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Root based responses account for *Psidium guajava* survival at high nickel concentration

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ABSTRACT

The presence of *Psidium guajava* in polluted environments has been reported in recent studies, suggesting that this species has a high tolerance to the metal stress. The present study aims at a physiological characterization of *P. guajava* response to high nickel (Ni) concentrations in the root-zone. Three hydroponic experiments were carried out to characterize the effects of toxic Ni concentrations on morphological and physiological parameters of *P. guajava*, focusing on Ni-induced damages at the root-level and root ion fluxes. With up to 300 μM $NiSO_4$ in the root-zone, plant growth was similar to that in control plants, whereas at concentrations higher than 1000 μM $NiSO_4$ there was a progressive decline in plant growth and leaf gas exchange parameters; this occurred despite, at all considered concentrations, plants limited Ni^{2+} translocation to the shoot, therefore avoiding shoot Ni^{2+} toxicity symptoms. Maintenance of plant growth with 300 μM Ni^{2+} was associated with the ability to retain K^+ in the roots meanwhile 1000 and 3000 μM $NiSO_4$ led to substantial K^+ losses. In this study, root responses mirror all plant performances suggesting a direct link between root functionality and Ni^{2+} tolerance mechanisms and plant survival. Considering that Ni was mainly accumulated in the root system, the potential use of *P. guajava* for Ni^{2+} phytoextraction in metal-polluted soils is limited; nevertheless, the observed physiological changes indicate a good Ni^{2+} tolerance up to 300 μM $NiSO_4$ suggesting a potential role for the phytostabilization of polluted soils.

Introduction

Nickel toxicity is a major environmental concern for plants and for other living organisms (Pyle et al., 2002; Seregin and Kozhevnikova, 2006). Either due to natural processes or as a result of human activities, such as mining activities, high amounts of heavy metals have been released in the environment and are contributing to the pollution of large areas of agricultural soils (Broadley et al., 2006; Di Baccio et al., 2009). Micronutrients are often challenging to plants, partly due to the fact that they have a narrow dynamic range between minimal requirement and toxicity, and partly because their concentrations and chemical speciation are subject to major fluctuations in the soil (Krämer et al., 2005, 2007). Although Ni^{2+} is crucial to the metabolism of plant cells at the μM level (0.05–10 $\mu g\, g^{-1}$ dry mass, Nieminen et al., 2007), Ni^{2+} is toxic to plants at higher concentrations; Ni^{2+} concentrations associated with phytotoxicity vary from as low as 8 to up to 14 $\mu g\, g^{-1}$ dry mass (Davis et al., 1978; Gupta and Gupta, 1998). Common Ni^{2+} toxicity symptoms include: reduced plant water content and stunted plant growth (Iori et al., 2013; Llamas et al., 2008); decreased stomatal conductance and photosynthesis (Velikova et al., 2011); changes in root growth and morphology (Samantaray et al., 1997; Seregin et al., 2003), severe nutrient imbalances (Barsukova and Gamzikova, 1999) and leaf chlorosis (Khalid et al., 1980; Piccini and Malavolta, 1992). Interestingly, it is still debated whether the inhibition of photosynthetic reactions or the increase in stomatal limitations is one of the primary causes of heavy metal toxicity at the shoot level (Bazihizina et al., 2014; Sagardoy et al., 2009, 2010). Multiple studies however support the hypothesis that exposure to toxic metal concentrations impairs, in the short- and long-term, shoot water relations (i.e., induce stomatal closure) as heavy metals negatively affects parameters important for plant–water relationships (e.g., reduce biomass allocation to the roots, increase cell membrane permeability,

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and reduce stem and root hydraulic conductivity, see Ryser and Emerson, 2007; Llamas et al., 2008; Michael and Krishnaswamy, 2011; Przedpelska-Wasowicz et al., 2011; de Silva et al., 2012).

The increased level of Ni^{2+} in the environment, as a result of anthropogenic pollution, is a growing concern for its impact on the food chain and the ecosystem; in the past decades increasing efforts have been made to develop technologies to reduce Ni^{2+} concentrations in contaminated soils and waters (Velikova et al., 2011; Iori et al., 2013). In particular, soil reclamation using plants has gained increasing attention in last decades, which is in part due to the high costs and side effects associated with most physicochemical approaches generally used for polluted soil and water, especially at large scale (Iori et al., 2013). The idea of phytoremediation originated from the fact that some plant species, i.e. metal "hyperaccumulators", can survive and even thrive in soils enriched with heavy metals and accumulate great amounts of toxic elements in their aboveground organs without visible injuries (Raskin and Ensley, 2000; Rascio and Navari-Izzo, 2011). On the other hand, plant-based in situ stabilization, or "phytostabilization," is another potential strategy for the reclamation of contaminated soils (Ali et al., 2013; Colzi et al., 2014). This technique relies on the ability of plants to stabilize contaminants by accumulating and precipitating toxic trace elements in the rhizosphere or by their adsorption on root surfaces, making this management strategy quite simple, non-invasive and cost effective (Colzi et al., 2014). While phytoremediation relies mainly on plants that can accumulate toxic elements in their shoots, plants able to tolerate high metal concentrations in the substrate by physiologically restricting their entry into the root and/or their transport to the shoot (i.e., excluders cf. Baker, 1981) are suitable for metal phytostabilization (Colzi et al., 2014). Today, due to the increasing soil and water heavy metal contamination, and its consequent effects on the food chain, the study of new species is of paramount importance in order to unravel their tolerance and their potentials for phytoremediation or phytostabilization purposes.

Psidium guajava is an important fruit tree of sub-tropical and tropical regions of the world and has attracted interest due to its widespread use for medicinal purposes and as food (Gutierrez et al., 2008). Recent studies have reported the presence of *P. guajava* in sites/environments affected by high heavy metal concentrations (Jacobi et al., 2007; Wang et al., 2007; Perry et al., 2010), suggesting a high tolerance to metal stress. Indeed, it has been reported that *P. guajava* leaves can accumulate Ni^{2+} in atmospherically polluted sites, with increasing leaf Ni^{2+} concentration as the distance from the emission source decreased (Perry et al., 2010). However it is important to stress that, in the above-mentioned study, the average leaf Ni^{2+} concentration varied from 0.16 to 3.01 $\mu g\,g^{-1}$, thus still within the optimal range for plants (0.05–10 $\mu g\,g^{-1}$ dry mass, Nieminen et al., 2007), which would explain why no phytotoxic effects were detected. Therefore, although there is some circumstantial evidence suggesting that *P. guajava* has a good tolerance to heavy metal stress, to our knowledge no studies have specifically evaluated the tolerance of *P. guajava* to toxic metal concentrations in the root-zone, unravelling eventual mechanisms underlying its ability to survive under heavy metal stress. The present study aims at a physiological characterization of *P. guajava* ability to tolerate high nickel concentrations in its root-zone. In order to evaluate *P. guajava* tolerance to increasing Ni^{2+} concentrations, we evaluated the effects of 3 Ni^{2+} concentrations (300, 1000, 3000 μM $NiSO_4$) on several physiological parameters. Furthermore, as heavy toxicity has been found to affect K^+ leakage in the short-term and this trait can be used as an indicator of heavy metal tolerance (Murphy and Taiz, 1997; Llamas et al., 2008), we conducted a second experiment to assess the effect of the above mentioned Ni^{2+} concentrations on K^+ and Ca^{2+} fluxes.

Materials and methods

Plant material and treatment

Three separate experiments (Expt) were conducted to investigate the Ni^{2+} tolerance of *Psidium guajava*. Seeds were germinated in fine peat in a growth chamber with 24 °C and 68% humidity, and a 10/14 h day/night cycle. In Expt 1, plants were initially irrigated with water (until 2 weeks from germination) and subsequently with aerated 25% (for the following week) and 50% Hoagland solution (for the remaining time). The half-strength solution contained: 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.50 mM $MgSO_4$, 20 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 nutrient solution was adjusted to 6.2 with KOH and solutions were changed weekly. Two months after germination, 40 plants were transferred in a glasshouse with a 15/29 °C night/day cycle and average relative humidity of max 60%. One-month after transferring the plants to the nutrient solution, uniform plants were selected and divided into four groups, with six replicates in each group, which received different treatments: 0 (control), 300, 1000, and 3000 μM $NiSO_4$.

Expt 2 was carried out to assess how toxic Ni^{2+} concentrations in the root zone affect K^+ and Ca^{2+} fluxes. In order to work with intact seedlings rather than with excised roots, 2 weeks after germination seedlings were transferred in 25% nutrient solution for 1 week and then fluxes were measured.

Expt 3 was specifically conducted to evaluate the Ni-induced damages at the root-system. The experiment design was similar to that of Expt 1 except that only 3 concentrations were used: 0, 1000 and 3000 μM $NiSO_4$.

Plant harvest (Expt 1)

Plants were sampled on day 0 (6 plants) and after two months of treatments by separating each plant into roots, leaves and stems. Fresh mass of all plants tissues was recorded. Roots were carefully washed three times and small subsamples were taken for subsequent transmission electron and light microscopy. At the shoot level, three leaf discs (diameter = 8 mm) were taken from young fully expanded leaves and stored in freezer at −20 °C to determine leaf pigment concentrations. Remaining shoot and root tissues were then oven-dried at 70 °C to determine their dry mass.

Leaf gas exchange parameters (Expt 1)

One day prior to the harvest, net photosynthetic rate, stomatal conductance and transpiration rates were calculated on young fully expanded leaves in each treatment, using the open gas exchange system Li-6400 (LiCor Inc., Lincoln, NE, USA). Leaf gas exchange measurements were taken on four plants from each treatment at ambient RH (40–50%), ambient CO_2, flow rate of 400 $\mu mol\,s^{-1}$, leaf chamber temperature at 20 °C and photosynthetically active radiation (PAR) of 1500 $\mu mol\,m^{-2}\,s^{-1}$. Intrinsic water use efficiency (WUE_i) was calculated as the ratio of photosynthetic rate to transpiration rate to quantify the amount of carbon assimilated per unit of water loss (Ehleringer et al., 1993).

Tissue impedance measurements (Expt 1)

The procedure for the electrical impedance measurement was previously described in Mancuso et al. (1999). Briefly, stem samples with similar length and diameter (20 mm long and 2–3 mm in diameter) were removed from the fifth node starting from the top of the plants. The sample was positioned between two Ag/AgCl electrodes covered with a conductive paste that allowed the passage of the

electric current. The impedance spectra were measured by using an impedance metre (QuadTech 1920 PrecisionLCRMeter). The device was calibrated using an OPEN/SHORT circuit correction to eliminate the polarization impedance of the electrode/paste interface. Absolute impedance value and phase angle were then measured within a frequency range from 10 Hz to 1 MHz. Impedance spectra were analyzed using an equivalent circuit that includes two DCE (Distributed Circuit Element) in series with a resistor, and the circuit parameters were calculated using the software LEVM 8.07 (Mancuso et al., 1999).

Determination of potassium, calcium and nickel concentrations in plants (Expt 1)

The K^+ and Ca^{2+} 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 analyzed for K^+ and Ca^{2+} concentrations (Digiflame DV710, NT Laboratory). Ni^{2+} 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). Ni^{2+} 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 Ni^{2+} was supplied by Sigma–Aldrich.

The tolerance index (TI) was calculated to measure the ability of plants to grow in the presence of high Ni^{2+} concentrations in the root zone with the following formula:

$$\mathrm{TI} = \left(\frac{\text{dry mass of treated plants}}{\text{dry mass of control plants}}\right)$$

The translocation factor (TF) or mobilization ratio was calculated with shoot and root Ni^{2+} concentrations to determine the relative translocation of metals from roots to shoot. TF < 1 and TF > 1 represent, respectively, a low and high capacity to translocate metals from the roots to the shoots (Zhang et al., 2002; Gupta et al., 2008a,b). Subsequently, to evaluate the capacity of *P. guajava* to absorb and accumulate Ni^{2+}, the bio-concentrations factor (BCF) was estimated according to Tanhan et al. (2007) and Liu et al. (2009). TF and BCF were calculated with the following formulas:

$$\mathrm{TF} = \frac{\text{shoot concentration}}{\text{root concentration}}$$

$$\mathrm{BCF} = \frac{Ni^{2+}\text{ concentration in the whole plant}(\mu g\ g^{-1}\text{ dry mass})}{Ni^{2+}\text{ concentration in growing media}(\mu g\ L^{-1})}$$

Leaf pigment analyses (Expt 1)

Total leaf chlorophyll and carotenoid concentrations were determined in young fully expanded leaves. Cold 100% methanol was added to 10–20 mg of the ground tissues and samples were then 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 leaf chlorophyll and carotenoid concentrations were calculated using the equations described in Wellburn (1994).

K^+ and Ca^{2+} fluxes (Expt 2)

Seedlings were placed in a measuring chambers filled with 4 mL of buffered (Tris/MES, pH = 6.2) basal salt media (BSM, 0.1 mM $CaCl_2$, 0.2 mM KCl) and roots were immobilized in the horizontal position. Plants were allowed to adapt to the new conditions for 2 h prior to the measurement. Net K^+ and Ca^{2+} fluxes were measured on roots using a vibrating probe non-invasive system (University of Florence, Italy). Details on fabrication and calibrations of microelectrodes were described previously in Mancuso et al. (2000). Basal K^+ and Ca^{2+} fluxes were measured for 15 min and then an adequate volume of 10 mM $NiSO_4$ was added to chamber's solution in order to have final concentrations of 300, 1000, 3000 μM $NiSO_4$. Net K^+ and Ca^{2+} fluxes were monitored for 40 min after $NiSO_4$ addition until stable values were obtained. Fluxes were then monitored 4 and 24 h after $NiSO_4$ addition in order to have a complete response of the plants to Ni^{2+} with time.

Microscopic analysis: transmission electron microscopy X-ray microanalysis (Expt 3)

Samples of the control and Ni^{2+}-treated roots were cut into pieces 3 mm long and immediately fixed in 2.5% glutaraldehyde, in 0.2 M phosphate buffer (pH 7.2) for 2 h at room temperature, washed twice in the same buffer, post-fixed in 2% OsO_4 in the same buffer for 2 h also at room temperature. Following dehydration in a graded ethanol series (30, 40, 50, 60, 70, 80, 95 and 100%), the specimen were gradually embedded in Spurr resin (Spurr, 1969) and polymerized at 70 °C for 24 h. Ultrathin (70–90 nm) transverse sections of the processed tissue were obtained with an LKB IV ultra microtome, collected on Formvar coated aluminium grids, stained with uranyl acetate and lead citrate and examined using Philips CM12 transmission electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV. The nature of dense material observed in root tissues was determined with an EDS-X-ray microanalysis system (EDAX, software EDAX Genesis, AMETEK, Mahwah, NJ, USA). The images were recorded by a Megaview G2 CCD camera (software iTEM FEI, AnalySIS Image Processing, Olympus, Shinjuku-ku, Japan).

Statistical analyses

Statistical analyses were conducted using GraphPad for Mac 6th Edition. Analysis of variance (ANOVA) was used to identify overall significant differences between treatments, depending on the data set. When significant differences were found, mean separations were calculated using the Turkey's multiple range test. Unless otherwise stated, the significance level was $P \leq 0.05$.

Table 1
Total dry weight (g), fresh and dry mass ratio in *P. gujava* plants grown with increasing Ni^{2+} concentrations in the root zone for two months. Values are mean ± S.E. ($n = 6$). The different letters indicate statistically significant differences. Initial shoot and root dry masses were respectively (g): 6.07 ± 0.64 and 4.34 ± 0.83.

$NiSO_4$ (μM)	Dry mass (g)		Fresh/dry mass ratio		
	Shoot	Roots	Leaves	Stem	Roots
0	16.51 ± 1.30[a]	7.65 ± 0.73[a]	3.17 ± 0.04[a]	2.62 ± 0.06[a]	6.25 ± 0.09[a]
300	14.33 ± 1.51[a]	5.74 ± 0.33[ab]	2.93 ± 0.20[a]	2.49 ± 0.12[a]	5.12 ± 0.17[b]
1000	10.05 ± 0.75[b]	4.72 ± 0.81[b]	2.04 ± 0.20[b]	2.08 ± 0.05[b]	4.23 ± 0.27[b]
3000	5.83 ± 0.58[c]	3.52 ± 0.30[c]	1.43 ± 0.05[c]	2.02 ± 0.04[b]	4.73 ± 0.06[b]

Results

Shoot and root growth (Expt 1)

Increasing concentration of Ni^{2+} in the nutrient solutions produced obvious toxicity symptoms, with substantial declines in plant dry mass (Table 1). For example, with 1000 and 3000 μM $NiSO_4$, shoot and root dry mass decline respectively by 40–65% and 39–54% compared to values in control plants. As initial dry masses were, on average, 7.48 g for the shoot and 4.80 g for the roots, with 3000 μM $NiSO_4$ both shoot and root dry mass declined by the end of the experimental period (Table 1). Although there were clear declines in plant growth with 1000 and 3000 μM $NiSO_4$, there were only small, and no significant ($P > 0.05$), declines in plant growth with 300 μM $NiSO_4$. Except with 300 μM $NiSO_4$, leaf fresh/dry mass ratio declined with increasing Ni^{2+} content in the root-zone (Table 1). By contrast, roots fresh/dry mass ratio declined and remained constant in all Ni^{2+}-treated plants independently of the Ni^{2+} concentrations in the root zone (Table 1).

Leaf gas exchange parameters (Expt 1)

Increasing Ni^{2+} concentrations reduced all gas exchange parameters (Fig. 1a–d) but increased the intrinsic water use efficiency (Fig. 1e). Interestingly, the photosynthetic activity of plants exposed to 300 μM Ni^{2+} did not differ compared to that in control plants, despite the significant declines in stomatal conductance and transpiration rates. At higher concentrations, a progressive decrease of all parameters was evident, although the decline in stomatal conductance was generally more extensive compared to that observed for the photosynthetic rate (Fig. 1a and b). For example, with 1000 μM Ni^{2+} in the root-zone, photosynthetic rate and stomatal conductance declined by 28% and 68% respectively. Given the greater inhibition of leaf stomatal conductance, the CO_2 concentration in the internal air space declined in all Ni treated plants (Fig. 1c).

K^+ and Ca^{2+} concentrations in plant tissues (Expt 1)

At the end of the experiment, both leaf and stem K^+ and Ca^{2+} concentrations did not vary between treated and control plants, independently of the treatment (Table 2). Similarly Ca^{2+} concentrations in the root did not differ in treated and control plants. By contrast, there was a significant decline in root K^+ concentration, which dropped from 439 μmol g^{-1} dry mass of the control to values of 115 μmol g^{-1} dry mass with Ni^{2+} at 3000 μM.

Ni^{2+} concentration in plant tissues, bioaccumulation and translocation factors (Expt 1)

Nickel accumulation in *P. guajava* varied in shoot and roots depending on the level of Ni^{2+} in the nutrient solutions (Table 2). In all Ni^{2+} treatments plants accumulated Ni^{2+} primarily in their roots. Indeed, compared with control treatments, Ni^{2+} concentrations in shoots of treated plants remained below 3 μmol Ni^{2+} g^{-1} dry mass (0.04 μmol Ni^{2+} g^{-1} dry mass in control plants vs 1.9–2.8 μmol Ni^{2+} g^{-1} dry mass in treated plants). On the other hand, in roots, the increases in Ni^{2+} concentration mirrored the Ni^{2+} increases in the growing media. For instance, when plants were exposed to 300 and 3000 μM Ni^{2+}, Ni^{2+} concentration in roots increased from 74 to 151 μmol Ni^{2+} g^{-1} dry mass.

We calculated BCF and TF to estimate Ni^{2+} uptake and translocation in *P. guajava* (Table 3). Although plants exposed to different Ni^{2+} concentrations accumulated Ni^{2+}, *P. guajava* limited Ni^{2+} translocation to the shoot, as clearly shown by the TF values (Table 3). Given that treated plants had very high BCF values

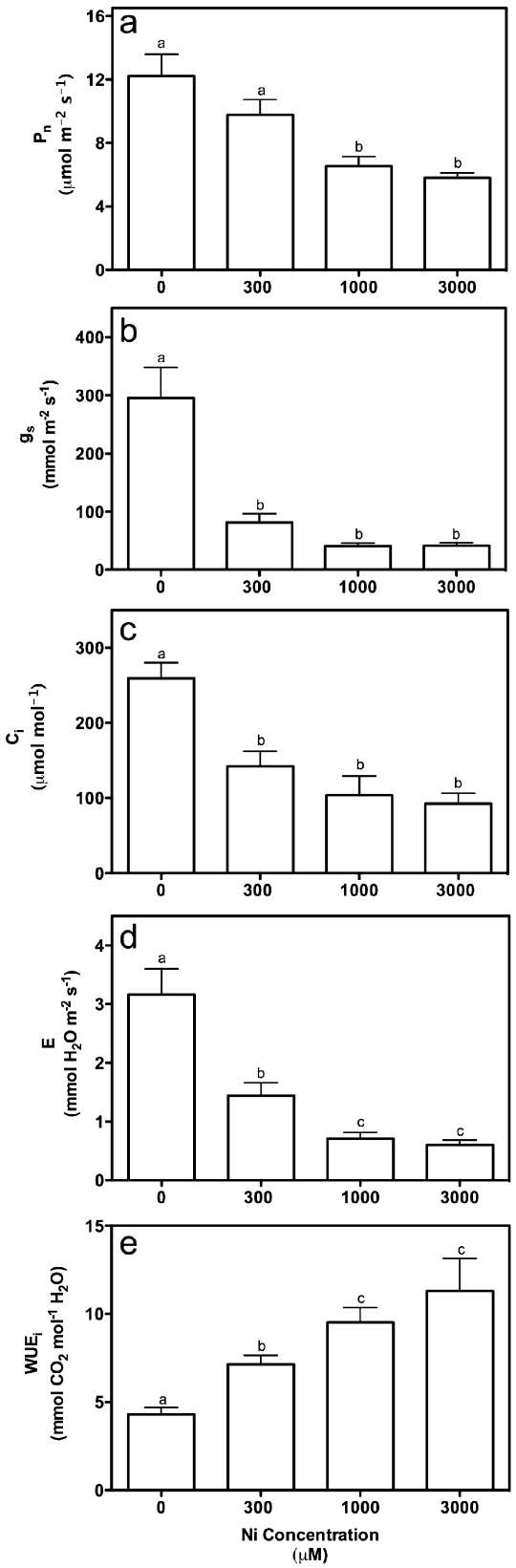

Fig. 1. Net CO_2 assimilation (a), stomatal conductance (b), CO_2 concentration in internal air space (c), transpiration (d) and intrinsic water use efficiency (e) in plants grown with increasing Ni^{2+} concentrations in the nutrient solution. In four treatments, the plant root systems were exposed to increasing Ni^{2+} concentrations (0, 300, 1000, 3000 μM Ni^{2+}). Values are mean ± S.E. ($n = 4$). The different letters indicate statistically significant differences ($P < 0.05$). Net CO_2 assimilation (P_n); stomatal conductance (g_s); CO_2 concentration in internal air space (C_i); transpiration (E); intrinsic water use efficiency (WUE_i).

Table 2
K^+, Ca^{2+}, and Ni^{2+} concentrations in the different parts of *Psidium guajava* plants grown at different Ni^{2+} concentrations. Values are mean ± S.E. ($n = 6$). The different letters indicate statistically significant differences.

$NiSO_4$ (μM)	K^+ (μmol g^{-1} dry mass)			Ca^{2+} (μmol g^{-1} dry mass)			Ni^{2+} (μmol g^{-1} dry mass)		
	Leaves	Stem	Roots	Leaves	Stem	Roots	Leaves	Stem	Roots
0	412.71 ± 13.94^a	214.14 ± 19.27^a	438.67 ± 34.28^a	117.33 ± 4.62^a	92.35 ± 7.63^a	47.22 ± 14.93^a	0.04 ± 0.02^a	0.10 ± 0.02^a	0.15 ± 0.02^a
300	457.45 ± 37.09^a	203.61 ± 22.50^a	202.10 ± 21.37^b	105.26 ± 16.16^a	89.78 ± 8.58^a	33.47 ± 13.31^a	1.92 ± 0.14^b	2.00 ± 0.19^b	73.83 ± 4.81^b
1000	503.56 ± 26.53^a	276.41 ± 34.16^a	178.46 ± 22.05^b	97.17 ± 23.28^a	85.89 ± 11.26^a	23.35 ± 8.68^a	2.86 ± 0.64^b	3.04 ± 0.85^{b,c}	127.61 ± 18.01^{b,c}
3000	453.57 ± 4.95^a	290.43 ± 40.62^a	114.76 ± 8.60^c	102.01 ± 8.94^a	84.01 ± 14.87^a	44.97 ± 19.79^a	2.79 ± 0.39^b	4.77 ± 0.71^c	151.06 ± 13.51^c

Table 3
Translocation factor (TF), shoot and root bioconcentration factor (BFC) and tolerance index (TI) in *Psidium guajava* plants grown at different Ni^{2+} concentrations. Values are mean ± S.E. ($n = 6$). The different letters indicate statistically significant differences ($P < 0.05$).

$NiSO_4$ (μM)	TF	BFC		TI
		Shoot	Roots	
300	0.056 ± 0.008	6.49 ± 0.45^a	246.10 ± 17.91^a	0.813 ± 0.028^a
1000	0.034 ± 0.005	3.31 ± 0.85^b	127.61 ± 18.01^b	0.582 ± 0.008^b
3000	0.035 ± 0.01	1.15 ± 0.22^b	46.50 ± 4.57^c	0.408 ± 0.017^c

(Table 3), the results indicate that *P. guajava* retained metals in their roots while limiting metal mobility from the roots to the shoots (Cui et al., 2007).

Tissue impedance measurements (Expt 1)

Although differences were not significant, it is possible to observe increments of the intracellular resistance values (Table 4) in plants grown at higher Ni^{2+} concentrations (1000 and 3000 μM). This may have been in part due to the fact that the entry of Ni^{2+} into the cells was limited, thus helping in maintaining the internal electrochemical properties unaltered. By contrast there was a substantial declines in the extracellular resistance of tissues in plants grown at 3000 μM Ni^{2+}.

Total chlorophyll and carotenoid concentrations (Expt 1)

After two months of exposure to increasing Ni^{2+} concentrations, total chlorophyll content in fully expanded leaves did not differ between treated and control plants (Fig. 2). On the other hand, carotenoid concentrations in leaves increased with increasing Ni^{2+} concentrations, with almost a two-fold increase in plants treated with 3000 μM Ni^{2+}.

Ion fluxes (Expt 2)

Exposure of *P. guajava* seedlings to different concentration of Ni^{2+} (300, 1000, 3000 μM) caused significant changes in the membrane transport activity depending on the Ni^{2+} concentration considered (Figs. 3 and 4). Maximum Ca^{2+} effluxes were seen in the first 25 min following the addition of Ni^{2+}; after the initial loss of Ca^{2+} observed in the first 25 min of treatment, no further effluxes were measured after 4 and 24 h (Figs. 3a and 4a).

Net K^+ fluxes were affected by the Ni^{2+} treatments (Figs. 3b and 4b). At 300 μM during the first 25 min a net K^+ uptake was recorded, with no significant differences between treated and control plants; after 4 and 24 h of treatment, however, root ability to maintain a net K^+ uptake was lost and a net K^+ efflux around 2–4 μmol m^{-2} s^{-1} became evident. At higher concentrations, the effect of 1000 and 3000 μM Ni^{2+} on K^+ fluxes were already visible after 25 min of treatment, with an initial net K^+ efflux of 6–17 μmol K m^{-2} s^{-1}, which increased to 21–39 μmol K^+ m^{-2} s^{-1} after 24 h of treatment.

Table 4
Intracellular and extracellular resistance values in *Psidium guajava* shoots of plants grown with increasing Ni^{2+} concentrations in the root zone. Values are mean ± S.E. ($n = 6$). The different letters indicate statistically significant differences ($P < 0.05$).

$NiSO_4$ (μM)	Intracellular resistance (Ω)	Extracellular resistance (Ω)
0	74.64 ± 10.56^a	10149.46 ± 1694.24^{ab}
300	73.12 ± 11.80^a	11939.68 ± 2957.47^a
1000	117.15 ± 24.85^a	10344.50 ± 1257.09^{ab}
3000	112.31 ± 12.30^a	6420.25 ± 705.30^a

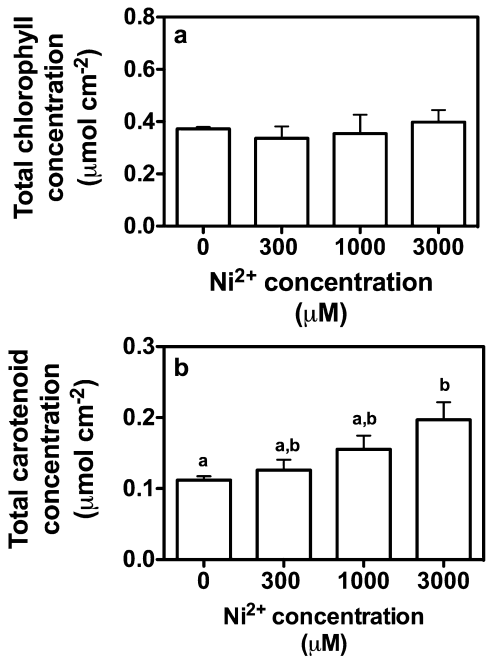

Fig. 2. Total chlorophyll (a) and carotenoids (b) concentrations in *Psidium guajava* in response to increasing Ni^{2+} concentrations in the nutrient solution. In four treatments, the plant root systems were exposed to increasing Ni^{2+} concentrations (the control treatment, and 300, 1000, 3000 μM Ni^{2+}). Values are mean ± S.E. ($n = 6$). The different letters indicate statistically significant differences ($P < 0.05$).

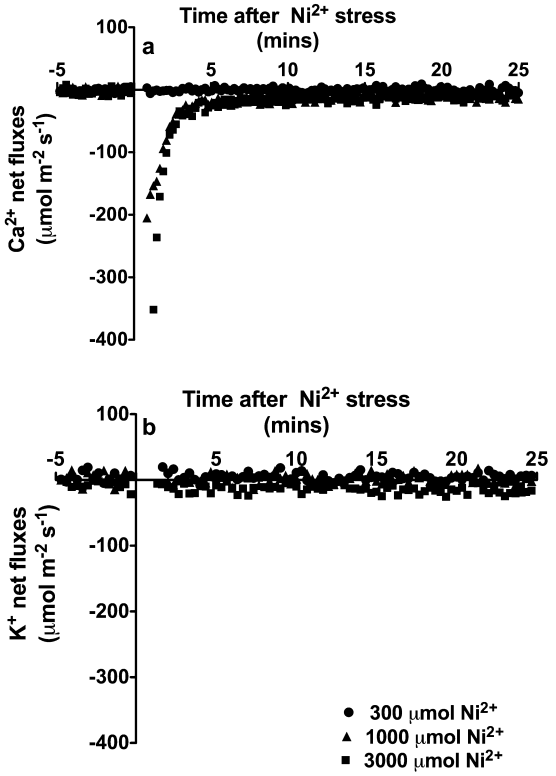

Fig. 3. Net Ca^{2+} (a) and K^+ (b) fluxes in roots of *Psidium guajava* seedlings in response to increasing Ni^{2+} concentrations in the nutrient solution in the first 25 min following the addition of Ni^{2+} stress. In (b) differences between treatments are not evident due to the scale used in the *y*-axis, and differences between treatments are highlighted in Fig. 4b.

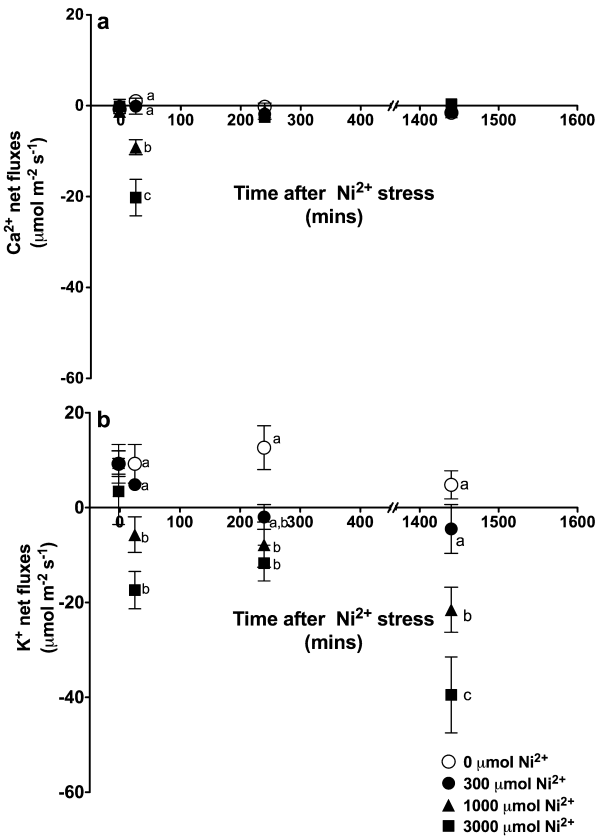

Fig. 4. Effects of Ni^{2+} addition on the net Ca^{2+} (a) and K^+ (b) fluxes from roots of *Psidium guajava* seedlings exposed for 24 h to increasing Ni^{2+} concentrations in the nutrient solution. Values are mean ± S.E. ($n = 6$). The different letters indicate statistically significant differences ($P < 0.05$).

Microscopic analysis: transmission electron microscopy (TEM) (Expt 3)

In control plants, there was an accumulation of vacuolar compounds in cells under the root epidermis (Fig. 5a). These shape granules or fine dispersal materials were also found scattered in the cortex, especially in the inner layer and in the stellar tissue. Fig. 5b shows epidermal and cortical cells of control roots with large nuclei and vacuole, with a thin layer of dense cytoplasm at the periphery of the cell.

Roots of treated plants exhibited an increasing number of cells with vacuolar dense depositions, particularly in the central cylinder (Fig. 5c and h). In epidermis and cortex of plants treated with Ni^{2+} 1000 μM some cells had irregular shape (Fig. 5d), with structureless cytoplasm (Fig. 5e), withdrawal of plasma membrane from the cell walls (Fig. 5f) and the vacuolation significantly increased (Fig. 5g). Finally, in Fig. 5i the disintegration of cytoplasm in root cells of plants grown at Ni^{2+} 3000 μM can be clearly observed.

The composition of the dense materials observed in vacuole of root cells was analyzed by EDX-microanalysis (Fig. 6). In plants treated with 1000 and 3000 μM Ni^{2+}, the analyses of the vacuolar compounds showed the presence of Ni^{2+} (Fig. 6b and c), whereas analyses performed on control roots did not show any Ni^{2+} peak (Fig. 6a).

Discussion

In the past decade, there has been growing interest in the use of plants for the reclamation of polluted soil. Several studies have reported the presence of *P. guajava* in sites/environments

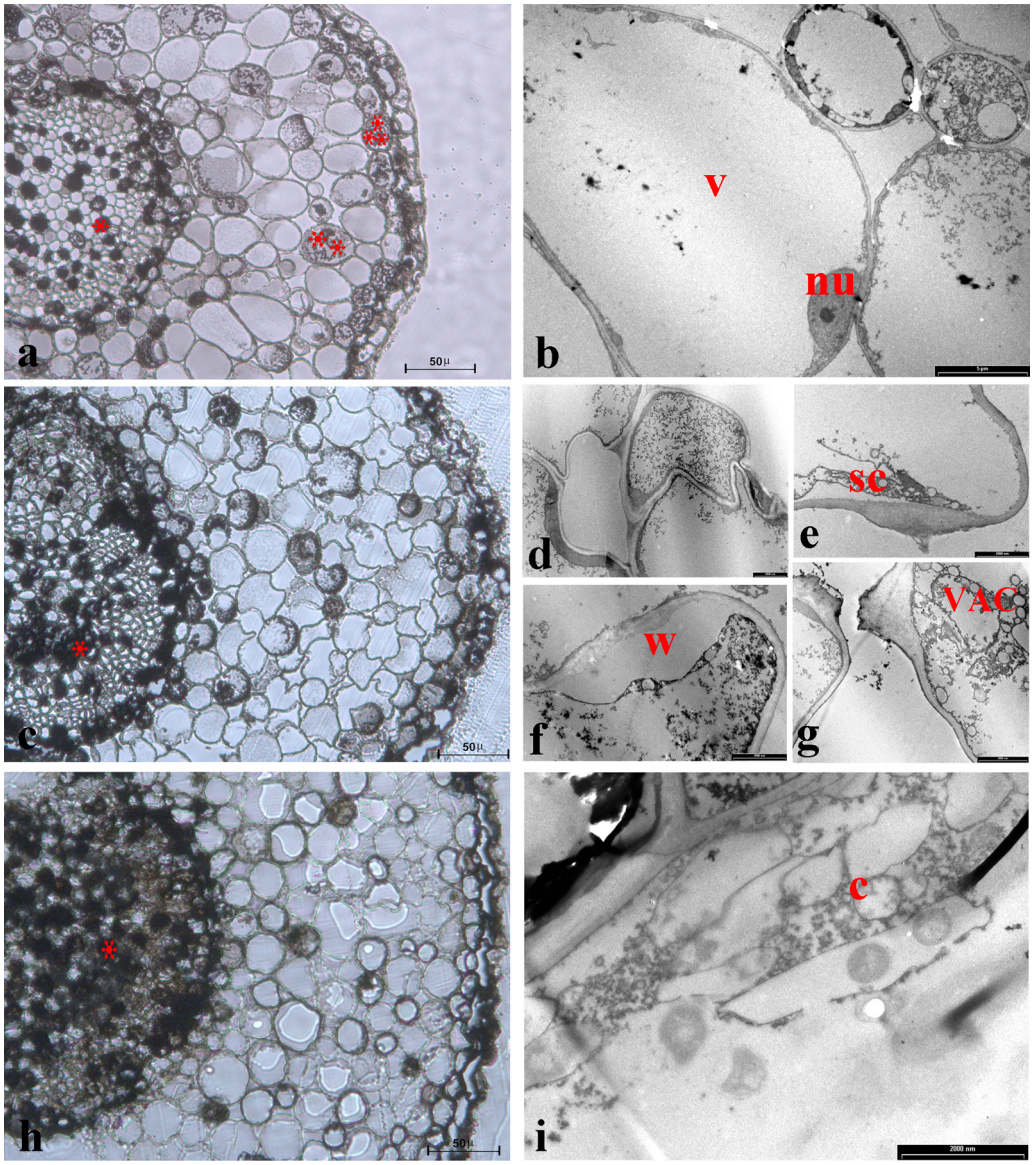

Fig. 5. Electron micrographs of transverse section of root cells of *Psidium guajava* plants exposed to increasing concentration of nickel in the nutrient solution. (a) Roots of control plants with vacuolar compounds under the epidermis (***), in the inner layer (**), and in the stellar tissue (*); (b) epidermal and cortical control root cells with large nuclei (nu) and vacuole (v); (c) roots of plants treated with Ni^{2+} 1000 μM with vacuolar dense deposition (*); (d) irregular shape of root cells treated with Ni^{2+} 1000 μM; (e) structureless cytoplasm of root cells treated with Ni^{2+} 1000 μM; (f) withdrawal of plasma membrane (w) in root cells treated with Ni^{2+} 1000 μM; (g) vacuolation (VAC) increase in root cells treated with Ni^{2+} 1000 μM; (h) roots of plants treated with Ni^{2+} 3000 μM with vacuolar dense deposition (*); (i) disintegrated cytoplasm (c) in root cells treated with Ni^{2+} 3000 μM.

affected by heavy metal contamination (Jacobi et al., 2007; Perry et al., 2010), indicating a potential high heavy metal tolerance. The present study indicates that *P. guajava* has a very good Ni^{2+} tolerance, with net dry mass production in plants exposed to 300 μM Ni^{2+}; this concentration can be considered elevated given that tree species are generally not able to adapt to high concentrations of heavy metals in their root-zone, with few exception e.g. *Salix* (Pulford and Watson, 2003; Drzewiecka et al., 2012). Plant growth at 300 μM $NiSO_4$ remained similar to that of control plants, which is likely to be associated with the ability to maintain net photosynthetic rates similar to those measured in control plants, despite significant (>50%) declines in stomatal conductance and transpiration. Our results indicate that Ni^{2+} homeostasis was tightly controlled, and Ni^{2+} shoot concentrations only increased marginally independently of the Ni^{2+} concentrations in the root-zone. The physiological changes following treatments indicate that the growth reduction in plants subjected to heavy metal stress resulted from direct effects (toxicity of heavy metals) at the root level as these were in direct contact with Ni^{2+}, and most likely from indirect effects at the shoot level, due to limitation in water and nutrient acquisition.

High Ni^{2+} concentrations in the root zone rapidly inhibited stomatal aperture (>50%), independently of the Ni^{2+} concentration in the growing media, meanwhile there were only small declines in net photosynthetic rates (<30%) and no declines at all in total leaf chlorophyll concentrations. Gas exchange data suggest that Ni^{2+} treatments affected mainly stomatal functioning rather than the photosynthetic machinery; indeed declines in C_i support the view that a limited CO_2 diffusion, associated with stomatal closure, was the main factor behind the reduction in CO_2 assimilation rates in all treated plants (e.g., Medrano et al., 2002). This result is in accordance with the studies that indicate that exposure to toxic metal concentrations substantially decreases stomatal and mesophyll conductances to CO_2 with no significant effects on leaf

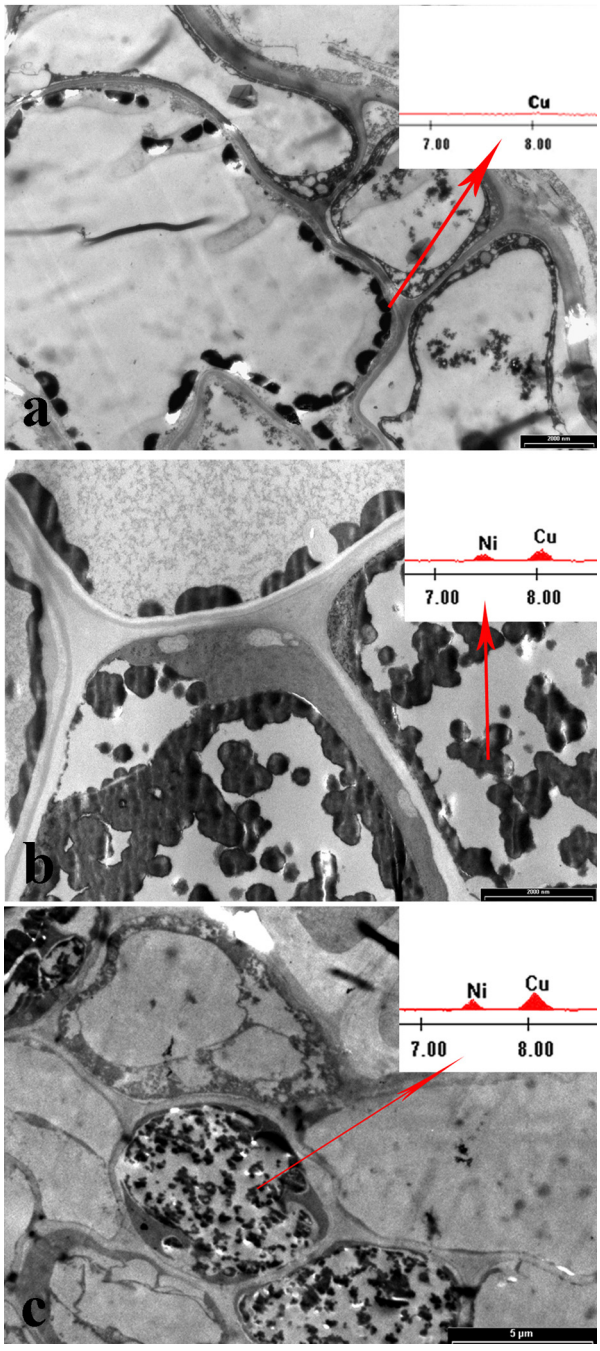

Fig. 6. EDX microanalysis of vacuolar dense deposition in (a) control root cells, (b) Ni^{2+} 1000 μM root cells and (c) Ni^{2+} 3000 μM root cells.

photochemistry and photosynthetic biochemistry (e.g. Sagardoy et al., 2010; Bazihizina et al., 2014). Several factors can explain the dramatic change in the stomatal aperture in response to a condition that seems to only be affecting the roots. For various abiotic stresses (e.g. salinity or drought) it is now well established that changes at the root level can quickly, through rapid systemic long-distance signals (hydraulic and/or non-hydraulic), mediate stomatal behaviour (Comstock et al., 2002; Christmann et al., 2007; Schachtman and Goodger, 2008; Choi et al., 2014). Similarly heavy metals have been found to quickly reduce or block water transport from roots to the above ground parts through effects at multiple points, from effects on stomatal opening to effect of root cell water permeability (Perfus-Barbeoch et al., 2002; Przedpelska-Wasowicz et al., 2011; Kholodova et al., 2011). For instance, in *Mesembryanthemum crystallinum* exposed to excess zinc and copper, long before visible symptoms of water status disturbance appeared in leaves, there were rapid reductions in leaf transpiration rates and aquaporin gene expression declined substantially in both leaf and root tissues (Kholodova et al., 2012).

The hypothesis that Ni^{2+} stress mainly affected plant water relations in *P. guajava* is further supported by the finding that *P. guajava* was very efficient in preventing the entry of large amounts of Ni^{2+} in shoots, thus avoiding typical toxicity symptoms associated with elevated Ni^{2+}. Indeed, several studies have shown that excess Ni^{2+} inhibits photosynthesis through various mechanisms, e.g., the disruption of the photosynthetic electron transport (Tripathy et al., 1981; Krupa et al., 1993; Velikova et al., 2011), negative effects on the water-splitting site of PSII (Velikova et al., 2011) and the inhibition of photosynthetic pigment biosynthesis (Küpper et al., 1996). Interestingly meanwhile total chlorophyll concentrations remained constant in all treatments, carotenoid concentrations increased with increasing Ni^{2+} concentrations in the root-zone, which could be ascribed to a defence strategy. Indeed, carotenoids are non-enzymatic antioxidants that play an important role in the protection of chlorophylls, as well as quenching or scavenging the free radicals, reducing the damage to cell, cell membrane, and their main genetic composition under heavy metals stress (Hou et al., 2007). In a previous study conducted with *Aeluropus littoralis*, it was found that carotenoid concentrations increased in plants exposed to 50 μM Pb^{2+} and Ag^{2+} meanwhile they decreased at higher concentrations (Rastgoo and Alemzadeh, 2011). Although reactive oxygen species (ROS) detoxification by carotenoid has not been investigated extensively in plants, it has been hypothesized that with lower heavy metal concentrations in the tissues, carotenoid contents increase to protect the cell against heavy metal damage meanwhile with higher heavy metals concentrations ROS levels exceed the capacity of the cell to cope with the oxidative stress (Pinto et al., 2003; Gratão et al., 2005; Singh and Agrawal, 2007; Rastgoo and Alemzadeh, 2011). It could therefore be speculated that, in the present study, the observed Ni^{2+} concentrations in the shoots of *P. guajava*, albeit small, were sufficient to increase oxidative stress in shoots therefore activating the non-enzymatic antioxidant defence mechanisms and therefore carotenoid synthesis, but never reached concentrations that would lead to degradation of these pigments.

P. guajava was able to withstand high Ni^{2+} concentrations in the growth medium. This tolerance was likely due to a vacuolar sequestration of the metal, as revealed by the presence of Ni^{2+} in the vacuoles of root cells of treated plants (Fig. 6), which would reduce toxic build-up of Ni^{2+} in the cytoplasm. Furthermore, shoot Ni^{2+} concentrations in all treated plants indicate that Ni^{2+} homeostasis was tightly controlled in this species, which contributed to *P. guajava* ability to withstand elevated concentrations in the root-zone. Indeed, the limited movement of Ni^{2+} to the leaves is to be considered a defence response aimed at avoiding the oxidative damage in the photosynthetic machinery. Therefore, although Ni^{2+} content exceeded many times its critical limits of plants toxicity (up to 16 times higher, Nieminen et al., 2007), it is logical to expect that the reduced plant growth was linked to the reduced supply water and ions from the roots to the shoot, rather than being associated with specific local symptoms of Ni^{2+} toxicity (Fig. 1, Table 1). In support of the hypothesis that shoot growth reduction in Ni^{2+}-treated plants was associated with a perturbation of water relations rather than to specific Ni^{2+} toxicity symptoms, it was observed that total chlorophyll contents and shoot concentrations of K^+ and Ca^{2+} were similar in treated and control plants, whereas leaf water content and stomatal conductance rates declined substantially with the increasing Ni^{2+} in roots. This would also explain why K^+ and Ca^{2+} content in shoots were not affected by the Ni^{2+} treatments. The result agrees with previous findings reported in the literature

showing that shoot-level perturbations of stomatal regulation and water relations are one of the main causes of heavy metal toxicity (Llamas et al., 2008; Sagardoy et al., 2010). We also observed no significant changes in intracellular resistance (i.e. membrane permeability) in treated and control plants, which may have been associated with a limited Ni^{2+} entry into the cells, which therefore helped in maintaining the internal electrochemical properties unaltered. By contrast there was a substantial decline in the extracellular resistance in the shoot of plants treated with 3000 μM Ni^{2+} (Table 4); this decrement could be linked to an ion extrusion by the cell in the apoplast, which would therefore avoid toxic Ni^{2+} build-up in the cytoplasm thus reducing its toxic effects (Kramer et al., 2000). Indeed it has been found that the hyperaccumulator *Thalaspi goesingense* has a higher capacity to bind Ni^{2+} to the cell wall when compared with the non-accumulator *T. arvense*, and this higher capacity to bind Ni^{2+} to the cell wall has been hypothesized to contribute to the higher Ni^{2+} tolerance in hyperaccumulator species (Kramer et al., 2000).

In the present study, for all Ni^{2+} treatments, K^+ content in roots declined compared to those in control plants. In plants exposed to 300 μM Ni^{2+}, K^+ ion fluxes data indicated that there is a progressively decline roots ability to take up K^+, although statistical analysis did not show any significance differences between this treatment and the control. It could therefore be argued that the decline in K^+ content in roots with 300 μM Ni^{2+} was mainly associated with lower uptake rates and dilution by growth (Table 1). By contrast, with 1000 and 3000 reduced K^+ content in roots was likely to be associated with the increased K^+ leakage, which were already evident after 24 h of treatments (see Figs. 3 and 4). It is therefore conceivable that the K^+ effluxes observed already after 24 h were a consequence of disturbed root membrane functionality, as Ni^{2+} has been found to interfere the functionality of cell membranes at various levels, from negative effects on the lipid composition of the plasma membrane, lipid peroxidation activity, membrane permeability and the plasma membrane H^+-ATPase activity (Pandolfini et al., 1992; Ros et al., 1992; Llamas et al., 2008). In a previous study in rice plants it was found that in short term treatments (up to 8 h) with 0.5 mM Ni^{2+} did not alter the membrane permeability of root cells (Llamas et al., 2008). However, when plants were grown for a longer time (up to 10 days) in presence of 0.5 mM Ni^{2+}, there was a progressive increase in membrane permeability and consequent K^+ efflux in roots. Conversely, the high Ca^{2+} effluxes observed immediately after Ni^{2+} addition (Figs. 3 and 4) could have been due to the replacement of Ni^{2+} with the extracellularly bound Ca^{2+} in the cell wall, resulting in the disruption of membrane integrity and the ionic homeostasis of the cells (Janicka-Russak et al., 2008). For instance, in *T. goesingense* a large proportion of total leaf Ni^{2+}, between 67% and 73%, was found to be localized in the apoplast or bound to cell wall material (Kramer et al., 2000). However, it cannot be ruled out that, as recently reported for pea roots exposed to polyamines (Pottosin et al., 2014), the observed Ca^{2+} effluxes were linked to a coupled Ni^{2+} effect on PM H^+ and Ca^{2+}-ATPase, with the latter being responsible for Ca^{2+} extrusion. Nevertheless, to date, information heavy metal, in particular Ni^{2+}, effects on PM H^+-ATPase is limited and even less is known about Ca^{2+} ATPase. Therefore conclusive statements cannot be made, which clearly warrants for further studies on this topic.

In conclusion, the present study was conducted to evaluate Ni^{2+}-induced changes in *P. guajava* and unravel its Ni^{2+} tolerance mechanisms. In this study, root responses mirrored all plant performances suggesting a direct link between root functionality, Ni^{2+} tolerance mechanisms and plant survival. Considering that Ni^{2+} accumulated mainly in the root system, the potential use of *P. guajava* for nickel phytoextraction in metal-polluted soils is very limited and the data in the present study strongly suggest that *P. guajava* is not suitable for extracting large amounts of metals. Nevertheless, *P. guajava* showed a very good tolerance to concentrations up to 300 μM Ni^{2+}, and therefore, although further studies on plant cultivation on real contaminated soils are of paramount importance, it is logical to expect that this species could be suitable for the phytostabilization of contaminated soils. Indeed, the process of phytostabilization depends on roots ability to limit the contaminant mobility and bio-availability in the soils, and heavy metals tolerant species with high BCF and low TF can be successfully used for phytostabilization purposes (Malik et al., 2010). Given that tree species are generally sensitive to high heavy metal concentrations with the exception of selected taxa, e.g. *Salix* (Pulford and Watson, 2003; Drzewiecka et al., 2012), further experiments with adult trees should be envisaged to understand the behaviour of this species in soils with elevated heavy metal concentrations and their potential use for the reclamation of contaminated soils.

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References

Ali H, Khan E, Sajad MA. Phytoremediation of heavy metals – concepts and applications. Chemosphere 2013;91:869–81.
Baker AJM. Accumulators and excluders – strategies in the response of plants to heavy metals. J Plant Nutr 1981;3:643–54.
Bazihizina N, Taiti C, Marti L, Rodrigo-Moreno A, Spinelli F, Giordano C, et al. Zn^{2+}-induced changes at the root level account for the increased tolerance of acclimated tobacco plants. J Exp Bot 2014., http://dx.doi.org/10.1093/jxb/eru251.
Barsukova VS, Gamzikova OI. Effects of nickel surplus on the element in wheat varieties contrasting in Ni resistance. Agrokhimiya 1999;1:80–5.
Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, et al. Biofortification of UK food crops with selenium. Proc Nutr Soc 2006;65:169–81.
Choi WG, Toyota M, Kim SH, Hilleary R, Gilroy S. Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci U S A 2014;111:6497–502.
Christmann A, Weiler EW, Steudle E, Grill E. A hydraulic signal in root-to-shoot signalling of water shortage. Plant J 2007;52:167–74.
Colzi I, Rocchi S, Rangoni M, Del Bubba M, Gonnelli C. Specificity of metal tolerance and use of excluder metallophytes for the phytostabilization of metal polluted soils: the case of *Silene paradoxa* L. Environ Sci Pollut R 2014;21:10960–9.
Comstock JP. Hydraulic and chemical signalling in the control of stomatal conductance and transpiration. J Exp Bot 2002;53:195–200.
Cui S, Zhou Q, Chao L. Potential hyper-accumulation of Pb, Zn, Cu and Cd in endurant plants distributed in an old smeltery, northeast China. Environ Geol 2007;51:1043–8.
Davis RD, Beckett PHT. Upper critical levels of toxic elements in plants. II. Critical levels of copper in young barley, wheat, rape, lettuce and ryegrass, and of nickel and zinc in young barley and ryegrass. New Phytol 1978;80:23–32.
de Silva NDG, Cholewa E, Ryser P. Effects of combined drought and heavy metal stresses on xylem structure and hydraulic conductivity in red maple (*Acer rubrum* L.). J Exp Bot 2012;63:5957–66.
Di Baccio D, Tognetti R, Minnocci A, Sebastiani L. Responses of the Populus × euramericana clone I-214 to excess zinc: carbon assimilation, structural modifications, metal distribution and cellular localization. Environ Exp Bot 2009;67:153–63.
Drzewiecka K, Mleczek M, Gańsecka M, Magdziak Z, Goliński P. Changes in *Salix viminalis* L. cv 'Cannabina' morphology and physiology in response to nickel ions – hydroponic investigations. J Hazard Mater 2012;217–218:429–38.
Ehleringer JR, Hall AE, Farquhar GD. Stable isotopes and plant carbon–water relations. Academic Press: New York; 1993.
Gratão PL, Polle A, Lea PJ, Azevedo RA. Making the life of heavy metal-stressed plants a little easier. Funct Plant Biol 2005;32:481–94.
Gupta N, Khan DK, Santra SC. An assessment of heavy metal contamination in vegetables grown in wastewater-irrigated areas of Titagarh, West Bengal, India. Bull Environ Contam Toxicol 2008a;80:115–8.
Gupta S, Nayek S, Saha RN, Satpati S. Assessment of heavy metal accumulation in macrophyte, agricultural soil, and crop plants adjacent to discharge zone of sponge iron factory. Environ Geol 2008b;55:731–9.
Gupta UC, Gupta SC. Trace element toxicity relationships to crop production and livestock and human health: implication for management. Commun Soil Sci Plant Anal 1998;29:1491–522.
Gutierrez RMP, Mitchell S, Solis RV. *Psidium guajava*: a review of its traditional uses, phytochemistry and pharmacology. J Ethnopharmacol 2008;117:1–27.

Hou WH, Song GL, Wang QH, Chang CC. Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemna minor*). Plant Physiol Biochem 2007;45:2–69.
Iori V, Pietrini F, Cheremisina A, Shevyakova NI, Radyukina N, Kuznetsov VV, et al. Growth responses, metal accumulation and phytoremoval capability in Amaranthus plants exposed to nickel under hydroponics. Water Air Soil Pollut 2013;224:1450.
Jacobi CM, do Carmo FF, Vincent RC, Stehmann JR. Plant communities on ironstone outcrops: a diverse and endangered Brazilian ecosystem. Biodivers Conserv 2007;16:2185–200.
Janicka-Russak M, Kabala K, Burzynski M, Klobus G. Response of plasma membrane H1-ATPase to heavy metal stress in *Cucumis sativus* roots. J Exp Bot 2008;59:3721–8.
Khalid BY, Tinsley J. Some effects of nickel toxicity on rye grass. Plant Soil 1980;55:139–44.
Kholodova V, Volkov K, Abdeyeva A, Kuznetsov V. Water status in *Mesembryanthemum crystallinum* under heavy metal stress. Environ Exp Bot 2011;71:382–9.
Krämer U. Phytoremediation: novel approaches to cleaning up polluted soils. Curr Opin Biotechnol 2005;2:133–41.
Kramer U, Pickering IJ, Prince RC, Raskin I, Salt DE. Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. Plant Physiol 2000;122:1343–53.
Krämer U, Talke IN, Hanikenne M. Transition metal transport. FEBS Lett 2007;581:2263–72.
Krupa Z, Siedlecka A, Maksymiec W, Baszynski T. In vitro responses of photosynthetic apparatus of *Phaseolus vulgaris* L. to nickel toxicity. Plant Physiol 1993;142:664–8.
Küpper H, Küpper F, Spiller M. Environmental relevance of heavy metal substituted chlorophylls using the example of water plants. J Exp Bot 1996;47:259–66.
Llamas A, Ullrich CI, Sanz A. Ni^2 toxicity in rice: effect on membrane functionality and plant water content. Plant Physiol Biochem 2008;46:905–10.
Liu Z, He X, Chen W, Yuan F, Yan K, Tao D. Accumulation and tolerance characteristics of cadmium in a potential hyperaccumulator – *Lonicera japonica* Thunb. J Hazard Mater 2009;169:170–5.
Malik RN, Husain SZ, Nazir I. Heavy metal contamination and accumulation in soil and wild plant species from industrial area of Islamabad, Pakistan. Pak J Bot 2010;42:291–301.
Mancuso S. Seasonal dynamics of electrical impedance parameters in shoots and leaves related to rooting ability of olive (*Olea europea*) cuttings. Tree Physiol 1999;19:95–101.
Mancuso S, Papeschi G, Marras AM. A polarographic, oxygen-selective, vibrating-microelectrode system for the spatial and temporal characterisation of transmembrane oxygen fluxes in plants. Planta 2000;211:384–9.
Medrano H, Escalona JM, Bota J, Gulias J, Flexas J. Regulation of photosynthesis of C-3 plants in response to progressive drought: stomatal conductance as a reference parameter. Ann Bot 2002;89:895–905.
Michael PI, Krishnaswamy M. The effect of zinc stress combined with high irradiance stress on membrane damage and antioxidative response in bean seedlings. Environ Exp Bot 2011;74:171–7.
Murphy A, Taiz L. Correlation between potassium efflux and copper sensitivity in 10 Arabidopsis ecotypes. New Phytol 1997;136:211–22.
Nieminen TM, Ukonmaanaho L, Rausch W, Shotyk N. Biogeochemistry of nickel and its release into the environment. In: Sigel A, Sigel H, Sigel RKO, editors. Metal ions in life sciences, vol. 2; 2007. p. 1–30.
Pandolfini T, Gabbrielli R, Comparini C. Nickel toxicity and peroxidase activity in seedlings of *Triticumaestivum* L. Plant Cell Environ 1992;15:719–25.
Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. Plant J 2002;32:539–48.
Perry CT, Divan AM, Rodriguaz MTR, Atz AL. *Psidium guajava* as a bioaccumulator of nickel around an oil refinery, southern Brazil. Ecotoxicol Environ Saf 2010;73:647–54.
Pinto E, Sigaud-Kutner TCS, Leitão MAS, Okamoto OK, Morse D, Colepicolo P. Heavy metal-induced oxidative stress in algae. J Phycol 2003;39:1008–18.
Pottosin I, Velarde-Buendí AM, Bose J, Fuglsang AT, Shabala S. Polyamines cause plasma membrane depolarization, activate Ca^{2+}-, and modulate H^+-ATPase pump activity in pea roots. J Exp Bot 2014;65:2463–72.
Przedpelska-Wasowicz EM, Wierzbicka M. Gating of aquaporins by heavy metals in *Allium cepa* L. epidermal cells. Protoplasma 2011;248:663–71.
Piccini DF, Malavolta E. Effect of nickel on two common bean cultivars. J Plant Nutr 1992;15:2343–50.
Pulford ID, Watson C. Phytoremediation of heavy-metal-contaminated land by trees – a review. Environ Int 2003;29:529–40.
Pyle GG, Swanson SM, Lehmkuhl DM. The influence of water hardness, pH, and suspended solids on nickel toxicity to larval fathead minnows (*Pimephales promelas*). Water Air Soil Pollut 2002;133:215–26.
Rascio N, Navari-Izzo F. Heavy metal hyperaccumulating plants: how and why do they do it? And what makes them so interesting? Plant Sci 2011;180:169–81.
Raskin I, Ensley BD. Phytoremediation of toxic metals: using plants to clean up the environment. New York: John Wiley and Sons; 2000.
Rastgoo L, Alemzadeh A. Biochemical responses of Gouan (*Aeluropus littoralis*) to heavy metals stress. Aust J Crop Sci 2011;5:375–83.
Ros R, Cooke DT, Burden RS, Jones CS. Effects of the herbicide MCPA and the heavy metals cadmium and nickel on the lipid composition, Mg^{2+}-ATPase activity and fluidity of the plasma membrane from rice, *Oryza sativa* (cv. Bahía) shoots. J Exp Bot 1992;41:457–62.
Ryser P, Emerson P. Growth, root and leaf structure, and biomass allocation in *Leucanthemum vulgare* Lam (Asteraceae) as influenced by heavy-metal containing slag. Plant Soil 2007;301:315–24.
Schachtman DP, Goodger JQD. Chemical root to shoot signaling under drought. Trends Plant Sci 2008;13:281–7.
Sagardoy R, Morales F, Lopez-Millan A, Abadia A, Abadia J. Effects of zinc toxicity on sugar beet (*Beta vulgaris* L.) plants grown in hydroponics. Plant Biol 2009;11:339–50.
Sagardoy R, Vázquez S, Florez-Sarasa ID, Albacete A, Ribas-Carbó M, Flexas J, et al. Stomatal and mesophyll conductances to CO_2 are the main limitations to photosynthesis in sugar beet (*Beta vulgaris*) plants grown with excess zinc. New Phytol 2010;187:145–58.
Samantaray S, Rout GR, Das P. Tolerance of rice to nickel in nutrient solution. Biol Plant 1997;40:295–8.
Seregin IV, Kozhevnikova AD. Physiological role of nickel and its toxic effects on higher plant. Russ J Plant Physiol 2006;53:257–77.
Seregin IV, Kozhevnikova AD, Kazyumina EM, Ivanov VB. Nickel toxicity and distribution in maize roots. Russ J Plant Physiol 2003;50:793–800.
Singh RP, Agrawal M. Effects of sewage sludge amendment on heavy metal accumulation and consequent responses of *Beta vulgaris* plants. Chemosphere 2007;67:2229–40.
Spurr AR. A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultra Mol Struct R 1969;26:31–43.
Tanhan MKP, Pokethitiyook P, Chaiyarat R. Uptake and accumulation of cadmium, lead and zinc by Siamweed [*Chromolaena odorata* (L.) King & Robinson]. Chemosphere 2007;68:323–9.
Tripathy BC, Bhatia B, Mohanty P. Inactivation of chloroplast photosynthetic electron-transport activity by Ni^{2+}. Biochim Biophys Acta 1981;638:217–24.
Velikova V, Tsonev T, Loreto F, Centritto M. Changes in photosynthesis, mesophyll conductance to CO_2, and isoprenoid emissions in *Populus nigra* plants exposed to excess nickel. Environ Pollut 2011;159:1058–66.
Wang HB, Wong MH, Lan CY, Baker AJM, Qin YR, Shu WS, et al. Uptake and accumulation of arsenic by 11 *Pteris* taxa from southern China. Environ Pollut 2007;145:225–33.
Wellburn AR. The spectral determination of chlorophyll a and chlorophyll b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 1994;144:307–13.
Zhang WH, Cai Y, Tu C, Ma LQ. Arsenic speciation and distribution in an arsenic hyper accumulating plant. Sci Total Environ 2002;300:167–77.