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Full Length Research Paper

Calcium enhances cadmium tolerance and decreases cadmium accumulation in lettuce *(Lactuca sativa)*

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We aimed at characterizing mechanisms controlling cadmium accumulation in lettuce, which is a food crop showing one of the highest capacities to accumulate this toxic compound. In this study, plants from three lettuce varieties were grown for eight days on media supplemented or not with cadmium (15 µM $CdCl_2$) and containing different concentrations of calcium (0.5, 1, 2.5, 5 and 10 mM $Ca(NO_3)_2$). Our results show that exposure to cadmium resulted in biomass reduction. The biomass reduction was particularly high at 0.5 mM calcium but supplementation of the medium with increasing calcium concentrations alleviated the toxic effect of cadmium on the growth and water status of lettuce plants. The three lettuce varieties displayed different abilities to accumulate cadmium. Interestingly, increasing the calcium concentration in the medium resulted in a strong decrease in cadmium contents. These results suggest that cadmium uptake in lettuce plants is negatively associated with the presence of calcium in the culture medium, maybe due to a competition between these two cations for binding and absorption sites in roots. In conclusion, the results suggest that fertilization with Ca^{2+} appears to be a promising strategy for decreasing risk associated with ingesting food crops grown on cadmium polluted soils.

Key words: Lettuce, food security, growth, cadmium accumulation, cadmium translocation, calcium.

INTRODUCTION

Since the industrial revolution, impact of industrial and agricultural activities on the environment has not stopped growing. While many organic molecules can be degraded, heavy metals remain in the environment, thus their concentrations are continuously increasing, particularly in upper horizons of the soils and in water resources. Cadmium (Cd) is an extremely toxic heavy metal that is often referred as the metal of the 20th Century. It is widely used in industry, principally in galvanizing and electroplating, in batteries, in electrical conductors, in the manufacture of alloys, pigments, plastics, and in the stabilization of phosphate fertilizers (Byrne et al., 2009). Cadmium is listed by the US Environmental Protection Agency as one of the 126 priority contaminants and as a human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Cadmium has well-established renal, bone, and pulmonary effects, with less conclusive evidence for neurotoxic, teratogenic, and endocrine-disrupting effects (Godt et al., 2006; Nordberg et al., 2007).

Plants growing in contaminated soils can absorb and accumulate cadmium in edible tissues, thereby introducing the metal into the food chain by trophic transfer, including the human diet. As a non-essential element for plants, cadmium has been assumed to be taken up by plants thanks to the lack of ion specificity of transporters involved in the uptake of essential elements (Clemens, 2006). For instance, cadmium, which is an analogue of calcium (Jacobson and Turner, 1980), was shown to be transported into or between plant cells through calcium transport systems (Hirschi et al., 1996; Perfus-Barbeoch et al., 2002; Antosiewicz and Hennig, 2004). Thus, it has been demonstrated that putative

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tonoplast Ca^{2+}/H^+ antiporters encoded by calcium exchanger 1 (CAX1) and calcium exchanger 2 (CAX2) from Arabidopsis are involved in the transport of cadmium from the cytoplasm to the vacuole (Pittman et al., 2004; Pittman et al., 2005; Manohar et al., 2011). Another non-selective trans-membrane transporter of Ca^{2+}, the low affinity cation transporter (LCT1) expressed in wheat, also appears to mediate Cd^{2+} transport into the cell (Clemens et al., 1998). Antosiewicz and Hennig (2004) showed that overexpression of LCT1 in tobacco enhances the protective action of calcium against cadmium toxicity and decreases cadmium accumulation in roots. Indeed, these results are the first to demonstrate the involvement of LCT1 in calcium acquisition and in diminishing Cd-toxicity by calcium. Exposure of mankind to cadmium mainly results from eating cadmium-contaminated food. Indeed, over 80% of the dietary cadmium intake has been estimated to come from cereals (especially rice and wheat), vegetables (especially leafy greens), and root vegetables (especially potatoes and carrots) (Järup and Åkesson, 2009). Among cultivated plant species, lettuce (*Lactuca sativa*) is known for displaying comparatively high cadmium contents in leaves (Mensch and Baize, 2004; Kim et al., 1988). Hence, it has been proposed as an indicator crop for testing the potential human dietary risk associated with ingesting food crop grown on cadmium polluted soils (Brown et al., 1996). In this context, lettuce is a good model both to study the mechanisms responsible for cadmium accumulation in tissues and to develop breeding strategies aiming at decreasing cadmium accumulation in crop plants. Up to now, researches are mainly focused on the effects of cadmium on growth of lettuce plants, as well as on the mechanisms of cadmium absorption by roots and cadmium distribution within different organs (Thys et al., 1991; Costa and Morel, 1994; Ramos et al., 2002). Moreover, interactions between cadmium and other elements such as manganese, calcium, zinc and iron, have been reported in lettuce (Ramos et al., 2002; Monteiro et al., 2009; Zorrig et al., 2010). This study aimed at analysing the impact of calcium on the accumulation of cadmium in three lettuce varieties exhibiting contrasting features for the characters of cadmium accumulation and cadmium translocation from roots to shoots.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of three lettuce varieties analysed in this study (Paris Island Cos, Fenja and Red Salad Bowl) were kindly provided by Dr. B. Maisonneuve (INRA Avignon, France). All accessions were pure lines. Seeds were germinated on sterile Whatmann paper humidified first with distilled water for five days and then with a nutrient solution for five additional days. The nutrient solution contained 2.5 mM KNO_3, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 0.1 $Fe_{III}NaEDTA$, 0.05 mM H_3BO_3, 0.05 mM $MnSO_4$, 15 µM $ZnSO_4$, 3 µM Na_2MoO_4, 2.5 µM KI, 0.05 µM $CuSO_4$ and 0.044 µM $CoCl_2$.

Different calcium treatments were applied (0.5, 1, 2.5, 5 and 10 mM Ca $(NO_3)_2$). Ten-day-old plantlets were transferred onto a floating support and grown in hydroponic condition. For every set of 24 plantlets, 8 L of aerated nutrient solution was used. Four days later, $CdCl_2$ was added to the culture medium at the final concentration of 15 µM and maintained for eight additional days. A control assay without cadmium addition was in parallel but only for the 0.5, 2.5, and 10 mM Ca $(NO_3)_2$ treatments. Nutrient solution was changed every four days over the course of the entire experiment. Growth conditions were adjusted to 20°C, 70% relative humidity and 16:8 h light-dark cycle with light intensity being 150 $\mu mol.m^{-2}.s^{-1}$. For each calciumXcadmiumXvariety condition, seven plants were considered and distributed following a randomized complete block design. At the end of the experiment, roots were dipped for a dozen of seconds in three separate ice-cold 0.5 mM $CaCl_2$ solutions to remove the cadmium adsorbed on the root surface. Then roots were gently dried between two layers of filter paper. Analyses were performed on individual plants, roots and shoots being analysed separately. There were thus seven repeats for each calcium x cadmium x variety condition.

Cadmium assays

After the fresh weight measurements, plant samples were dried at 80°C for 48 h. Samples were incubated in 1 N H_2SO_4 at 80°C for 30 min to extract cadmium (Zorrig et al., 2010). Concentration of cadmium in the extracts was determined by atomic absorption spectrophotometry (SpectrAA 220, Varian and Australia) using the deuterium background correction. Following preliminary assays, extracting cadmium from lettuce tissues using this procedure was proven to be as efficient as recovering cadmium following a complete acidic digestion (data not shown). A series of standard solutions was prepared (0, 0.3, 0.5, 0.7, 1, 2, 3, 4; and 5 mg/l of Cd^{2+}). The absorbances of the standard solutions were measured (228.8 nm) and used to prepare a calibration curve, which is a graph showing how the experimental observable (the absorbance in this case) varies with the cadmium concentration. The points on the calibration curve should yield a straight line (Beer's Law). The slope and intercept of that line provide a relationship between absorbance and cadmium concentration. The data obtained with the Varian SpectrAA 220 was manipulated by the SpectrAA software. The precision of the value of the cadmium concentration is given by the SpectrAA software. The “bad” samples were passed after the adaptation of dilution. Cadmium concentration (expressed in µg/g of dry weight) was calculated on entering the parameters; final volume after dilution, dilution factor and mass of the digested sample.

Statistical analyses

One-way ANOVA was used for parametric or nonparametric comparison of means. Significant differences were further analysed using Turkey’s parametric or nonparametric tests to identify differences between accessions. All these tests used an alpha of 0.05 and were done with XLSTAT software v. 2011 (www.xlstat.com). A principal component analysis (PCA) was done using XLSTAT, considering variables centred on their means and normalized with a standard deviation of 1.

RESULTS

Effect of cadmium on lettuce growth according to calcium concentration in the medium

In order to analyse the effects of cadmium on lettuce growth according to calcium concentration in the medium,

plants from the Paris island Cos, Fenja and Red Salad Bowl varieties were exposed to different calcium treatments (0.5, 1, 2.5, 5 and 10 mM Ca $(NO_3)_2$) with or without cadmium at the final concentration of 15 µM. Exposure to increasing calcium concentrations resulted in both shoot and root biomass stimulation, especially for the Paris Island Cos variety (Figure 1). This stimulation was observed in whatever cadmium in the culture medium. The three varieties displayed similar overall responses regarding cadmium toxicity symptoms. Indeed, exposure to cadmium resulted in both shoot and root biomass reduction. However, the cadmium-induced biomass reduction was particularly high at 0.5 mM calcium (Figure 1); the cadmium-treated plants exhibiting exceptionally severe symptoms of dehydration, chlorosis and necrosis (data not shown). Our results clearly show that lettuce plants are more challenged by cadmium at low calcium concentrations in the culture medium than at higher ones: increasing calcium concentration in the medium protected plants against cadmium toxicity (Figure 1).

Cadmium accumulation and translocation

Cadmium contents were measured in roots and shoots for the three lettuce varieties. Root and shoot cadmium contents decreased in response to increasing concentrations of calcium in the growth medium (Figure 2A, B). However, the three varieties displayed a differential ability to accumulate cadmium in their roots as well as in their shoots: Paris Island Cos displayed the lowest cadmium contents in roots and shoots in nearly all conditions while Fenja displayed the highest ones. The greatest between-variety differences were observed for plants grown in the presence of 0.5 mM calcium. We then checked whether the inter-varietal differences in root and shoot cadmium contents could be related to different strategies with respect to cadmium translocation from roots to shoot. Cadmium translocation from roots to shoots was very stable in Fenja and Red Salad Bowl across all calcium treatments: ~ 57% of the cadmium present in Fenja plants was in the shoot, while this proportion was ~ 72% for Red Salad Bowl plants. In contrast, Paris Island Cos plants show cadmium translocation from roots to shoots slightly decreased from 70 to 62% in response to increases in external calcium concentration (Figure 2C). This might reveal different strategies developed by the three lettuce varieties to cope with cadmium.

Combined analyses of the different cadmium-related traits

The trait by trait analyses were completed by a (PCA) as well as by a correlation analysis, which took into account all the analysed traits characterising plants submitted to the cadmium treatment: water and cadmium contents, cadmium translocation from the roots to the shoot, as well as of calcium concentration in the medium. These analyses confirmed the previously observed negative correlation linking calcium concentration in the culture medium and root and shoot cadmium contents, which were themselves highly correlated to each other (Figure 3 and Table 1). These analyses also confirmed the lack of effect of the calcium concentration in the culture medium on the translocation of cadmium from the roots to the shoot. The new information is the positive correlation linking calcium concentration in the medium and the shoot water content. Differences between the three lettuce varieties clearly appeared. Paris Island Cos displayed rather average performances even if it was characterised by an ability to limit cadmium accumulation in roots. In contrast, Red Salad Bowl and Fenja displayed more contrasted phenotypes. Red Salad Bowl was for instance characterized by a high translocation of cadmium from roots to shoot, in contrast to Fenja.

DISCUSSION

This study aimed at characterising the impact of the calcium concentration of the culture medium on the cadmium response of lettuce plants. Three varieties were comparatively analysed; Paris Island Cos, Red Salad Bowl and Fenja. The three varieties displayed similar overall responses to cadmium that is shoot and root biomass reduction, chlorosis, necrosis and decreases in relative water content. This was in agreement with previous reports showing that cadmium displays a large panel of toxic effects: restriction of photosynthesis, decrease in chlorophyll contents, induction of oxidative stress, or alteration of plant water status (Mobin and Khan, 2007; López-Millán et al., 2009; Razinger et al., 2008; Szőllősi et al., 2009; Perfus-Barbeoch et al., 2002; Zorrig et al., 2010). Our results reveal that calcium counteracted the toxic effect of cadmium on growth as well as on the water status in lettuce. A similar result had already been obtained in beet or in *Trifolium repens* (Greger and Bertell, 1992; Wang and Song, 2009). Given the known broad spectrum of calcium contribution to the regulation of metabolic processes (Bush, 1995), it is not known whether the observed calcium-induced reduced cadmium toxicity might result from less cadmium uptake or from more efficient detoxification. In our study, the presence of calcium in the culture medium leads to a decrease in the cadmium content in tissues.

Numerous authors have described that calcium may decrease the uptake, translocation and accumulation of cadmium in plants (Jarvis et al., 1976; Kawasaki and Moritsugu, 1987; Wallace et al., 1980; Tyler and McBride, 1982; Österås and Greger 2003; Österås and Greger, 2006). Some of these results were shown on soil (Österås and Greger, 2003; Österås and Greger, 2006).

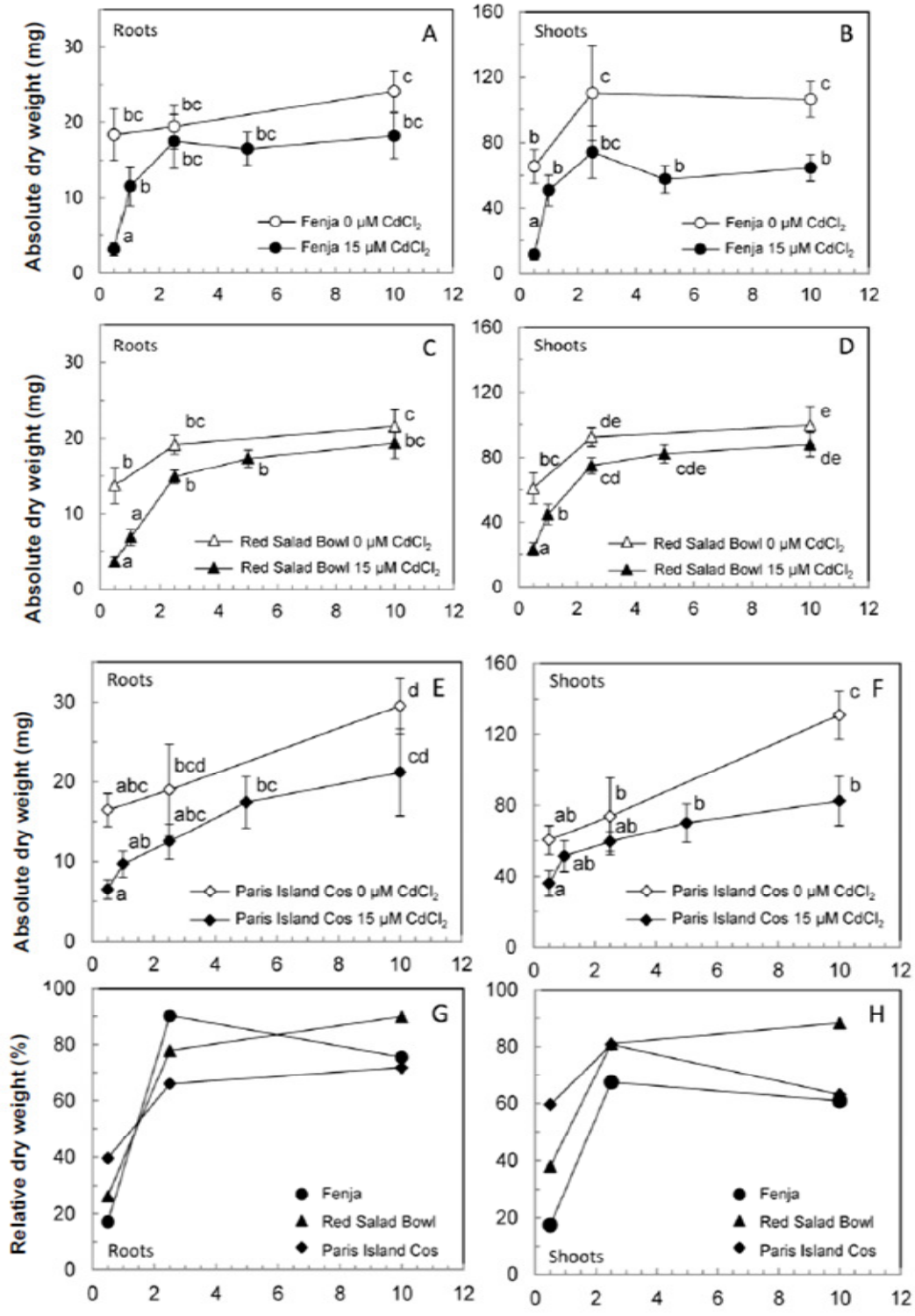

Figure 1. calciumium protection of growth of lettuce plants from cadmium. Absolute root and shoot dry weight (A-F) and relative root and shoot dry weight (G,H) were measured in Fenja (A,B,G,H), Paris Island Cos (C,D,G,H) and Red Salad Bowl (E-H) lettuce plants grown in media supplemented or not with $CdCl_2$ at the final concentration of 15 µM and harbouring increasing calcium concentrations. In G and H, relative dry weights correspond to the ratio between dry weight of plants grown in the presence of cadmium and dry weight of control plants. Measurements were performed on individual plants (Mean ± S.E) n=7. Bars marked with same letter are not significantly different at p = 0.05.

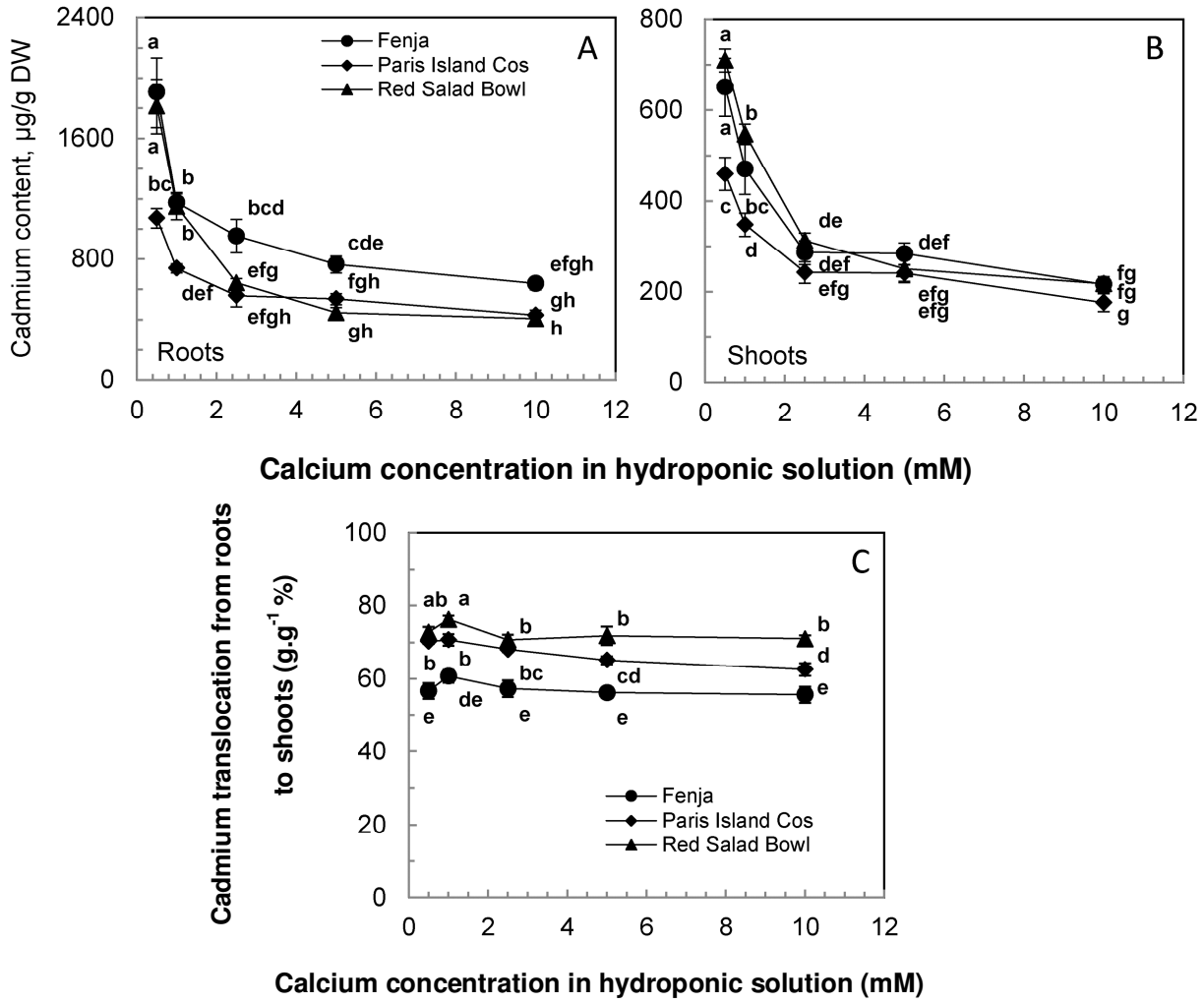

Figure 2. calcium prevention of cadmium accumulation in lettuce plants. The effect of increasing the calcium concentration in the culture medium on the cadmium content of roots (A) and shoots (B) as well as on the cadmium translocation from roots to shoots (C) is reported for Fenja, Paris Island Cos and Red Salad Bowl lettuce plants. Cadmium translocation from roots to shoots is expressed as the amount of cadmium in the shoot relative to the total amount of cadmium accumulated in the plant; it is expressed in percentage. Measurements were performed on individual plants (mean± S.E.; n=7). Bars marked with same letter are not significantly different at p = 0.05.

In fact, because Ca^{2+} and Cd^{2+} ions compete for binding sites, their concentration in the uptake solution are decisive for the resulting transport across a membrane (Kim et al., 2002; Lu et al., 2008; Lu et al., 2010). Cd^{2+} (and other non-essential metal ions) is supposed to enter into plant cells through systems devoted to the uptake of essential cations. Cadmium and calcium are chemically very similar (Jacobson and Turner, 1980). Raising the Ca^{2+} concentration was shown to block both Cd^{2+} transport into rice roots as a result of the competition of Cd^{2+} with Ca^{2+} for calcium transporters (Clemens et al., 1998; Clemens, 2006). Ca^{2+} channels have for long been shown to be involved in Cd^{2+} uptake into mammalian cells (Hinkle et al., 1992). Furthermore, Perfus-Barbeoch et al. (2002) showed that guard cell Ca^{2+} channels are permeable to Cd^{2+}. More recently, it has been demonstrated that putative tonoplast Ca^{2+}/H^{+} antiporters encoded by calcium exchanger 1 (CAX1) and calcium exchanger 2 (CAX2) from Arabidopsis are involved in the transport of cadmium from the cytoplasm to the vacuole (Pittman et al., 2004; Pittman et al., 2005; Manohar et al., 2011). Alternatively, calcium may protect cells against cadmium through other mechanisms. Choi et al. (2001) described the contribution of calcium in immobilizing cadmium as co-precipitates with calcium and phosphorous. Recently, Tian et al. (2011) demonstrated that calcium protects *Sedum alfredii* plants against cadmium-induced oxidative stress.

Paris Island Cos and Fenja plants exhibited a significant difference with respect to cadmium

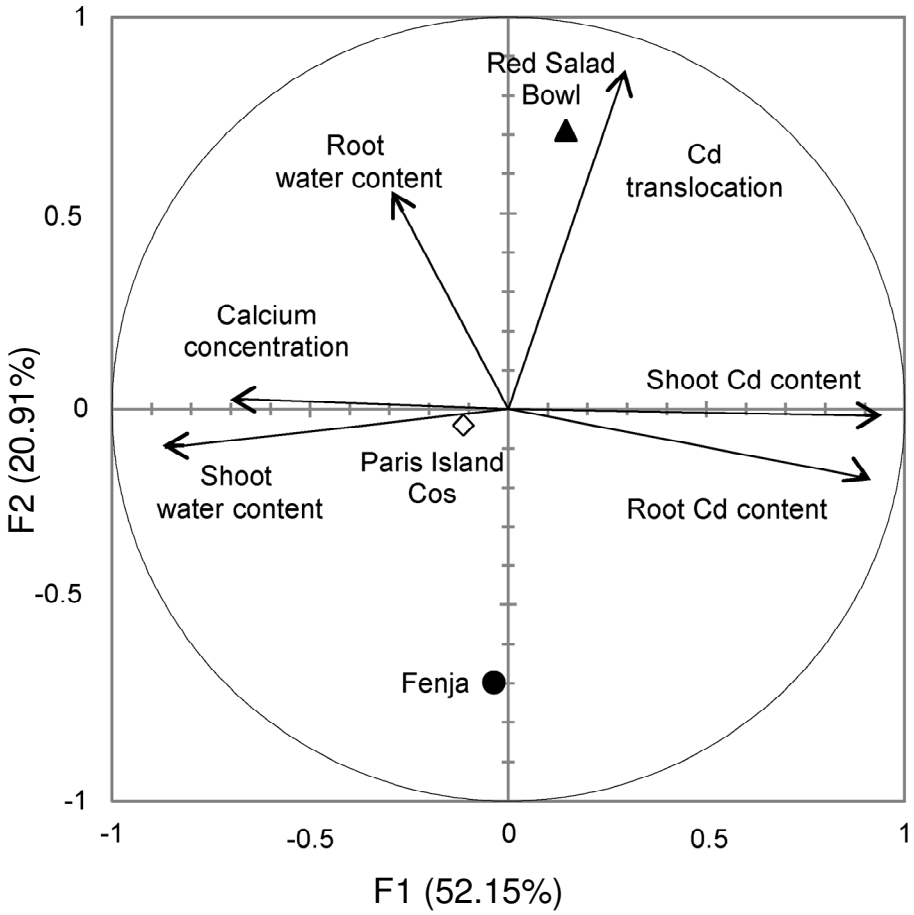

Figure 3. Principal component analysis of water content, cadmium content and cadmium translocation from the roots to the shoot for the three *L. sativa* varieties as well as of calcium concentration in the medium. The six variables (arrows) as well as the three different varieties are projected onto the F1-F2 principal factorial plane that explains 73% of the variation.

accumulation as well as to tolerance to cadmium. This variability might be explained by different abilities to limit cadmium net uptake at the root level, as already suggested in lettuce (Zorrig et al., 2010). However, since increasing the calcium concentration in the culture medium resulted in a similar effect on the cadmium content in the tissues for the two varieties, it seems that these varieties do not differ with respect to the mechanism by which calcium interacts with cadmium. The lettuce varieties also differed in their translocation of cadmium from the roots to the shoot. Changing the calcium concentration in the culture medium had however almost no impact on this trait. In Arabidopsis, cadmium tran-slocation from roots to shoots is known to depend mainly on the activity of the HMA2 and HMA4 heavy metal P_{1B}-type ATPase, which control cadmium loading into, and unloading from the

xylem sap (Verret et al., 2004; Wong et al., 2009). No report describes any role of calcium in influencing the transport properties of these ATPases. If the lettuce orthologues of *AtHMA2* and *AtHMA4* also play a critical role in the root to shoot transport of cadmium and display the same characteristics as *AtHMA2* and *AtHMA4*, it is probably not surprising that calcium had no impact on the translocation of cadmium from the roots to the shoot.

Conclusion

The results show that calcium enhances cadmium tolerance and decreases cadmium accumulation in lettuce. Fertilization with Ca^{2+} could thus be a promising strategy for decreasing risk associated with ingesting food crops grown on cadmium polluted soils.

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Table 1. Pearson's correlation matrix analysing the water content, the cadmium content, and the cadmium translocation from the roots to shoot in three *L. sativa* varieties and the calcium concentration in the medium.

Variable[a]	Water content		Cadmium content		Cd translocation	Calcium concentration	Lettuce variety		
	Shoot	Root	Shoot	Root			Fenja	Paris Island Cos	Red Salad Bowl
Shoot water content	**1**[b]	0.228	**-0.663**[b]	**-0.682**[b]	**-0.306**[b]	**0.461**[b]	0,171	-0.061	-0.107
Root water content		**1**[b]	-0.164	-0.125	0.026	0.261	-0.027	**-0.348**[b]	**0.362**[b]
Shoot cd content			**1**[b]	**0.896**[b]	0.212	**-0.702**[b]	0.036	-0.241	0.198
Root Cd content					0.009	**-0.652**[b]	0.255	**-0.276**[b]	0.020
Cd translocation					**1**[b]	-0.230	**-0.769**[b]	0.098	**0.646**[b]
Calcium concentration						**1**[b]	0.005	0.072	-0.074

[a] Variables were centred around their means and normalized with a standard deviation of 1; [b] Figures in bold represent significant correlations at 0.05 level.

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