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Polyamine and tyramine involvement in NaCl-induced improvement of Cd resistance in the halophyte *Inula chrithmoides* L.

Rim Ghabriche[a,b,c], Tahar Ghnaya[a,*], Mejda Mnasri[a], Hanen Zaier[a], Raoudha Baioui[a], Delphine Vromman[c], Chedly Abdelly[a], Stanley Lutts[c]

[a] Laboratoire des Plantes Extremophiles (LPE), Centre de Biotechnologies de la Technopole de Borj Cedria, BP 901, Hammam Lif 2050, Tunisia
[b] Faculté des Sciences de Bizerte, Université de Carthage, 7021 Jarzouna, Tunisia
[c] Groupe de Recherche en Physiologie Végétale (GRPV), Earth and Life Institute, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

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ABSTRACT

The aim of the present work was to analyze the impact of salinity on the plant response to Cd toxicity in the Mediterranean halophyte species *Inula crithmoides*. For this purpose, cuttings were cultivated hydroponically during 21d in the presence of 0, 25 or 50 µM $CdCl_2$ combined or not with 0, 100, 200 and 400 mM NaCl. The obtained data demonstrated that, in the absence of Cd, NaCl strongly increased plant growth (the maximal dry weight being observed at 100 mM) and enhanced the Na^+/K^+ ratio in the shoot. Cd alone strongly affected plant growth in this halophyte. However, in Cd-treated plants, NaCl protected *Inula crithmoides* from Cd toxicity and contributed to reduce Cd absorption and translocation. Small aliphatic polyamine (putrescine, spermidine, spermine) increased in response to both NaCl and $CdCl_2$, the highest concentration in plants being observed when both agents are present in the medium. The recorded increase preferentially concerned the polyamine bound fraction, which might be related to their involvement in the protection of endogenous cellular structures. The aromatic monoamine tyramine also strongly increased in response to Cd toxicity and its putative role is discussed in relation to conjugation processes. Salinity and Cd increased ammonium/nitrate ratio in leaves and roots and the involvement of stress-induced modification of N nutrition on polyamine oversynthesis is also discussed.

1. Introduction

Heavy metal pollution is an important environmental constraint that compromises ecosystem stability and human health. Cadmium is a highly toxic elementmainly produced by metallurgic industries and mining activities. It disturbs cell metabolism even at low concentration and induces oxidative stress responsible for protein damages and DNA injuries (Viehweger, 2014). In plants Cd also induces ionic imbalances alteration of the plant water status and photosynthesis (Ghnaya et al., 2007; Lefèvre et al., 2014; Viehweger, 2014). The use of plants to restore heavy-metal polluted areas received considerable attention since several years. Many experiments have been conducted with the final aim to remove heavy metals from polluted soils. Regarding the phytoextraction process there are many limitations to overcome among which the low biomass of hyperaccumulating plants and their low deep penetrating roots. Beside phytoextractionphytostabilisation consists in the use of resistant rustic plants to reduce erosion and avoid dispersion of the contaminated substrate (Ali et al. 2013). Phytoremediation of coastal areas and salt-affected soils is an exciting challenge for scientists. These areas are indeed characterized by the simultaneous presence of heavy metals on the one hand and high salt levels on the other hand. It has been postulated that halophyte plant species may constitute a promising material for phytomanagement in those areas (Ghnaya et al., 2007; Manousaki et al., 2008; Lefèvre et al., 2009; Lokhande et al., 2013). Indeed some of them are able to cope with different types of salts and are therefore expected to display physiological adaptations allowing them to overcome various types of ionic toxicities (Lutts and Lefèvre, 2015).

Inula crithmoides (syn. *Limbarda crithmoides*) is a common halophyte in Mediterranean area. It is a perennial coastal species found growing on salt marsh and sea cliffs and it is able to grow at extremely high salinity. It belongs to the Asteraceae family and is commonly known as Golden samphire. It may be grow up to 1 m tall and exhibit narrow fleshy leaves and large flower heads (Zurayk and Baalbaki, 1996). Exhaustive data are available on numerous metabolites presenting a putative interest for medical applications (Abdel-Wahhab et al., 2008) and

Abbreviations: PAs, polyamines; Put, putrescine; Spd, spermidine; Spm, spermine; Cad, cadaverine; Tyr, tyramine

* Corresponding author.
E-mail address: Tahar.ghnaya@gmail.com (T. Ghnaya).

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some reports detailed the involvement of mycorrhiza in the plant development (Roda et al., 2008). This species may be found in ruderal zones where different types of ionic toxicities are simultaneously present. Zurayk et al. (2001), reported that it is able to cope with cadmium and nickel contamination in salt affected areas but these authors did not provide information regarding the physiological basis of traits involved in this tolerance.; The higher heavy metal tolerance of halophytes comparatively to glycophytes may be related to the fact that toxic trace elements and Na^+ excess are acting on similar physiological targets and that similar biochemical strategies may therefore be triggered to provide resistance to both constraints (Lutts and Lefèvre, 2015). Among the myriad of compounds involved in resistance to ionic toxicities, polyamines (PAs) may assume crucial protective functions and play key roles in heavy metal resistance (Lutts et al., 2013).

At cellular pHPAs are low molecular weight organic cations that are implicated in plant growth and development processes (Tiburcio et al., 2014). A tight regulation of polyamine homeostasis is required for optimal metabolism. The three major polyamines (putrescine (Put) spermidine (Spd) and spermine (Spm)) indeed control the rate of cell division but also differentiation pattern (Sobieszczuk-Nowicka and Legocka, 2014). These polyamines assume crucial functions in stress tolerance (Minocha et al., 2014; Liu et al., 2015; Pál et al., 2015) ; they regulate cation transport across plant membrane (Pottosin et al., 2014), control senescence and programmed cell death (Del Duca et al., 2014), act as free radical scavengers (Alcázar et al., 2010) and directly or indirectly regulate gene expression (Lutts et al., 2013). Beside the three major polyamines, cadaverine (Cad) was also reported to be involved in plant growth and development, although its role in stress resistance is still unclear (Tomar et al., 2013). Similarly, tyramine (Tyr) is an aromatic amine present in numerous plant species but its putative involvement in plant resistance to ion toxicities remains poorly documented. Salinity has an influence on PAs metabolism. Salt-induced modification in PAs concentration may occur as a result of salt impact on enzymes involved in PAs synthesis (Hummel et al., 2004) or in PAs degradation (Quinet et al., 2010). Polyamine concentration may also be affected by heavy metals such as Cd (Lefèvre et al., 2014; Qiao et al., 2015) lead (Legocka et al., 2015; Qiao et al., 2015) manganese (Yao et al., 2012) and chromium (Scoccianti et al., 2006). ; Within plant tissuesPAs may remain in a soluble free form but may also be conjugated to phenolic acids or even bound to macromolecules such as protein and DNA. In some cases the protective functions of PAs on cellular structure were clearly demonstrated to be due to bound rather than free fraction (Legocka et al., 2015). As nitrogenous molecules the homeostasis of polyamines in plants correlates with the nitrogen status. Accumulating experimental evidences clearly suggest that the PAs titers are directly influenced by nitrate absorption and ammonium assimilation (Chen et al. 2011; Moschou et al., 2012). Salinity may directly influences nitrogen assimilation through an impact on glutamine synthase/glutamate synthase cycle (Ghanem et al., 2011) and may thus influence by this way PAs synthesis and thus the plant's ability to cope with an additional constraint such as heavy metals. Nevertheless to the best of our knowledge data concerning polyamine accumulation in halophyte plant species exposed to heavy metal stress under saline conditions are crucially lacking. The present study focuses on the halophyte plant species Inula crithmoides exposed to increasing doses of Cd in the absence or in the presence of NaCl. It aims to i) determine the influence of NaCl on the plant response to Cd stress and ii) to precise the involvement of free conjugated and bound fractions polyamines and tyramine on this influence.

1. Material and methods

1.1. Plant material and growth conditions

The plant material was propagated by cutting. Three-centimeter-long stem segments with two nodes and two leaves were taken from mother plants in natural habitats (Tunisia; 36° 44' 33" N 10° 19' 22" E) in the coastal region of Borj Cedria (34 km South-East of Tunis). These cuttings were placed into plastic pots filled with tap water. Rhizogenesis occurred within 3 week. The rooted cuttings were cultivated for 7 days in an aerated half strength Hewitt (1966) solution, supplemented with Fe EDTA (45 μM) and micronutrients according to Arnon and Hoagland (1940) in a growth chamber with a mean constant temperature of 25 °C ± 2 under a photoperiod of 16 h (250 μmol $m^{-2} s^{-1}$ PAR) and a relative humidity of 70–80%. Young seedlings were then transferred to plastic pots filled with 5L of an aerated nutrient solution (Hewitt, 1966). Each treatment contains 10 replicates. Twelve treatments were applied for 21 days: control plants were cultivated on Hewitt solution without NaCl and cadmium, two concentrations of $CdCl_2$ (25 and 50 μM $CdCl_2$) three doses of NaCl (100, 200 and 400 mM NaCl) and six treatments combining cadmium (25 or 50 μM) and salt (100, 200 or 400 mM). The nutrient solution was continuously aerated and was renewed every 2 d. The harvest was performed after 21 d of treatment. At harvest, shoots were separated from roots, rinsed three times with cold distilled water and blotted between two layers of filter paper. Roots were dipped in a 0.01 M HCl cold solution to eliminate most external heavy metals adsorbed at the root surface, then rinsed three times with cold distilled water and blotted with filter-paper. The fresh weight was immediately determined, and the dry weight was measured after desiccation for 48 h in an oven at 60 °C on 5 plants per treatment. Five other plants were quickly frozen in liquid nitrogen and subsequently stored at −80 °C for polyamine analysis and N analysis.

1.2. Sodium and cadmium determination

Dried samples were ground to a fine powder using a porcelain mortar and a pestle, digested in 35% HNO_3 and evaporated to dryness on a sand bath at 80 °C. The minerals were incubated with a mix of 37% HCl and 68% HNO_3 (3:1); they were slightly evaporated and were dissolved in HCl 0.1N. Na^+ and Cd^{2+}concentrations were determined by SOLAAR S4 atomic absorption spectrophotometry (Thermo Scientific, Cambridge, UK). For each treatment, five separated plants were considered and each analysis was performed on technical triplicates.

1.3. Determination of NH_4^+ and NO_3^- content

Three hundred mg of freeze-dried material was incubated with 15 mL of distilled water at 70 °C for 1 h with stirring. The samples were then centrifuged at 5000 *g* for 15 min at 20 °C and the supernatent was collected. Ammonium was quantified according to Weatherburn (1967) at 625 nm and nitrate according to Cataldo et al. (1975) at 410 nm using a UV Spectrophotometer (SHIMADZU, UV spectrophotometer (UV-1800)). The standard curves were established using ammonium from 0 to 300 μM NH_4Cl solutions and nitrate from 0 to 5 mM $NaNO_3$. For each treatment, five separated plants were considered and each measurement was made in technical triplicates.

1.4. Extraction and analysis of polyamines

For polyamine extraction, tissues were frozen in liquid nitrogen and ground in a pre-chilled mortar: samples (approximately 500 mg lyophilized material for shoots and 250 mg for roots) were then homogenized with 500 μL of cold HCl (1 M), kept on ice for 1 h and then centrifuged at 23,100*g* at 4 °C for 20 min. Pellets were re-extracted with 500 μL HCl (1 M) and re-centrifuged. The two supernatants were used to determine free polyamines. Dansylation was performed according to Smith and Davies (1985). Samples were re-suspended in 1 mL methanol, centrifuged at 13,000*g* for 15 min and filtered through microfilters (Chromafil PES-45/15, 0.45 μm; Macherey-Nagel). Aliquots (20 μL) were injected into a Bio-Rad HPLC system equipped with a

Nucleosil 100-5 C18 MN 250/04 column (particle size: 5 µm, 4.6 × 250 mm^2). Elution was performed at 35 °C at a flow rate of 1 mL min^{-1} using a methanol/water stepped gradient programme changing from 60% to 100% methanol over 25 min. The column was washed with 100% methanol for 15 min. Detection of dansylated polyamines was performed with a Shimadzu RF-10Axl fluorimeter, with an excitation wavelength of 320 nm and an emission wavelength of 510 nm. The rate of free polyamine recovery at the end of the procedure was higher than 95%.

For conjugated and bound polyamine analysis, 200 µL of the supernatant was mixed with 200 µL of 12N HCl and heated at 110 °C for 16 h in tightly capped glass tubes. After acid hydrolysis, HCl was evaporated from the tubes by further heating at 80 °C and the residue was resuspended in 200 µL of 10% PCA and used for dansylation. To extract PCA- insoluble bound PAs, the pellet was dissolved by vigorous vortexing in 5 mL of 1N NaOH. The mixture was centrifuged at 23,100*g* at 4 °C for 20 min, and the supernatant, including the solubilized bound PAs, was hydrolysed under the same conditions as above. Aliquots of 200 µL of supernatant (free PAs), supernatant hydrolysed (conjugated PAs) and pellet hydrolysed (bound PAs) were dansylated, along with PAs standards, as previously described by Lefèvre et al. (2001). Polyamines were then quantified as described above for free polyamines. For a given treatment, each quantification was performed on three independent samples.

1.5. Statistical analysis

All data were analyzed using a SPSS statistical package. One-way ANOVA was used to determine the significant difference of among the treatments (*F*-test, at 0.05 significance level).

2. Results

2.1. Plant morphology and growth

After 10 d of treatment, chlorosis was visible in young leaves of *I. crithmoides* exposed to 25 and 50 µM of $CdCl_2$ alone. At the end of the experiment, these visual symptoms were accentuated and accompanied by a reduction in whole plant length. In contrast, addition of salt (NaCl) in Cd-treated plants alleviated significantly Cd-induced toxicity symptoms (Fig. SM1). *Inula crithmoides* presented its whole plant maximum biomass production in the presence of 100 mM NaCl as shown in Fig. 1, thus confirming that *I. crithmoides* is an obligate halophyte.

Results related to the biomass accumulation showed that, for all treatments, plant were able to grow and produced additional matter to that measured at the beginning of the treatment (Fig. 1). However, plant growth was significantly and differently influenced depending on treatment. Hence, exposure to cadmium alone resulted in a marked reduction in the whole plant dry weight, especially at the highest concentration (50 µM of $CdCl_2$). Plants exposed to Cd^{2+} (25 µM or 50 µM) in the absence of NaCl, produced respectively 40% and 24% dry matter compared to control plants. Addition of moderate doses of salt (100 and 200 mM) to the Cd^{2+} containing medium restored the whole plant growth (Fig. 1), although the whole plant dry weight was strongly reduced by the highest NaCl dose of salt in the presence as well as in the absence of Cd.

2.2. Ratio of Na^+/K^+ and cadmium accumulation

The Na^+/K^+ ratio was lower than 1 for plants cultivated in the absence of salt (Table 1). This ratio increased in response to NaCl and was always higher in the leaves than in the roots, reflecting the ability of *Inula crithmoides* to efficiently absorb and specifically translocate Na^+ to the aerial parts. In plants exposed to NaCl, the ratio was always higher than 1. In the absence of NaCl, increasing Cd concentration induced an augment in Na^+/K^+ ratio in all organs. This increase reached

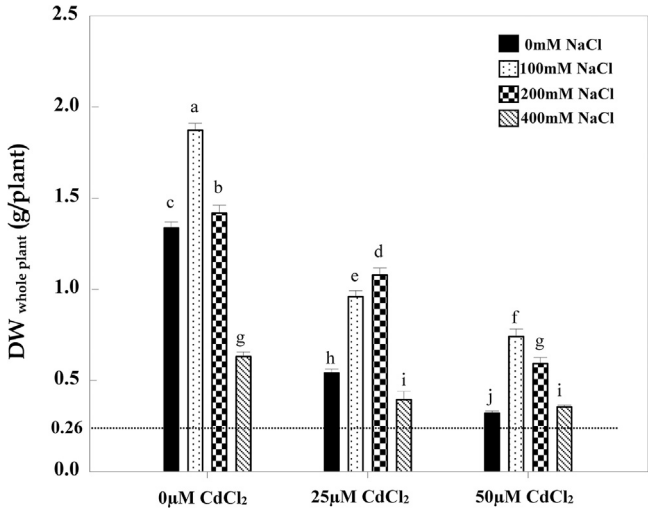

Fig. 1. Whole plant dry weight of *Inula crithmoides* exposed for 21 d to control nutrient solution or to nutrient solution containing 25 or 50 µM $CdCl_2$ combined or not combined with 100 or 200 or 400 mM NaCl in the nutrient solution. The horizontal line indicates the value of dry weight of plantlets at the beginning of treatments. Each value is the mean of 5 replicates and vertical bars are S.E. of the means. Values sharing a common letter are not significantly different at $P < 0.05$.

170% as compared to that of control plants in the shot of plants subjected to 50 µM Cd without NaCl. However, when cultivated in the presence of all NaCl doses, the increase of Cd concentration in the medium does not influenced significantly the ration Na^+/K^+ in both organs (Table 1).

Cadmium was not detected in control plants but it accumulated in response to Cd treatment proportionally to the exogenous Cd concentration. Cadmium concentration was higher in the roots than in the shoots. The presence of NaCl significantly decreased Cd accumulation, especially for the highest NaCl dose.

2.3. Polyamine analysis

Exposure to NaCl clearly increased the endogenous Put concentration in both leaves and roots (Fig. 2). As far as leaves are concerned, such an increase mainly concerned the bound and free polyamine fractions, while the conjugated fraction was not modified by Cd stress or NaCl exposure. Cadmium alone slightly, but significantly, increased the Put concentration in the leaves. Plants subjected to the combined effects of Cd and NaCl exihibted the more elevated Put concentration in the leaves. In contrast, In the roots, NaCl alone increased all fractions of Put including the conjugated. In these organs, total Put was the highest in salt-treated plants exposed to 25 µM Cd.

In the absence of NaCl, Spermidine concentration (Fig. 3) strongly increased in response to Cd. The presence of NaCl alone increased Spd concentration mainly through an impact on the bound fraction in the leaves, and on both conjugated and bound fractions in the roots. Combined treatments induced the more important increase of Spd concentration in both organs. Similarly, NaCl in the absence of Cd as well as Cd in the absence of NaCl obviously increased the endogenous Spm concentration (Fig. 4). The effects of these treatments appear to be additive since the highest Spm concentration was observed in plants exposed concomitantly to Cd and NaCl. In the presence of Cd, the free Spm remained the smallest fraction and this heavy metal mainly increased the conjugated and bound Spm.

Salinity alone slightly increased Cad concentration in the absence of Cd (Fig. 5), once again mainly through an impact on the conjugated fraction. The highest total Cad was observed in response to 25 µM Cd + 400 mM NaCl. Cadmium alone slightly increased Cad concentration in both organs. In contrast, in the absence of salt, Cd strongly increased tyramine concentration in leaves and roots: at the highest Cd dose

Table 1
Sodium/potassium ratio (Na^+/K^+) and Cd^{2+} concentration in roots and leaves of *Inula crithmoides* exposed for 21 days to 0, 25 or 50 µM $CdCl_2$ combined with 0, 100 (S1), 200 (S2) or 400 (S3) mM NaCl. Each data is the mean of 5 replicates ± S.E. Values followed by similar letters are not significantly different at $P < 0.05$. ND: not detected.

	Na^+/K^+		Cd^{2+}, µg/g DW	
	Leaves	Roots	Leaves	Roots
Control	0.15 ± 0.06 a	0.34 ± 0.06 a	ND	ND
S1	2.91 ± 0.37 b	1.34 ± 0.29 bc	ND	ND
S2	5.06 ± 0.88 c	1.72 ± 0.25 cd	ND	ND
S3	10.97 ± 1.76 f	1.76 ± 0.16 d	ND	ND
25 µM Cd	0.26 ± 0.09 a	0.43 ± 0.17 a	335 ± 14 b	1288 ± 145 b
25 µM Cd + S1	5.83 ± 1.76 cd	1.57 ± 0.10 bcd	143 ± 25 e	765 ± 82 de
25 µM Cd + S2	7.62 ± 1.25 de	2.32 ± 0.39 e	114 ± 18 f	656 ± 89 e
25 µM Cd + S3	12.27 ± 1.17 f	4.23 ± 0.49 f	40 ± 12 h	382 ± 34 g
50 µM Cd	0.36 ± 0.09 a	0.39 ± 0.03 a	534 ± 20 a	3039 ± 72 a
50 µM Cd + S1	5.13 ± 1.73 c	1.22 ± 0.26 b	203 ± 9 c	1042 ± 77 c
50 µM Cd + S2	8.80 ± 2.27 e	2.43 ± 0.30 e	175 ± 9 d	867 ± 36 d
50 µM Cd + S3	14.70 ± 1.63 fg	4.38 ± 0.17 f	75 ± 30 g	522 ± 101 f

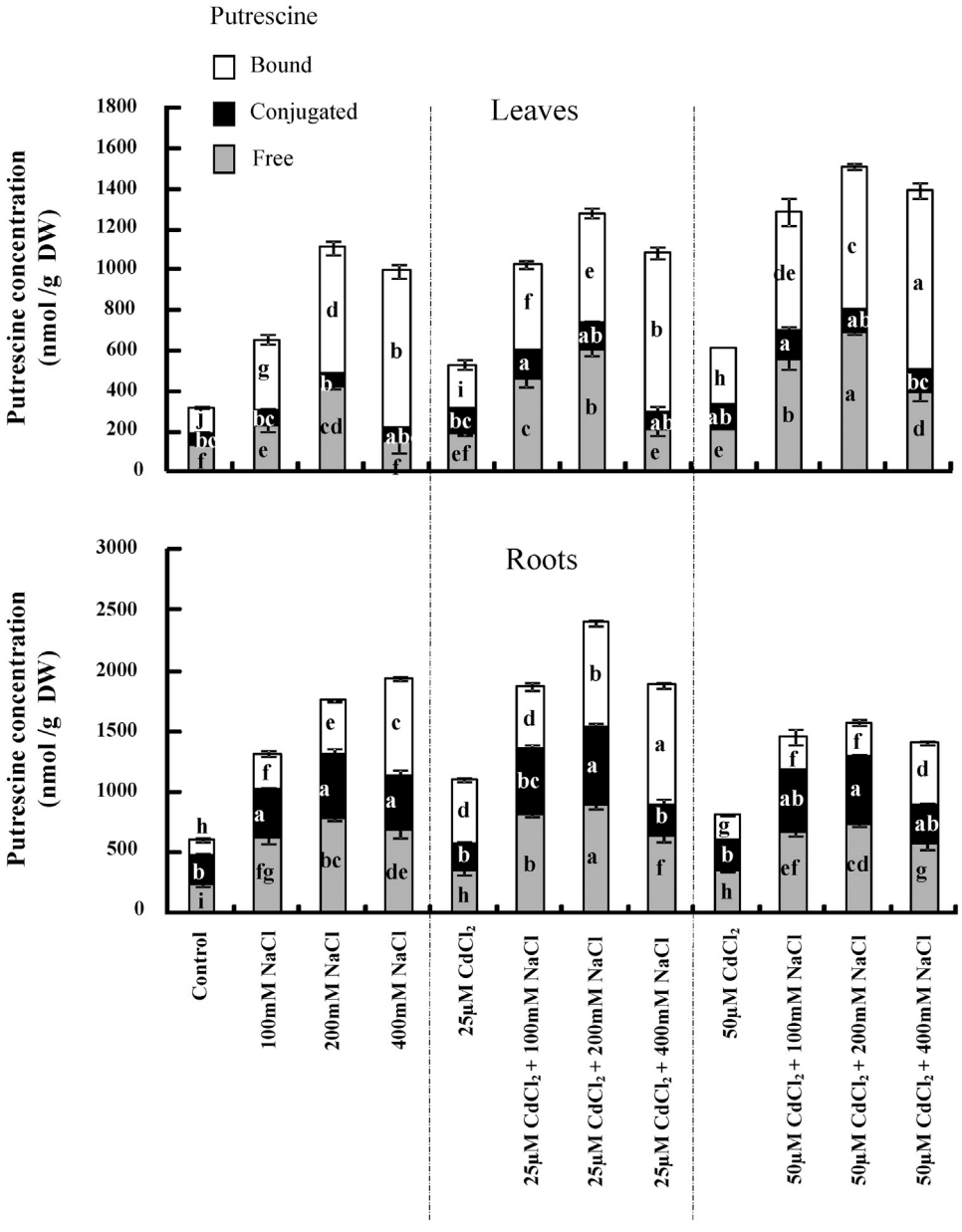

Fig. 2. Putrescine concentration in leaves and roots of *Inula crithmoides* exposed for 21 d to control nutrient solution or to nutrient solution containing 25 or 50 µM $CdCl_2$ combined or not combined with 100 or 200 or 400 mM NaCl in the nutrient solution. Each value is the mean of 3 replicates and vertical bars are S.E. of the means. For each fraction, values marked with the same letter are not significantly different at $P = 0.05$ according to Tukey HSD test. Note that vertical scales are not the same for leaves and roots.

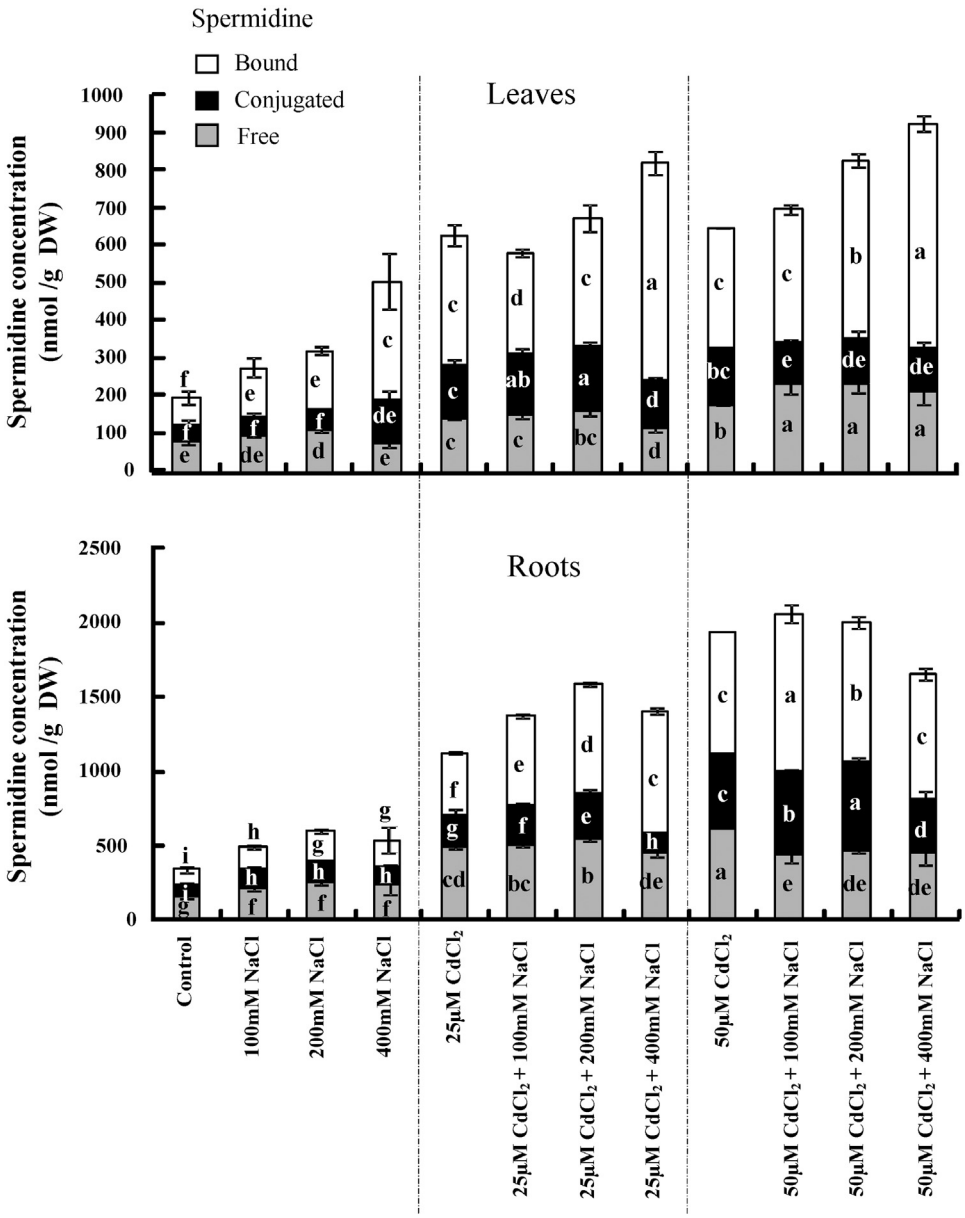

Fig. 3. Spermidine concentration in leaves and roots of *Inula crithmoides* exposed for 21 d to control nutrient solution or to nutrient solution containing 25 or 50 µM $CdCl_2$ combined or not combined with 100 or 200 or 400 mM NaCl in the nutrient solution. Each value is the mean of 3 replicates and vertical bars are S.E. of the means. For each fraction, values marked with the same letter are not significantly different at P = 0.05 according to Tukey HSD test. Note that vertical scales are not the same for leaves and roots.

(50 µM), total Tyr was increased by more than 900% in both organs and such an increase involved all fractions. Tyramine accumulation in both organ was reinforced in the concomitant presence of NaCl and Cd in the nutrient solution.

2.4. Ammonium and nitrate content

The leaf nitrate concentration was reduced in response to salinity and Cd stress (Fig. 6A). These effects were however not additive and plants exposed to the highest NaCl dose exhibited a similar NO_3^- content, whatever the exogenous Cd concentration. The leaf NH_4^+ was reduced by NaCl in the absence of Cd but salinity had no significant impact on Cd-treated plants (Fig. 6B). As an overall consequence, the leaf NH_4^+/NO_3^- ratio strongly increased in response to salinity while Cd had only a limited impact on this parameter (Fig. 6C).

Salinity also reduced NO_3^- concentration in the roots (Fig. 6D) while 25 µM Cd increased the root NO_3^- concentration in the presence of NaCl. Conversely 50 µM Cd significantly decreased the root NO_3^- in the absence of salt. Cadmium strongly increased the root NH_4^+ (Fig. 6E) concentration in the absence of NaCl while the presence of NaCl increased the root NH_4^+ concentration in the absence of Cd and decreased it in the presence of this heavy metal. Cadmium increased the root NH_4^+/NO_3^- ratio in the absence of NaCl but had a limited impact in salt-treated ones (Fig. 6F).

3. Discussion

3.1. Salinity plant growth and Cd resistance in Inula crithmoides

The present work confirms that *Inula crithmoides* typically behaves as an obligate halophyte plant species. High NaCl doses did not compromise plant survival while low NaCl level (100 mM) strongly improved plant growth. Such plant growth stimulation by exogenous NaCl was already reported in this species by Zurayk and Baalbaki (1996) and these authors even recommended *Inula chrithmoides* for the production of medicinal compounds in saline agriculture.

Salinity resistance is a complex property that requires the combination of several traits. Plant species regulate their internal Na^+ concentration through the control of influx and efflux. Our data show that salinity resistance in *Inula crithmoides* is not related to Na^+ exclusion

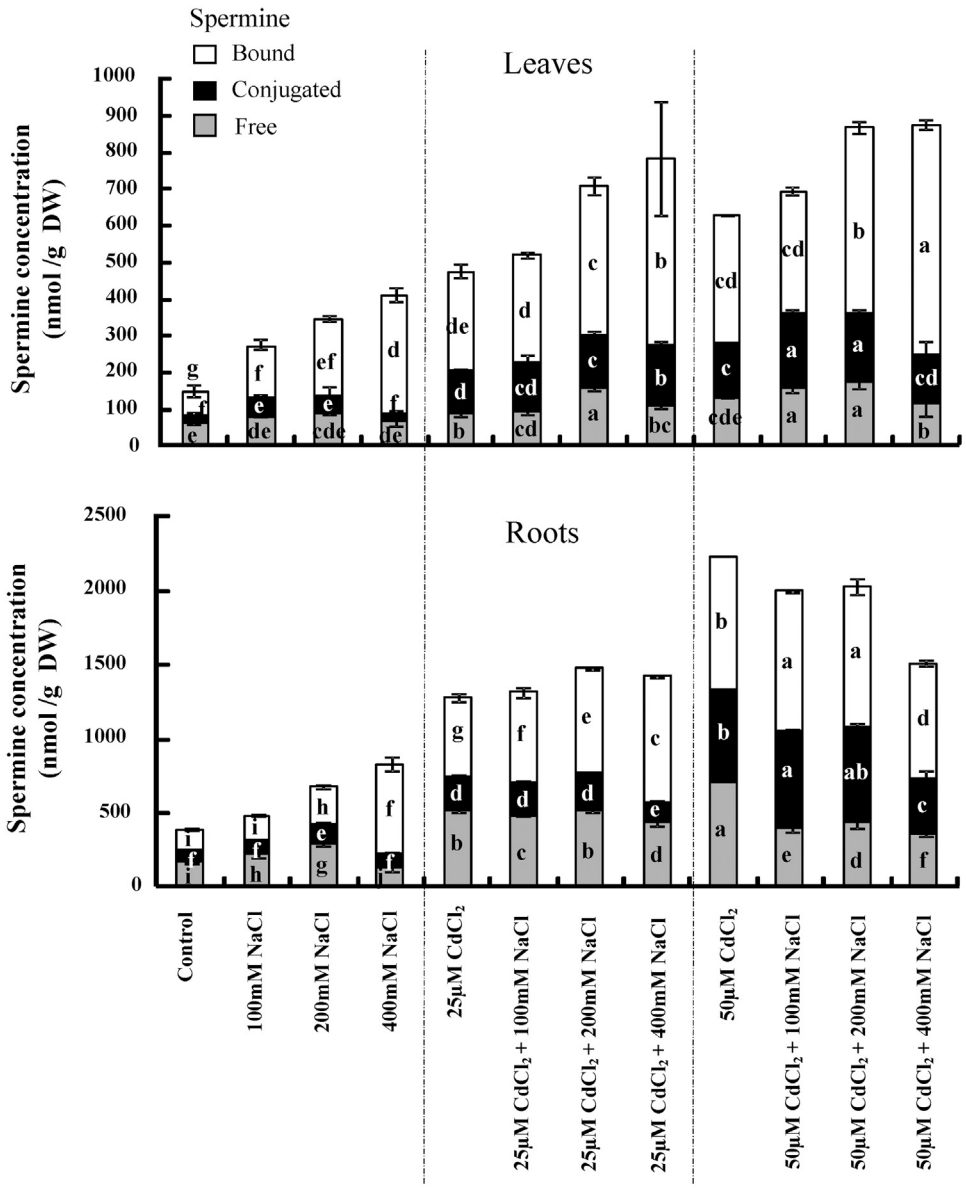

Fig. 4. Spermine concentration in leaves and roots of *Inula crithmoides* exposed for 21 d to control nutrient solution or to nutrient solution containing 25 or 50 µM $CdCl_2$ combined or not combined with 100 or 200 or 400 mM NaCl in the nutrient solution. Each value is the mean of 3 replicates and vertical bars are S.E. of the means. For each fraction, values marked with the same letter are not significantly different at P = 0.05 according to Tukey HSD test. Note that vertical scales are not the same for leaves and roots.

and maintenance of K^+ selectivity. Although we have not added NaCl in the nutrient solution, plants cultivated in control conditions exhibited low Na^+ content in the shoot (0.23 mmol/gDW), mainly due the presence of Na^+ in medium as impurity or due to the fact that the cuttings were harvested from field conditions and that the initial Na^+ content was slightly diluted during plant growth in the absence of salt. Under this condition (Without NaCl), the increase of the ration Na^+/K^+ with increasing Cd concentration could be attributed to the Cd-induced reduction of K^+ absorption and translocation in these plants as suggested by Ghnaya et al., 2007 in other halophytes as *Sesuvium portulacastrum* and *Mesembryanthemum crystallinum*. In the presence of NaCl, the Na^+ endogenous content strongly increased in all plant parts concomitantly with a K^+ decrease and growth stimulation, suggesting that salt tolerance in *I. crithmoides* is related to Na^+ tolerance mechanisms. In NaCl-treated plants, the shoot Na^+ was even higher than in roots, and Na^+ thus appears to be specifically translocated to the aerial parts in this plant species.

Plant nutrition depends on the activity of numerous mineral transporters and the homeostasis of K^+ and Na^+ is under a complex regulation network (Pardo et al., 2006). Some members of the CHX (Cation Proton Exchanger) family, such as CHX21, have been reported to specifically translocate Na^+ (Hall et al., 2006). Identification of similar transporters in *I. crithmoides* may provide information on the mechanisms involved in Na^+ accumulation and localization in this species. Potassium play key role in plant metabolism; even in halophyte plant species, Na^+ is not supposed to substitute efficiently to K^+ in essential functions (Ruan et al., 2010). Sodium, however, may accumulate in some specific compartments such as vacuoles and thus contribute to cell turgor allowing cellular expansion and plant growth (Lutts and Lefèvre, 2015).

Salinity not only increased plant growth in uncontaminated solution but also obviously increased plant resistance to toxic Cd concentrations. Such an increase was directly related to a decrease in Cd absorption and accumulation in plant tissues. In the presence of salt, Cd may partly be present in the solution as $CdCl^+$ and even undissociated $CdCl_2$ and these chemical species are less available for plant absorption than Cd^{2+} (Lefèvre et al., 2009). However, the recorded decrease in salt-treated plants was by far higher than the decrease in Cd^{2+} availability estimated by VisualMinteq software (Lutts et al., unpublished results). We therefore postulate that NaCl may improve the capacity of *I. crithmoides* to exclude Cd but also to cope with accumulated Cd in plant organs.

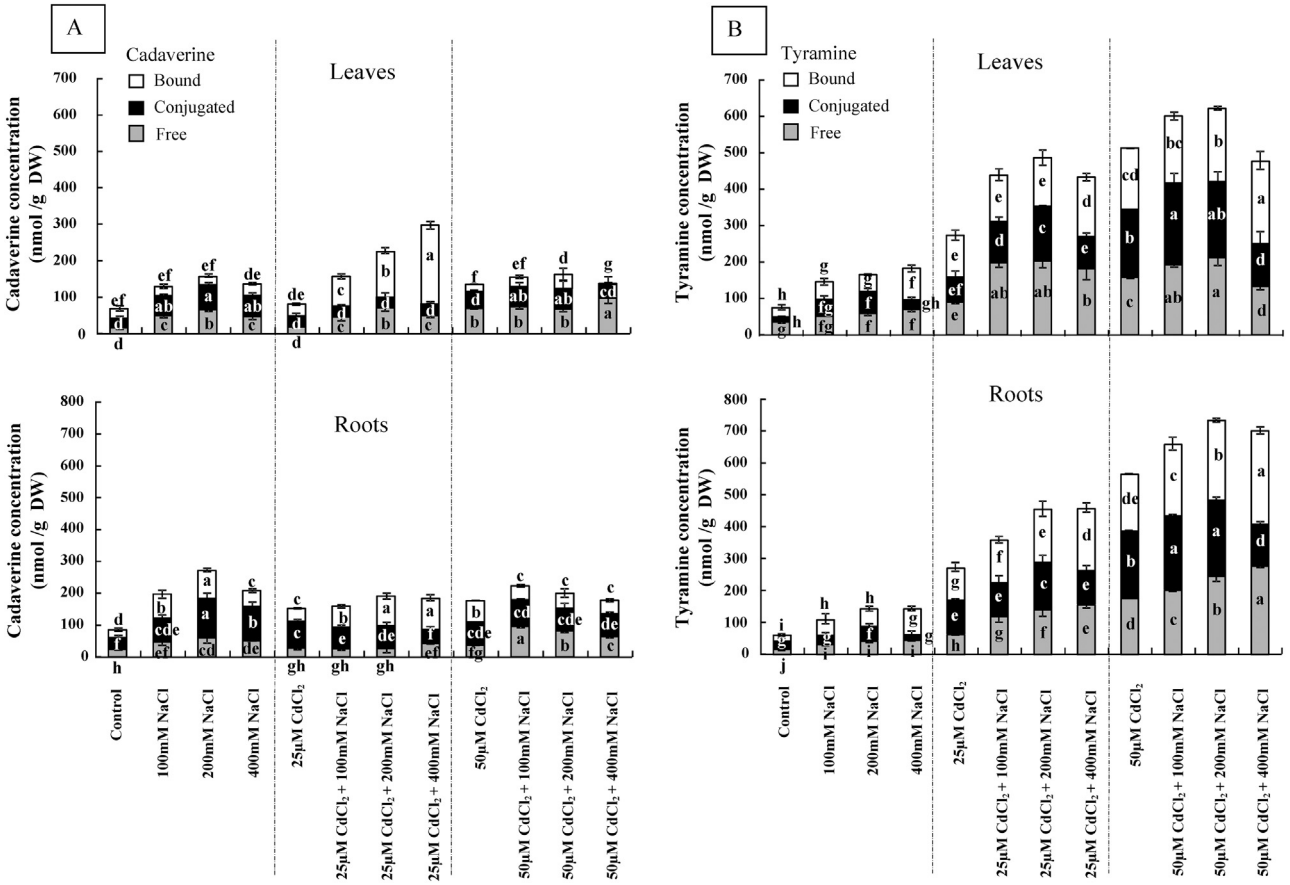

Fig. 5. Concentration of Cadaverine (A) and Tyramine (B) in leaves and roots of *Inula crithmoides* exposed for 21 d to control nutrient solution or to nutrient solution containing 25 or 50 μM $CdCl_2$ combined or not combined with 100 or 200 or 400 mM NaCl in the nutrient solution. Each value is the mean of 3 replicates and vertical bars are S.E. of the means. For each fraction, values marked with the same letter are not significantly different at P = 0.05 according to Tukey HSD test. Note that vertical scales are not the same for leaves and roots.

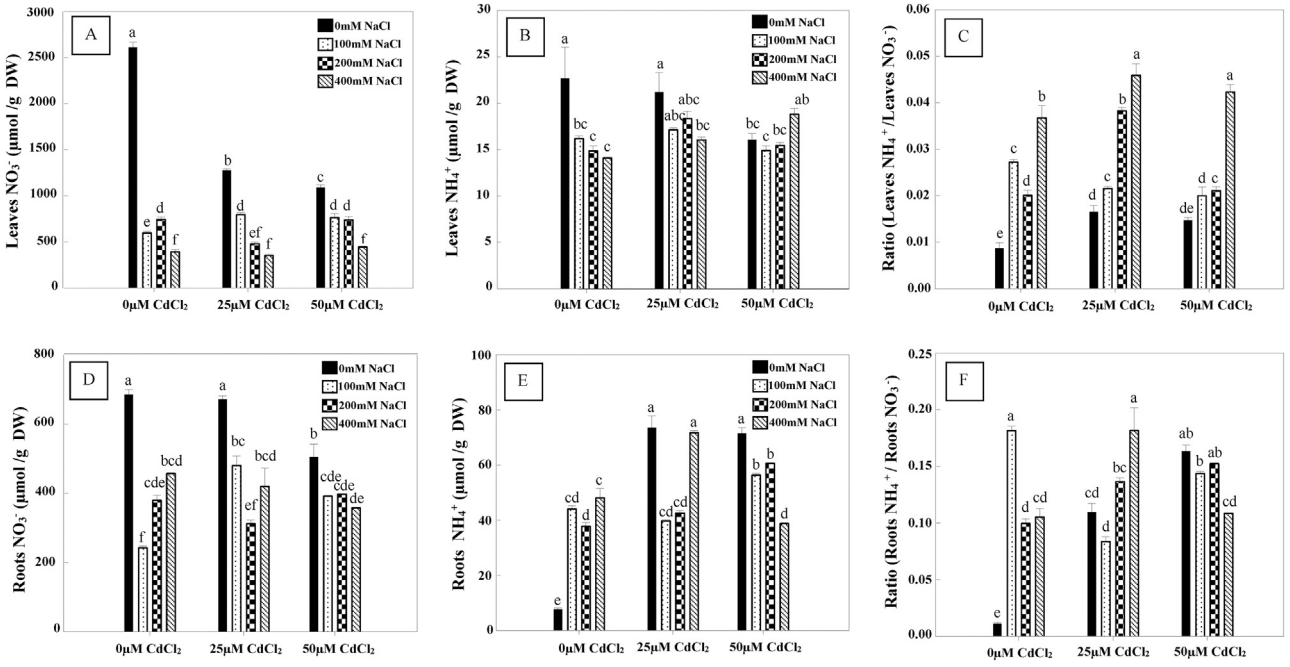

Fig. 6. Leaves nitrate (A) ammonium (B), ammonium/nitrate ratios (C) as well as root nitrate (D), ammonium (E) and ammonium/nitrate (F) *Inula crithmoides* exposed for 21 d to control nutrient solution or to nutrient solution containing 25 or 50 μM $CdCl_2$ combined or not combined with 100 or 200 or 400 mM NaCl in the nutrient solution. Each value is the mean of 3 replicates and vertical bars are S.E. of the means. Values sharing a common letter are not significantly different at P < 0.05.

3.2. Modulation of polyamine synthesis by salt and cadmium toxicity

Endogenous polyamines appear as interesting candidates for the modulation of plant response to NaCl. Salt-induced plant growth stimulation was indeed associated to an increase in Put, Spd and Spm concentrations. These low-molecular-weight aliphatic amines are involved in plant survival to adverse environmental conditions (Gupta et al., 2013; Lutts et al., 2013; Minocha et al., 2014; Tiburcio et al., 2014; Liu et al., 2015; Pál et al., 2015). Polyamines were reported to regulate antioxidant system and contribute to decrease ROS production (Liu et al., 2015; Sudhakar et al., 2015), to be directly involved in abiotic stress signaling (Pál et al., 2015), to influence plant growth and development (Lutts et al., 2013; Quinet et al., 2010) and to trigger various adaptations of hormonal synthesis to stress conditions (Radhakrishnan and Lee, 2013). Because of their polycationic nature at physiological pH, PAs bind strongly to negative charges in cellular components such as nucleic acids, proteins and phospholipids. It may thus be hypothesized that the protective functions of PAs should be related to a stress-induced increase of the bound fraction. Indeed, NaCl-induced increase in endogenous PAs titters in *I. crithmoides* mainly concerned bound fraction not only in the leaves but also in the roots. A similar response was observed in the presence of Cd toxicity and it may therefore be argued that *I. crithmoides* used similar responses to cope with distinct toxic ions. Owing to their protective functions and stabilizing properties, bound PAs may trigger a wide range of adaptation in stressed plants. Spermine was found to regulate the stabilization of Cd-accrued DNA methylation (Kumar et al., 2012). Polyamines may also regulate plant membrane transport through a direct interaction with plasmamembrane or vacuolar transporters (Pottosin and Shabala, 2014; Pottosin et al., 2014) and might thus be involved in Cd^{2+} exclusion and in Na^+ vacuolar compartmentation. It has also been demonstrated that PAs afford specific protection to photosynthetic tissues. Spermine has been shown to allow fluorescence quenching in light-harvesting antenna and it therefore contributes to photoprotection through non-photochemical quenching (Malliarakis et al., 2015). Bound Spd and Spm are commonly found in PSII and LHCII of higher plants, whereas free Put accumulates in the lumen during photosynthesis (Ioannidis et al., 2012). Legocka et al. (2015) recently demonstrated that PAs play a role in the structural organization and functional activity of thylakoids in chloroplasts of Pb-stressed tissues.

Beside PAs, the aromatic monoamine tyramine was also reported to strongly increase in response to NaCl and Cd. The highest concentration of Tyr was observed when both salt and Cd are present. A salt-induced increase in Tyr was also observed in tomato where it appears as a specific consequence of Na^+ accumulation (Aziz et al., 1999). Tyramine accumulation may trigger the synthesis of osmocompatible proline (Aziz et al., 1998) and its conjugation was reported in response to pathogen (Hohlfeld et al., 1996), UV-C (Back et al., 2001) and wounding (Borg-Olivier and Monties, 1993). To the best of our knowledge, this is the first report mentioning Tyr accumulation in response to Cd stress. Conjugated tyramine is directly involved in cell wall fortification and may directly regulate lignifications and suberization processes (Borg-Olivier and Monties, 1993; Back et al., 2001). It may thus influence turgor properties. Cell wall may also act as a binding compartment which efficiently contributes to Cd detoxification through fixation of toxic divalent cations on negatively charged polymers (Krzesłowska, 2011). Further studies are required to determine the precise role of Tyr in *I. crithmoides* adaptation to NaCl and Cd stress.

The diamine Cad is a lysine catabolite which also influences plant growth and development (Tomar et al., 2013). It was reported to accumulate in salt-treated tomato (Aziz et al., 1999) while its accumulation remained limited in *I. crithmoides*, except in roots exposed to NaCl in the absence of Cd, and in shoots exposed concomitantly to salt and 25 μM $CdCl_2$ stress where bound Cad was reported to increase.

3.3. Impact of Cd and NaCl on N nutrition and link with polyamine synthesis

In the absence of Cd or in the presence of the lowest Cd dose, salinity tended to increase NH_4^+/NO_3^- ratio. Salt-induced decrease in NO_3^- may be related to Cl^- competition at the absorption sites. Toxic levels of ammonium in plant tissues accumulate when the overall rate of conversion of ammonium to amino acids become lower than the uptake rate. Ammonium may also be produced by amino acid catabolism, nitrate reduction, phenylpropanoid metabolism and photorespiration (Bittsánsky et al., 2015). *Inula crithmoides* displays a C3 photosynthetic pathway (Atia et al., 2014) and may thus present high level of photorespiration especially in the presence of low external water potential such as those occurring in salt conditions and which are responsible for partial stomatal closure. A similar decrease in stomatal conductance is also reported in response to Cd stress as a direct consequence of alteration in the overall plant water status (Lutts and Lefèvre, 2015).

The PAs (Put, Spd, Spm) are major sinks of assimilated N (Moschou et al., 2012). It is tempting to speculate that stress-induced increase in NH_4^+ may trigger the recorded increase in endogenous PAs. Excess NH_4^+ often results in increased Put biosynthesis and accumulation and N flow towards PAs may thus limit NH_4^+ excess and toxicity (Moschou et al., 2012). Excess ammonium may be integrated in carbamoyl phosphate which is converted to urea, this compound being partly converted to arginine, one of the Put precursors. Cadmium was reported to decrease glutamine synthetase and glutamate synthase activities involved in NH_4^+ assimilation, and to concomitantly increase NH_4^+ and arginine content in rice (Li et al., 2013). According to these authors, the Cd-induced alteration in N cycle was the main responsible for modification of PAs in this species. On the other hand, nitrogen may be recycled via polyamine oxidation. Copper amine oxidase catalyzes the oxidation of diamines Put and Cad at their primary amino group. It has been suggested that salinity may influence PAs translocation from cytosol to apopalsm, where diamine and polyamine oxidases are located. Further work are needed to clearly assess to which extent PAs accumulation in salt- and Cd-treated *Inula crithmoides* is related to inhibition of PAs catabolism and/or to stimulation of PAs synthesis.

4. Conclusions

Inula crithmoides displays a fascinating capacity to cope with high external doses of salt and to tolerate high Na^+ concentration in its aerial part. Salinity affords protection against $CdCl_2$ through a decrease in Cd absorption and accumulation, and through the synthesis of protective polyamines and tyramine. Bound polyamines may assume positive functions in stressed tissues and their highest concentration was observed for plants simultaneously exposed to NaCl and $CdCl_2$. Ammonium accumulation may assume functions in triggering polyamines synthesis in stressed tissues.

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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.jplph.2017.05.018.

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