledonous *B. natalensis*, the negative effect of Cd on plant growth was not as pronounced as in *R. crispus* (Fig. 4). Cd exposure reduced the seedlings' fresh weight by more than 30 % compared to the control (Fig. 4a) but did not affect the number of roots produced at the investigated concentration. However, the root length was reduced by about 20 % compared to the control (Fig. 4b, c). Cd exposure also reduced the length of the shoots by around 25 % (Fig. 4d). Cytokinin (BA) treatment intensified the negative effects of Cd on plant performance,

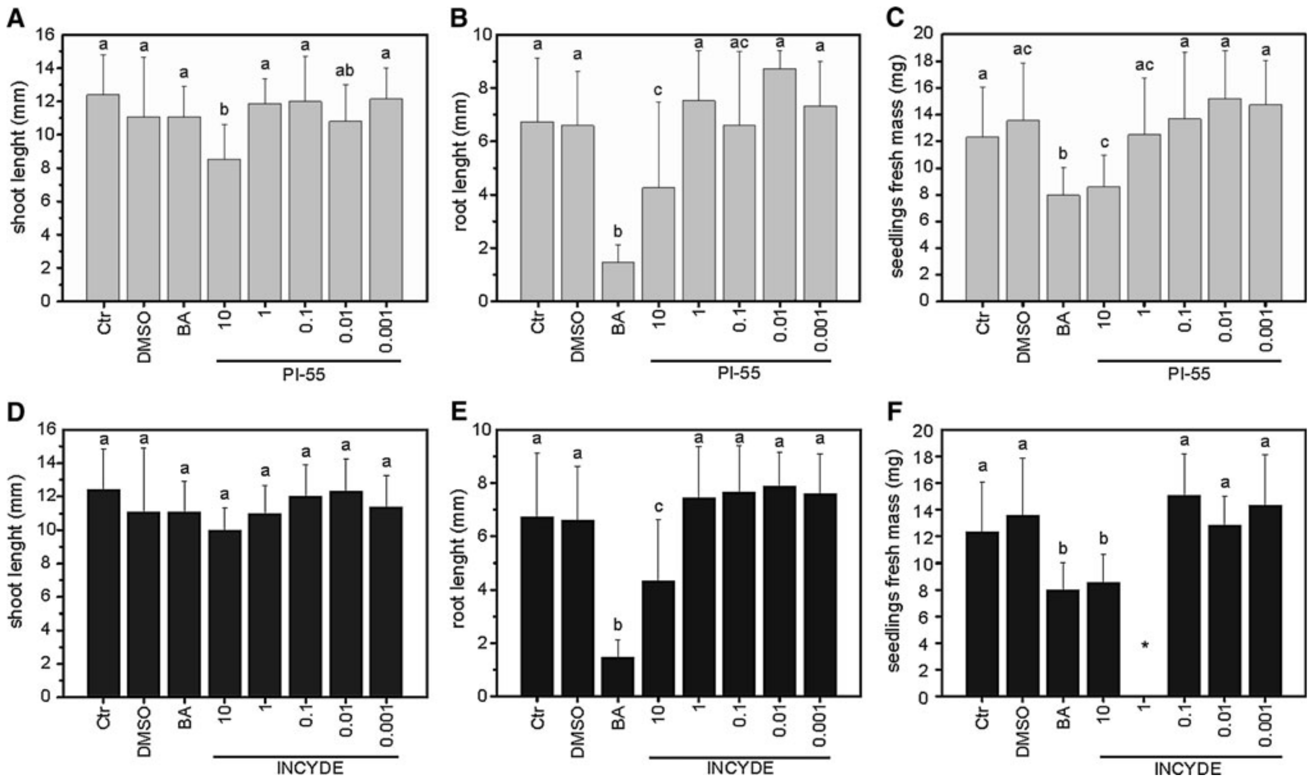

Fig. 2 The effects of the cytokinin antagonist PI-55 (**a**–**c**) and the inhibitor of cytokinin degradation INCYDE (**d**–**f**) on *B. natalensis* growth. Seeds were germinated in water containing 0.001–10 µM of one of the two compounds and the effects of the treatment on the seedlings' shoot length (**a**, **d**), root length (**b**, **e**), and overall weight (**c**, **f**) were estimated. Results for control (Ctr), N^6-benzyladenine (BA) and solvent control (DMSO) treatments are also shown. *Bars* show mean values and SD (n = 45); standard error bars with *different letter(s)* denote results that differ significantly ($p < 0.05$). The effect of treatment with 1 µM INCYDE on seedling fresh weight could not be determined due to contamination (indicated by an *asterisk* in **f**)

primarily by further reducing the length of the seedlings' roots (Fig. 4c). Although treatment with PI-55 alone increased seedling fresh weight by about 15 %, it failed to overcome the negative effects of Cd (Fig. 4a). However, PI-55 treatment did provide significant protection against the retardation of root and shoot growth regardless of whether it was applied once or repeatedly (Fig. 4c, d). The effects of INCYDE were very similar to those of PI-55 (Fig. 4): although it did not overcome the effect of Cd on seedling fresh weight, it did protect the plants against Cd-induced retardation of shoot and root growth (Fig. 4c, d).

Discussion

Environmental pollution of soils and water by heavy metals has become a serious problem in recent times due to human activities. Low concentrations of heavy metals such as Fe, Cu, Zn or Ni are essential for some physiological processes in plants, but other metals such as Cd, Pb, Hg and Cr are phytotoxic (Clemens 2001). Heavily polluted areas may contain up to 100 µg g^{-1} Cd, giving soil concentrations of up to 32 µM (Alloway 1990). We performed experiments to determine whether modulating plants' cytokinin status using a cytokinin antagonist (PI-55) and/or an inhibitor of cytokinin degradation (INCYDE) could promote germination and seedling establishment in the presence of Cd at concentrations that are common in polluted environments.

The rationale behind the use of the cytokinin antagonist to increase plant resistance against heavy metal stress was that decreased cytokinin perception has been shown to help plants in coping with osmotic, drought and salt stress (Tran et al. 2007). We have previously shown that the cytokinin antagonist PI-55 blocks the binding of natural cytokinins to their receptors, and that plants treated with this compound phenocopied cytokinin receptor mutants (Spíchal et al. 2009). As expected, PI-55 treatment stimulated root development in *R. crispus* and *B. natalensis* seedlings and remained effective in the presence of Cd (Figs. 3, 4). It is possible that PI-55 affected cytokinin perception in the treated seedlings and thereby conferred enhanced stress tolerance similar to that observed in plants with mutated cytokinin receptors. PI-55 also positively affected the growth of the root system and thus certainly improved nutrient uptake and utilization, which is otherwise limited in plants growing under Cd stress due to the retardation of

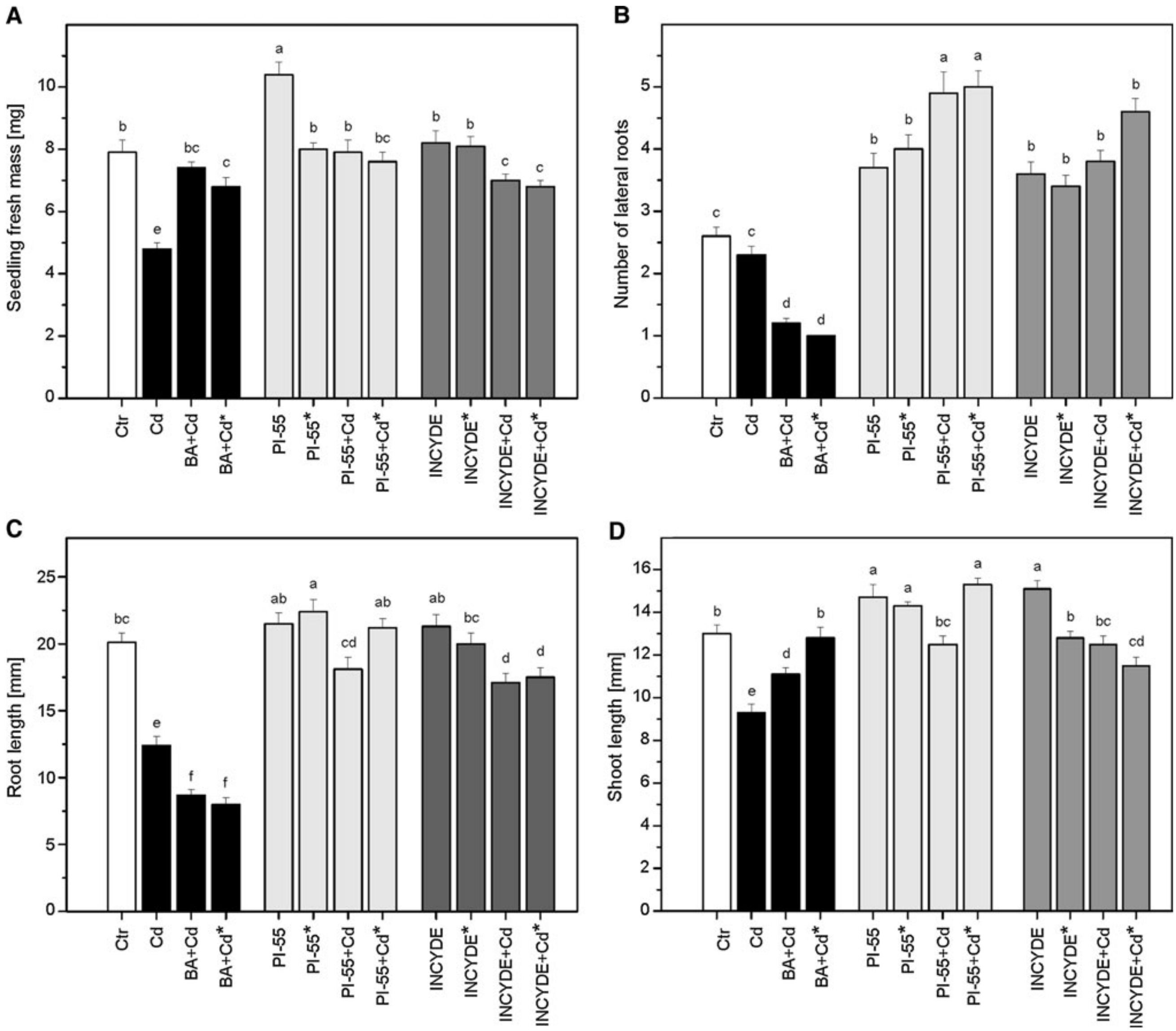

Fig. 3 The effects of treatment with the cytokinin N^6-benzyladenine (BA; 10 μM), the cytokinin antagonist PI-55 (10 nM) and the inhibitor of cytokinin degradation INCYDE (100 nM) on *R. crispus* growth in the presence of 5 mg L^{-1} Cd. The tested substances were either applied once at the begining of germination or continously at every watering (indicated with an *asterisk*) and the effects of each treatment on seedling weight (**a**), the number of lateral roots (**b**), root length (**c**), and shoot length (**d**) were determined. Control (Ctr) seedlings were treated with water containing 0.1 % DMSO. Bars show the mean values obtained in each case and the SD (n ≥ 23); standard error bars with *different letter(s)* denote results that differ significantly ($p < 0.05$)

root system growth. Even a single application of PI-55 at the start of germination was sufficient to protect root growth in *R. crispus* against the detrimental effects of Cd exposure.

The mechanism of PI-55 action is complicated by the fact that PI-55 is only a partial receptor antagonist. Although it blocks AHK4, it weakly activates AHK3 (Spíchal et al. 2009). Weak cytokinin activity may also have important protective effects. Exogenous cytokinin treatment reportedly has positive effects on the growth of Cd-stressed plants (Wozny et al. 1995; Lukatkin et al. 2003, 2007; Ul'yanenko et al. 2004). This is partially consistent with the results obtained in this work, which demonstrated that BA has protective effects on seedling shoot length and overall biomass formation in *R. crispus* (Fig. 3a, d) and to a lesser extent in *B. natalensis* (Fig. 4a, d). On the other hand, cytokinins negatively regulate root growth (Werner et al. 2001); in keeping with this function, we observed that Cd exposure in conjunction with BA treatment had an additive adverse effect on root system development. Cd is generally toxic to plants because it induces oxidative stress (Briat and Lebrun 1999), but treatment with exogenous cytokinin-derived compounds can protect against the oxidative damage caused by

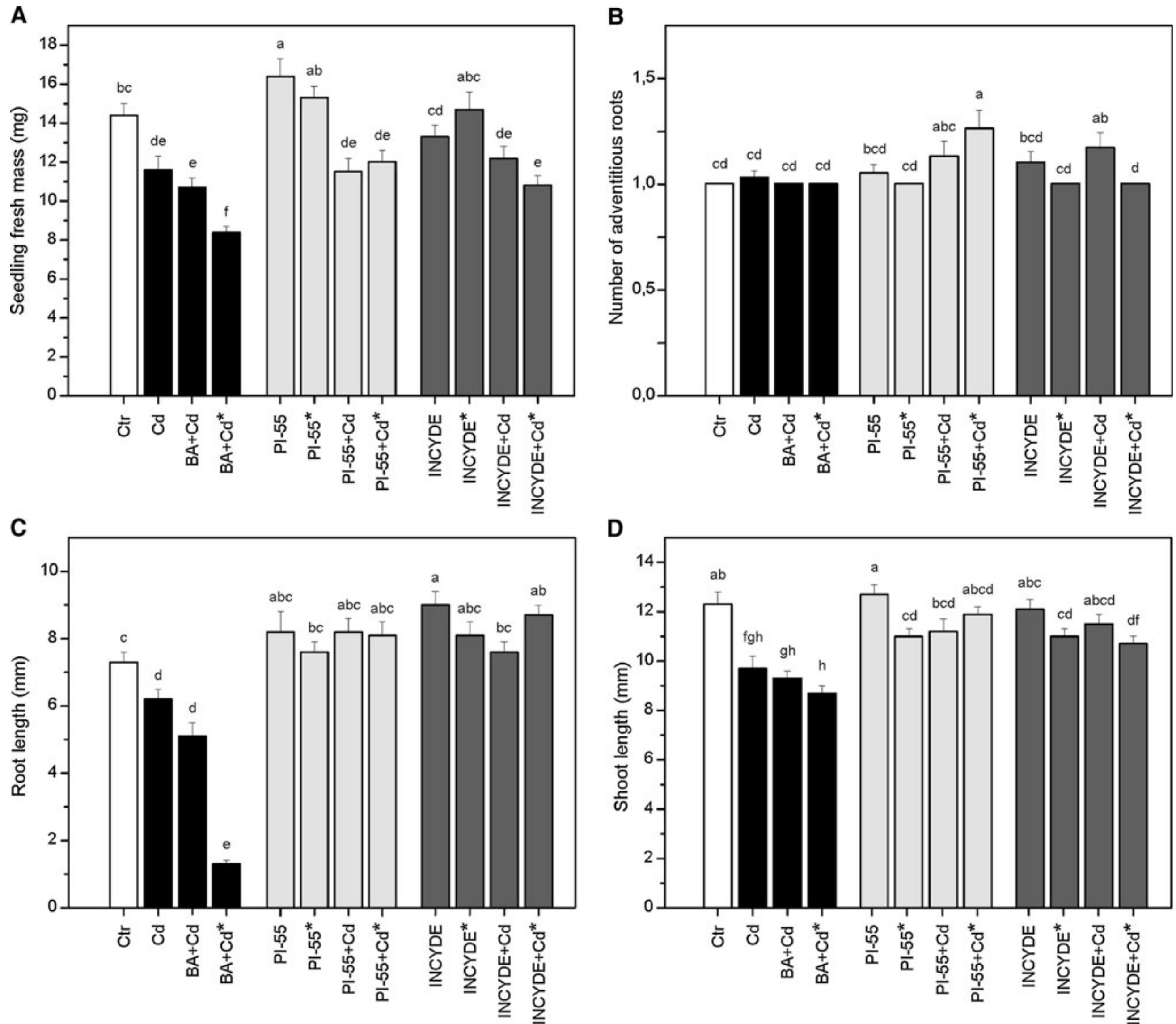

Fig. 4 The effects of treatment with the cytokinin N^6-benzyladenine (BA; 10 μM), the cytokinin antagonist PI-55 (10 nM), and the inhibitor of cytokinin degradation INCYDE (10 nM) on *B. natalensis* growth in the presence of 5 mg L^{-1} Cd. The tested substances were either applied once at the begining of germination or continously at every watering (indicated with an *asterisk*) and the effects of each treatment on seedling weight (**a**), number of roots (**b**), root length (**c**), and shoot length (**d**) were determined. Control (Ctr) seedlings were treated with water containing 0.1 % DMSO. *Bars* show the mean values obtained in each case and the SD ($n \geq 23$); standard error bars with *different letter(s)* denote results that differ significantly ($p < 0.05$)

exposure to transition metals such as Cd (Lukatkin et al. 2007). The apparent difference in the effect of BA on *B. natalensis* and *R. crispus* may be due to the higher resistance of *B. natalensis* to Cd. Whereas only a single application of Cd inhibited root growth of *R. crispus* (Fig. 3c), continuous Cd application was needed to inhibit root growth in *B. natalensis* (Fig. 4c). In addition, *B. natelensis* forms adventitious roots that appear later during root system development and thus this morphological trait may not have been affected by Cd applied under the experimental conditions. Previous works have shown that treatment with the synthetic cytokinin kinetin or the CKX inhibitor thidiazuron (Chatfield and Armstrong 1986) can mitigate the negative effects of high concentrations of heavy metals such as Zn and Ni by increasing the activity of ascorbate peroxidase, one of the key antioxidant defence enzymes (Lukatkin et al. 2007). This finding explains the protective effect of INCYDE, an inhibitor of CKX that blocks cytokinin degradation in vivo (Gemrotová et al. 2009). Moreover, we recently showed that INCYDE treatment can significantly increase antioxidant capacity *in plants* (Gruz and Spíchal 2011). Overall, our data indicate that both approaches to modulating cytokinin status (i.e. decreasing it by blocking cytokinin signalling, or causing finely tuned increases in endogenous cytokinin levels by inhibiting their degradation) can protect against the adverse effects of Cd exposure. It was clearly shown that these protective effects are cytokinin-dependent. However, they are also likely to be influenced by a complex mixture of primary and secondary factors.

Rumex crispus is a plant that is known to tolerate high levels of heavy metals (Zhuang et al. 2007). Together with hyperaccumulators such as *Viola baoshanensis*, *Thlaspi caerulescence* and *Sedum alfredii*, it is suitable for phytoextraction (Zhuang et al. 2005). While its tolerance is lower than that of other hyperaccumulators, it accumulates high levels of Cd (Zhuang et al. 2007), has a relatively high biomass and can be grown worldwide. Recent studies performed under field conditions indicated that due to its high biomass, *R. crispus* can extract 26.8 kg ha^{-1} Zn and 0.16 kg ha^{-1} Cd from metal-contaminated soil with high efficiency (Zhuang et al. 2007). The phytoextraction potential of *R. crispus* is thus high and could be further improved by treatment with PI-55 or INCYDE at sub-micromolar concentrations (10–100 nM) to protect the roots against Cd stress. This may even increase the number of roots produced relative to untreated plants (Fig. 3). Moreover, even a single application of PI-55 or INCYDE at an early stage of development was sufficient to induce strong positive effects, further enhancing the practicality of these compounds. Overall, the results suggest that phytoextraction facilitated by these compounds could be useful in plant-based environmental remediation technologies for removing Cd from polluted areas or in biofortification processes.

Many medicinal plants are exposed to heavy metals in their natural habitats due to mining activities and the disposal of processed and unprocessed wastes (Street et al. 2008). Cd is one of the major sources of contamination and is hazardous to plants, affecting their growth and development (Dong et al. 2006; Patra et al. 2004). This work examined the responses of the monocotyledonous plant *B. natalensis*, which is harvested from the wild for use in traditional medicine, to Cd exposure. The number of roots and the lengths of the roots and shoots produced by Cd-exposed *B. natalensis* were significantly improved in plants treated with PI-55. This clearly shows the potential utility of PI-55 in alleviating the detrimental effects of Cd exposure. As such, it may be useful for protecting medicinal plants against the effects of heavy metal contamination.

Conclusion

Heavy metal pollution is a serious environmental problem in many countries. New substances such as PI-55 and INCYDE could potentially be used to protect crop plants against heavy metal accumulation or to increase the effectiveness of desirable processes such as biofortification and phytoremediation. In order to use these processes to their full potential, it will be necessary to understand the molecular mechanisms of metal efflux and uptake in plants, and so these should be investigated further. While the exact mechanism by which modulation of cytokinin status protects plants against Cd stress still needs to be resolved, the results presented herein demonstrate that treatment with substances that modulate plants' cytokinin status can significantly improve seedling establishment under high-Cd conditions.

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