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Abstract

The effectiveness of plant growth promoting bacteria (PGPB) in improving metal phytoremediation is still limited by stunted plant growth under high soil metal concentrations. Meanwhile, mixed planting with leguminous plants is known to improve yield in nutrient deficient soils but the use of a metal tolerant legume to enhance metal tolerance of a phytoremediator has not been explored. We compared the use of *Pseudomonas brassicacearum*, *Rhizobium leguminosarum* and the metal tolerant leguminous plant *Vicia sativa* to promote the growth of *Brassica juncea* in soil contaminated with 400 mg Zn kg^{-1}, and used synchrotron based microfocus X-ray absorption spectroscopy to probe Zn speciation in plant roots. *B. juncea* grew better when planted with *V. sativa* than when inoculated with PGPB. By combining PGPB with mixed planting, *B. juncea* recovered full growth while also achieving soil remediation efficiency of >75%, the maximum ever demonstrated for *B. juncea*. μXANES analysis of *V. sativa* suggested possible root exudation of the Zn chelates histidine and cysteine were responsible for reducing Zn toxicity. We propose the exploration of a legume-assisted-phytoremediation system as a more effective alternative to PGPB for Zn bioremediation.

Keywords: leguminous plant; metal phytoremediation; plant growth promoting bacteria; μXANES analysis; Zn bioremediation

Introduction

Metals and metalloids like cadmium, zinc, chromium, lead and arsenic are widely associated with environmental contamination and biological toxicity (Shahid et al. 2012). Although they can be released into the environment through geogenic processes, much of the environmental contamination originates from anthropogenic activities, including industrial production, energy production, mining and agricultural activities (Hu and Cheng 2013). The large inventory of metal contaminated sites (Lado, Hengl and Reuter 2008), the cross media environmental contamination and persistence of metals have necessitated the development of chemical and mechanical remediation methods which, although effective, are also environmentally destructive, expensive and ultimately unsustainable (Mulligan, Yong, and Gibbs 2001).

By contrast, plants are natural miners of nutrients and other elements from the environment (Sheoran, Sheoran, and Poonia 2009). The unique ability of plants to remediate metals has been widely investigated, leading to identification of metal hyperaccumulators, plants that accumulate metals in their tissue (Cappa and Pilon-Smits 2014; Lorestani, Cheraghi, and Yousefi 2012). Unfortunately, metal accumulating plants are not necessarily tolerant and may be subject to metal toxicity at high metal concentrations, leading to poor remediation efficiency (Ebbs and Kochian 1997). Inoculating hyperaccumulators with plant growth promoting bacteria (PGPB) has been demonstrated to promote growth in contaminated environments (de-Bashan, Hernandez, and Bashan 2012; Jing et al. 2014), potentially offering a sustainable remediation technology for toxic metal contaminants. However, the

effects of PGPB on plant growth promotion under high soil metal contamination are not always satisfactory and remediation efficiency is still relatively low (Zhuang et al. 2007).

An alternative to PGPBs for enhancing phytoremediation has been to use mixed planting or co-cropping to improve plant growth, on the assumption that interaction of roots from two or more plants modifies root physiology as well as root surface properties and rhizospheric chemical dynamics (Bashan et al. 2012; Gove et al. 2002). Although a large number of such studies have been reported, the outcomes are often variable and plant- as well as metal-specific. In some cases, the co-crop increases both the biomass and the metal accumulation capacity of the phytoremediator (Lucisine et al. 2014; Wu, Wei, and Ouyang 2007), whereas other studies have shown reduced growth of the phytoremediator whilst increasing metal accumulation due to the concentration effect (Wu et al. 2007). Further still, some studies have shown no effect of the co-crop on both growth/biomass and soil metal remediation (Wu et al. 2013; Zou 2015).

Significantly, most of these studies do not provide a biochemical basis for the observed changes. Upon exposure to toxic metals, most plants produce amino acids and low molecular weight peptides, which are used in metal binding, antioxidant defences and signaling (Sharma and Dietz 2006). Specifically, hypertolerant plants are capable of growing in metal-contaminated environments with little or no sign of stress, although they do not necessarily have metal-remediating abilities (Broadley et al. 2007). Studies suggest that this tolerance is linked to the production of metallothioneins and phytochelatins that act as metal chelates, leading to reduced metal phytotoxicity (Benatti et al. 2014; Blindauer 2008). Therefore, a mixed planting system, involving a metal remediating plant (target

plant) and a metal-tolerant plant that does not necessarily have metal remediating ability, may offer a better approach to improve soil metal remediation.

This work presents results of an experiment in which *Brassica juncea*, an established multiple metal phytoremediator but with relatively poor tolerance to high concentrations of zinc (Zn), was co-planted under Zn contamination with the leguminous plant *Vicia sativa.* The novelty of our study lies in the use of a plant with no known metal accumulation attributes but established to be tolerant to Zn in our preliminary studies, leading us to erect two complimentary hypotheses. Firstly, that the hypertolerance of *V. sativa* to Zn and low Zn accumulation was due to synthesis of low molecular weight peptides that acted as either efflux or external binding mechanisms. Secondly, that the hypertolerant *V. sativa* would confer its metal-tolerance benefits to *B. juncea*, leading to improved growth and soil Zn remediation. We also compared the effects of our mixed planting system with the use of plant growth promoting *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* bacteria to enhance soil Zn remediation.

Materials and Methods

Materials

We used *B. juncea* L. *Czern* (Indian mustard) and *Vicia sativa* subsp. *sativa* L. (cultivated vetch), with seeds sourced from Sow Seeds Ltd., UK. The plant growth promoting bacteria used were *Pseudomonas brassicacearum* subsp**.** *brassicacearum* (strain DBK11) isolated from a *Brassica* plant (Leibniz Institute DSMZ, Germany) and *Rhizobium leguminosarum* bv. *trifolii* (strain WSM1325) isolated from the rhizosphere of a leguminous plant (School

of Biological Sciences, University of Edinburgh, UK). The bacteria strains have been demonstrated to have plant growth promoting abilities under Zn contamination (Adediran et al. 2015). A standard soil for pot experiments, Scotts Levington F2+S Seed & Modular growth medium (Green-tech Ltd., UK) (Vicente et al. 2012), was used. The soil has pH 5.5-5.7, conductivity 210-290 μS cm^{-1} and nitrogen, phosphorus and potassium content of 350, 650 and 550 mg kg^{-1}, respectively. The soil was sterilized and dried in an autoclave at the start of the experiment. Zn was chosen as an essential micronutrient that is also phytotoxic at high concentration, and is one of the most abundant and most studied metal contaminants (Broadley et al. 2007; Dinh et al. 2015). The background soil Zn content was 48±10 mg kg^{-1}. The soil was spiked using Zn sulfate solution instead of Zn nitrate to avoid the confounding effects of nitrate as a macronutrient.

B. juncea and V. sativa growth and Zn toxicity tolerance assessment

The experiments presented in this paper were conducted in parallel with those of sole planted *B. juncea* under Zn contamination reported in Adediran et al. (2015), and the methods are summarized here to provide additional information on Zn tolerance assessment of *V. sativa* and mixed-planting procedures. To assess the growth of sole planted *B. juncea* and sole planted *V. sativa* in un-contaminated and Zn contaminated soil, a pot experiment was conducted in a glasshouse at the University of Edinburgh with mean 21°C daytime and 18°C night-time temperatures, and artificial lighting providing a photoperiod of 18 h d^{-1} and photo levels of ~150 μmol m^{-2} s^{-1}. 0.5 kg of soil was placed in 5 L plastic pots located in a saucer for each experimental replicate. Contaminated treatments were spiked with Zn sulfate solution at the rate of 400 mg Zn kg^{-1} soil dry weight, a concentration typical of

contaminated agricultural soils in the UK (Baker et al. 1994). The same volume of deionized water was added to the controls. Pots were then watered to field capacity and allowed to stand in the glasshouse for a week before seed planting. Seeds of the two plants were surface sterilized in 0.05 M sodium hypochlorite for 30 min, washed in sterile deionized water and dried under aseptic conditions (Kumar et al. 2008). Five seeds were planted per pot. Seedlings were weeded to two plants per pot 5 days after emergence. Each treatment was replicated in 3 pots which were randomly distributed in the glasshouse space. To prevent water stress the pots were kept moist throughout the experiment by adding deionized water in saucers placed under the pots. Plant height was measured weekly for 6 weeks after seed planting when plants under Zn contamination were still alive and those in uncontaminated treatments were almost 1.5 m in height.

Evaluation of growth and Zn remediation promotion systems with legume-mixed planting and PGPB

The ability of the leguminous *V. sativa* plant to promote the growth of *B. juncea* in a mixed planting system was evaluated and compared to the use of PGPB under the same experimental conditions as described above. For the mixed planting treatments, one *B. juncea* plant and one *V. sativa* plant were established 1.5 cm apart in the same 5 l pot. This was deemed sufficient to provide space for each plant to grow whilst maximizing rhizospheric interaction between the two plants to test the hypothesis of *V. sativa* conferring Zn toxicity tolerance to *B. juncea*. For the bacteria inoculated treatments, cells of *P. brassicacearum* and *R. leguminosarum* were cultured in nutrient broth at 30°C to an

exponential growth stage and treated as described in Adediran et al. (2015). Surface sterilized seeds of *B. juncea* plants were incubated under aseptic conditions at 30°C in a suspension of bacteria in sterile water (absorbance of 0.5 at 600 nm) for 3 h. We have previously investigated the toxicity of Zn to the bacteria strains used and reported a sufficient population of live bacteria cells under Zn 400 – 600 mg l^{-1} (Adediran et al. 2015). This inoculation system has been shown to provide active root colonization until the end of the 6 week plant growth experiment under Zn (Adediran et al. 2015). Seeds for the control treatments were soaked in sterile deionized water in the same conditions.

The soil in each of the three replicates was sampled at the end of the 6 week growing period and two 1 g oven-dried sub-samples were extracted using a modified BCR sequential extraction method followed by aqua regia extraction to determine soil Zn concentrations in exchangeable, reducible, oxidizable and residual fractions (Rauret et al. 1999). Extracts were analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Perkin Elmer Optima 5300 DV. Zn calibration standards were prepared from analytical grade AAS stock standards. Use of a certified multi-element M6 (VWR) standard among sample runs showed the error on Zn determination was less than 5%. Extraction blanks were also analysed and subtracted from analytical results. Total Zn content was calculated as the sum of the concentration in the four fractions. Phytoremediation efficiency was estimated by subtracting total soil Zn content in pots under phytoremediation from contaminated pots without plants. This method has been used in similar experiments conducted to estimate soil metal removal efficiency by plants (Bennett et al. 2003).

Analysis of Zn speciation in the plant roots

Live plants were transported to the Diamond Light Source, UK, at 5 weeks after planting. Plant roots were used for Zn speciation analysis on the microfocus spectroscopy beamline I18 (Mosselmans et al. 2009) since they are the first plant organ in contact with Zn, roots of other plants and soil bacteria, and the site where the first reaction to metal toxicity and sequestration takes place (Zhou, Tang, and Wu 2013). Due to extensive root interactions in pots where *B. juncea* was co-planted with *V. sativa,* we could not confidently separate the root of one plant from another without causing physical damage and possibly influencing the nature of metal accumulation and speciation. Only *B. juncea* and *V. sativa* roots from the mono-cropped pots were therefore analyzed. Two primary root samples of uniform sizes were excised from each plant, rinsed with sterile deionized water and immediately cryofixed on the beam sample holder mounted on an x-y-z stage at an angle of 45° to the incident beam. The beamline energy was calibrated using a Zn foil (9661 eV).

Micro X-ray fluorescence (μXRF) maps of Zn were collected from the root tip to about 120 μm towards the stem at cryogenic temperature of -80°C in fluorescence mode using a nine-element germanium solid state detector. The collected μXRF data were processed into images using PyMCA 4.4.1 (Solé et al. 2007). μXANES data were collected from several Zn hot spots identified in the element maps, examined for possible beam damage and at least 8 good μXANES datasets selected for each treatment and merged. μXANES data were also collected for 12 model Zn compounds (see Supplementary material Table S1) potentially involved in Zn dynamics within the plant-soil system studied (Kopittke et al. 2011; Terzano et al. 2008). μXANES data from the root samples were compared to the

model compounds using a least-squares algorithm involving Linear Combination Fitting (LCF) in Demeter 0.9.18. The goodness of fit was estimated as the residual *R* factor of the fit, Σ_i (experimental-fit)^2 / Σ_i (experimental)^2, where the sums are over 103 data points as flattened mu (E). A lower *R* factor represents a better match between the fitted standard spectra and the sample spectrum (Terzano et al. 2008).

Statistical analysis

The mean weekly plant heights (of the two plants per pot) and the Zn concentrations of the two soil sub-samples from each pot 6 weeks after planting were found to be normally distributed using Anderson-Darling's normality test and of equal variance. Significant ($p<0.05$) differences between the treatment means of three replicate pots were identified by applying either two-sample t-tests or one-way Analysis of Variance followed by Tukey's multiple comparison tests. All statistical analyses were conducted using Minitab 16 software (Minitab^{TM} Inc., USA).

Results

B. juncea and V. sativa plant growth and remediation abilities under Zn

The height of *B. juncea* and *V. sativa* plants exposed to Zn contamination (BZn and VZn, respectively) was evaluated and compared over a period of 6 weeks. The significant differences in plant height shown for week 6 (Fig. 1a) were maintained throughout the previous growth period (see Supplementary material Figures S1-S2). After 6 weeks the height of BZn plants was significantly lower compared to the control uncontaminated

plants (Bo) while there was no significant difference in plant height between VZn and the control (Vo).

FIGURE 1.

These results show that *V. sativa* is tolerant to Zn added at 400 mg kg^{-1} (a total of 448 mg kg^{-1} Zn including the background soil content) whereas *B. juncea* is susceptible. In our preliminary investigation, we observed *V. sativa* to exhibit maximum Zn tolerance of about 800 mg kg^{-1} Zn (data not shown). Despite the vulnerability of *B. juncea* plants to this level of Zn contamination, they have a significantly higher soil Zn remediation efficiency than the tolerant *V. sativa* plants (Fig. 1b) and also remove significantly more exchangeable soil Zn (Fig. 1c). The observed metal remediation ability of *B. juncea* is well known (Seth, Misra, and Chauhan 2012) but the Zn hypertolerance of *V. sativa* reported in this study is new.

Growth and Zn remediation promotion of B. juncea with PGPB and V. sativa B. juncea

Growth and Zn remediation ability was enhanced by inoculation with PGPB, as shown in our previous study (Adediran et al. 2015), but the use of leguminous plants to enhance soil phytoremediation has not previously been compared with PGPB. Hence we compared the use of PGPB to promote the growth and phytoremediation efficiency of *B. juncea* with the use of the leguminous plant *V. sativa*. *V. sativa* was chosen because of its demonstrated tolerance to Zn contamination (Fig. 1a) and its reported association with diverse species of bacteria (Lei et al. 2008). Although inoculation with PGPB promoted plant growth and

resulted in improved soil Zn remediation compared to uninoculated contaminated treatments (BZn) (Figs. 2a-2c), the growth of inoculated *B. juncea* was still significantly retarded, with a mean BRZn plant height of 60 cm compared to 129 cm for *B. juncea* growing in uncontaminated soil (Bo).

At 6 weeks after planting, *B. juncea* co-planted with *V. sativa* (BVZn) was significantly taller than sole planted *B. juncea* (BZn) as well as plants inoculated with bacteria (BRZn and BPZn) upon exposure to Zn contamination (Fig. 2a).

FIGURE 2

The amount of Zn removed from the contaminated soil was significantly higher in the BVZn than the BZn treatment (Fig. 2b). Plants in the mixed planting treatment (BVZn) also remediated significantly more exchangeable and reducible soil Zn than *B. juncea* on its own (BZn) (Fig. 2c). Significantly, the higher Zn remediation observed under mixed planting (mean = 259 mg kg^{-1}, Fig. 2b) exceeds the sum of the individual remediating effects of the two plants (mean = 199 mg kg^{-1}, Fig. 1b).

Our results show clearly that co-planting with a leguminous plant (BVZn, mean plant height after 6 weeks = 103 cm) outperforms the plant growth promoting effects of bacteria (BRZn and BPZn, mean plant height after 6 weeks = 60 and 22 cm, respectively) for *B. juncea* under Zn contamination. Nevertheless, plant height in the BVZn treatment is still significantly lower than *B. juncea* plants grown in uncontaminated soil (Bo, mean plant

height after 6 weeks = 129 cm). When PGPB were combined with co-planting with the leguminous *V. sativa* (BPVZn and BRVZn treatments) growth of *B. juncea* plants in Zn contaminated soils was significantly better than any other treatments 6 weeks after planting (Fig. 2a). A weekly analysis of plant height under the experimental treatments showed similar differences throughout the growth period (see Supplementary material Figure S2).

Remarkably, there was no significant difference between *B. juncea* plant heights in the BPVZn and BRVZn treatments and plants grown in uncontaminated soil (Bo) at 6 weeks after planting. As a result, BPVZn and BRVZn plants exhibited significantly higher Zn remediation efficiencies, removing as much as 75% of the added Zn (Fig. 2b) and remediating more exchangeable soil Zn than the PGPB and mixed planting only treatments (Fig. 2c). It is clear that the BPVZn and BRVZn treatments combine the benefits of inoculation with PGPB with the plant growth promoting effects of the leguminous *V. sativa* plants. Although the use of co-planting has been demonstrated in the remediation of contaminants (Gove et al. 2002; Hechmi et al. 2014), to our knowledge, the legume assisted-microbial phytoremediation method reported here is the first to demonstrate complete growth recovery in plants exposed to 400-450 mg kg^{-1} soil Zn contamination within a 6 week period.

Zn content and speciation in B. juncea and V. sativa roots

The Zn content and speciation in *B. juncea* and *V. sativa* roots was investigated through a comparative synchrotron based XAS analysis of root biomass from BZn and VZn plants. μXRF imaging of Zn in the plant roots showed that *B. juncea* accumulated significantly

more Zn than *V. sativa* (Fig. 3), consistent with the relative differences in soil remediation efficiencies of the two plants.

FIGURE 3

We are confident of this inference because, although the counts also depend on thickness of the mapped root, and this was not controlled during analysis, there is close to a 100:1 ratio in the maximum counts between the root samples of the two plants. Thus, there would have to be a similar difference in the thickness of the two roots shown in Figure 3 for the concentrations to be roughly similar. In addition to differences in the amount of Zn accumulation, the spatial distributions of Zn in the root of the two plant species are also conspicuously different. In *B. juncea* accumulated Zn appears to have been transported away from the cell walls and deposited inside the root endodermis, probably in the root vascular tissue. In contrast, there is a narrow band of Zn concentration at the epidermis of the *V. sativa* root with Zn more uniformly distributed inside the root.

Differences in Zn speciation between the *B. juncea* and *V. sativa* root samples are indicated by conspicuous differences in normalized Zn K edge in the LCF of Zn μXANES spectra (Fig. 4a and 4c). As previously demonstrated (Adediran et al. 2015), BZn has a higher *R* value than for BRZn and VZn, such that our fit with Zn oxalate (~52%) and Zn sulfate (~39%) can potentially be improved with addition of another species for which we do not have a standard spectrum, such as Zn malate which has been observed to accumulate in other phytoremediating plants (Sarret et al. 2002). Our tentative interpretation of Zn

speciation in BZn plant roots is supported by other studies that showed Zn oxalate accumulation in the root of Zn-resistant ecotypes of *Silene cucubalus* and *Rumex acetosa* planted on Zn spiked nutrient medium (Mathys 1977). Zn in the BRZn roots is stored predominantly as Zn polygalacturonate (~54%), Zn phytate (~20%), Zn carbonate (~14%) and Zn cysteine (~12%). In contrast to the BZn plant root, Zn accumulated mostly in the forms of Zn histidine (~60%) and Zn cysteine (~32%) with small traces of Zn sulfate (~6%) in *V. sativa* root.

FIGURE 4

Discussion

In this study, we compared the use of *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* bacteria with the use of a leguminous plant *Vicia sativa* in promoting the growth and metal remediation ability of *Brassica juncea* under Zn contamination. We then used synchrotron μXANES to constrain the speciation of Zn in *B. juncea* and *V. sativa* as a basis for understanding their different tolerances to Zn contamination.

Upon exposure to toxic metals, most plants and particularly known hyperaccumulators upregulate genes that are responsible for the synthesis of amino acids and low molecular weight peptides that perform several functions, including metal binding, antioxidant defense and signaling (Sharma and Dietz 2006). Metallothioneins and phytochelatins are both cysteine-rich peptides that are closely associated with metal detoxification and

tolerance by chelating and sequestering the metal in the cytosol, thereby removing the metal from sensitive plant organs (Benatti et al. 2014; Blindauer 2008). We have previously shown (Adediran et al. 2015), based on speciation analysis, that *B. juncea* does not produce significant quantities of these peptides when planted on its own in Zn contaminated soil (Figure 4a). Nevertheless, possible thiol detoxification was detected, as represented by cysteine complexation, upon inoculation of *B. juncea* with *Rhizobium leguminosarum* (Figure 4b). This observation invited the possibility that synthesis of thiol-containing ligands could be enhanced through co-planting *B. juncea* with a leguminous plant, allowing for natural colonization with rhizoids and hence conferring thiol production onto *B. juncea*. Unfortunately, the unexpectedly vigorous growth under mixed planting led to such an extensive root interaction that we could not independently determine the speciation of Zn in *B. juncea* co-planted with *V. sativa*. Thus, the hypothesis of *V. sativa* conferring Zn tolerance to *B. juncea* through biochemical means remains unproven and requires further investigation.

Speciation analysis of the hypertolerant *V. sativa* revealed Zn histidine complexes as the dominant form of Zn in the root tissue, followed closely by Zn cysteine complexes. Metal tolerance and hyperaccumulation *via* histidine complexation has been demonstrated with Ni studies (Salt et al. 1999a) but has also been implicated in Cu and Zn response (Sharma and Dietz 2006). Thus, reported Zn histidine complexation in the hyperaccumulator *Thlaspi caerulescens* has been regarded as the dominant Zn tolerance mechanism in roots, while in the xylem sap, most Zn was transported in the form of organic acid complexes (Salt et al. 1999b). More significantly, it has been shown that histidine was part of the root exudate

pool produced as a response to Ni exposure that led to Ni accumulation in both accumulating and non-accumulating species of *Thlaspi* (Salt et al. 1999a). This observation suggests that histidine may detoxify metals by exclusion from sensitive tissues and specifically by complexing metals outside the root and/or in the epidermis. Such an inference is consistent with our observation that in *V. sativa* roots, higher Zn concentrations localize in the epidermis (Figure 3b).

It appears that, by using Zn sulfate in a mixed planting system involving a legume, we created ideal conditions for the simultaneous exploitation of sulfur and nitrogen metabolisms based on the common glycolysis and citric acid cycle (Sharma and Dietz 2006), as outlined in a conceptual model (Figure 5).

FIGURE 5

Cysteine synthesis in plants is linked to sulfur metabolism (Saito 2000), whereby sulfur is taken up by plants in its inorganic sulfate form, and enzymatically converted to cysteine through several steps to produce sulfide, which reacts with O-acetylserine to form cysteine (Saito 2000; Tavares et al. 2015). The combination of cysteine with glutamate and glycine leads to the formation of glutathione, which forms phytochelatins under the influence of phytochelatin synthase. Studies with multiple plants have shown that the formation of phytochelatins is triggered by exposure to toxic metals, and likely forms mostly in the roots (Cobbett 2000). The development of this tolerance mechanism is shown on the left arm of Figure 5, occurs in most plants even in the absence of PGPB, and leads to the sequestration

of Zn (and other metals) in the cytosol of cells. However, and as shown in Figure 4b, inoculation with rhizobia enhances cysteine synthesis and promotes production of other chelates, which are included in the speciation model of the root in Figure 5.

Similarly, histidine is synthesized from glucose *via* the pyruvate route, and involves multiple enzymatic steps that essentially add nitrogen (NH_4^+), glutamine and glutamate to the 3-phosphoglycerate intermediate (Bromke 2013). Unlike phytochelatins, however, the production of histidine does not appear to be triggered by exposure to toxic metals (Krämer et al. 1996; Persans et al. 1999). Apparently, the whole histidine biosynthetic pathway in plants occurs in chloroplasts (Stepansky and Leustek 2006) and histidine must thus be translocated to the roots in order to form part of the exudate pool reported for *Thlaspi* species (Salt et al. 1999b). Our conceptual model accounts for this by locating Zn histidine complexes in the rhizosphere/epidermis, and this applies to both *B. juncea* and *V. sativa*.

Although such a model may provide a reasonable explanation for the reduced Zn uptake in *V. sativa* roots, it need not necessarily apply to *B. juncea* as histidine has also been shown to enhance phytoextraction of metals in other plants (Salt et al. 1999b). Since Zn binds more strongly to hard O- and N-containing ligands relative to soft S-containing ligands (Kopittke et al. 2011), production of histidine within the rhizosphere of the mixed planted *B. juncea* is likely to augment the already efficient thiogenic accumulation mechanism operating in *B. juncea*. Hence, we would argue that the use of leguminous plants to produce metal chelates naturally within the rhizosphere of the contaminated soil system is a more sustainable alternative to synthetic chelates such EDTA (Ebbs and Kochian 1998). A key

element of this approach, both in terms of plant growth promotion and enhanced soil remediation, is the symbiotic relationship between rhizoids and legumes, as shown in other studies of better yields in crops co-planted with legumes (Dakora 2003). Although not tested in our experiment, it is likely that the diverse bacteria population associated with the leguminous *V. sativa* plants contributed to the better growth and Zn remediation observed in BVZn plants (Lei et al. 2008). This interpretation is possible because, in order to replicate natural conditions, sterile conditions were not maintained in the glasshouse and it is expected that natural rhizobia colonized the roots of the leguminous plant. It is also supported by the data, which showed that there was no statistical difference in the growth between *B. juncea* inoculated with *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* under mixed planting (Figure 2a), suggesting that rhizoids associated with *V. sativa* override those of the inoculant.

Conclusions

This study utilized a combination of synchrotron based X-ray fluorescence imaging and μXANES to unravel the mechanism of Zn accumulation and toxicity tolerance in a non-remediating but Zn tolerant leguminous plant, *V. sativa*, and a well-known metal remediating plant, *B. juncea*, with poor tolerance to high Zn contamination. It then compared the use of a legume mixed planting system to the use of PGPB in promoting the growth and phytoremediation potential of *B. juncea*. Secretion of phytochelatins - histidine by the legume and enhanced cysteine synthesis by PGPB - was judged to be responsible for the better growth and soil Zn remediation in the system where PGPB was combined with *V.*

sativa. We therefore recommend a legume-phytoremediator mixed planting system as a more effective and sustainable remediation alternative for Zn contaminated soil. However, further studies are required to establish if histidine synthesis conferred by *V. sativa* to *B. juncea* is the only factor responsible for the improved growth and soil Zn remediation observed in BVZn treatments. Moreover, we utilized artificial spiked soil in this study in order to understand the specific biochemical metabolism of Zn in the studied plants. There is a need to extend the scope of the research to screen more leguminous plants for chelate secretion and to evaluate various combinations of tolerant leguminous species co-planted with metal remediating plant species under different forms of Zn and mixtures of metal contaminants.

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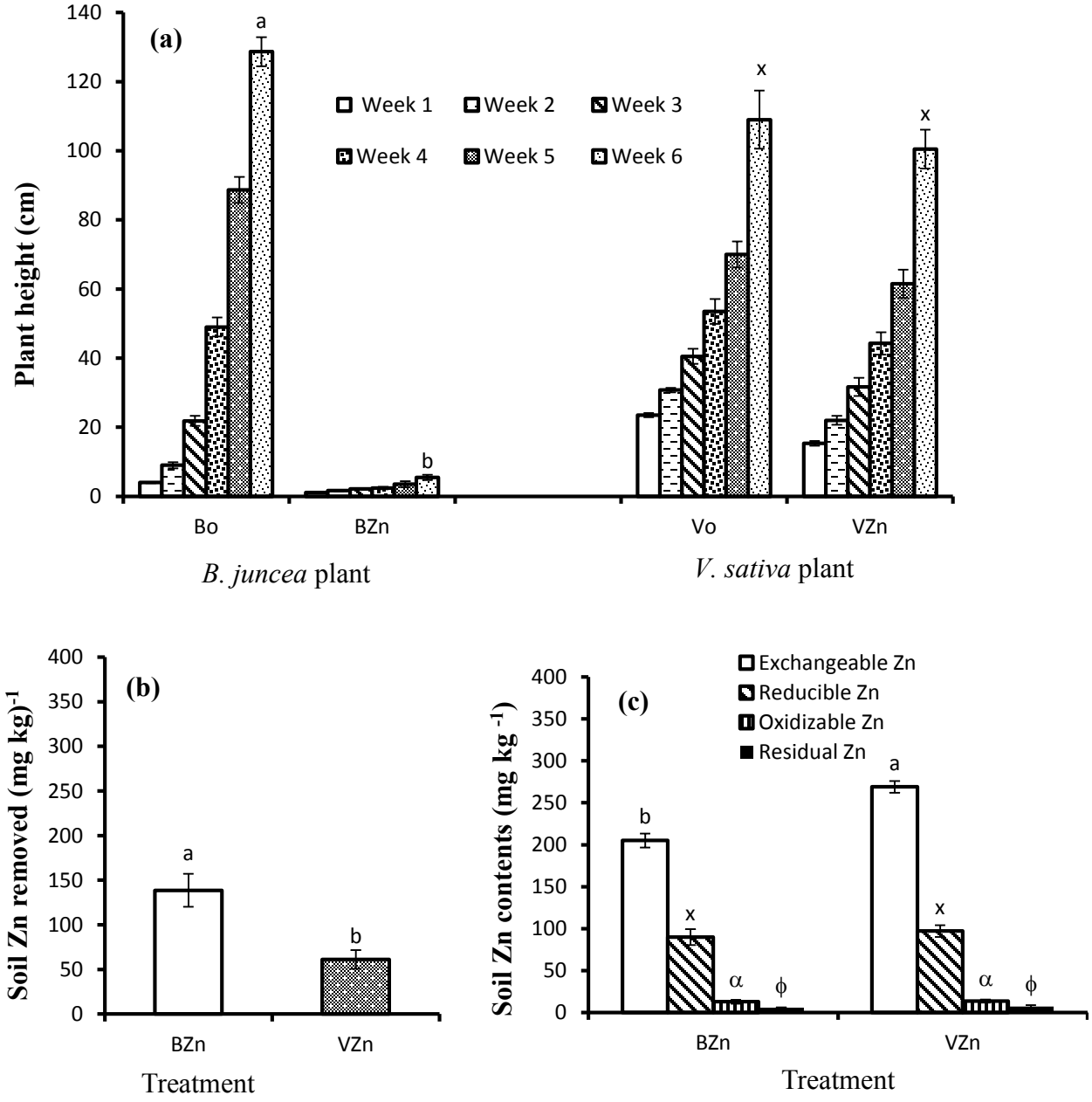
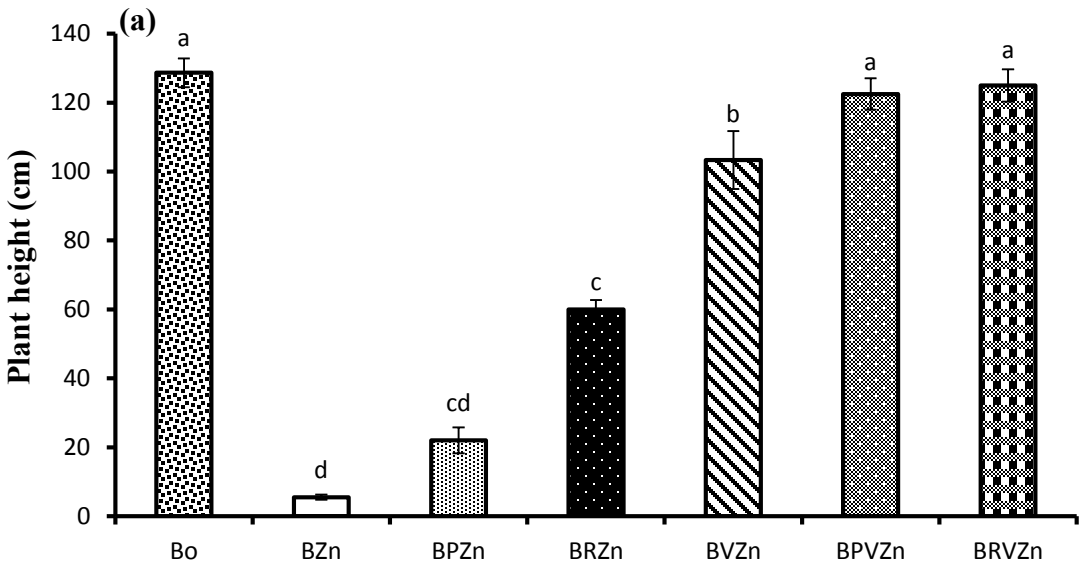
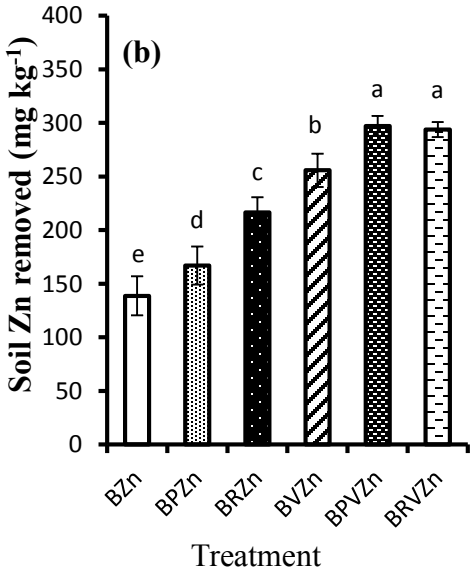

(a)
Plant height (cm)
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Bo
BZn
Vo
VZn
B. juncea plant
V. sativa plant
(b)
Soil Zn removed (mg kg)-1
Treatment
(c)
Soil Zn contents (mg kg -1)
Exchangeable Zn
Reducible Zn
Oxidizable Zn
Residual Zn
Treatment

Figure 1. (a) Weekly plant height of *B. juncea* and *V. sativa* in uncontaminated soil (Bo and Vo) and in Zn contaminated soil (BZn and VZn), (b) Zn removed per mass of soil from the total soil Zn concentration of 448 mg kg^{-1}, showing *B. juncea* as a better phytoremediator and (c) Zn concentrations in different fractions of remediated soil under BZn and VZn. Bars are mean values at 6 weeks after planting and error bars show standard errors (n=3). Different letters/symbols indicate significant ($p<0.05$) differences between treatments for week 6 only.



B. juncea plant growth at 6 weeks after planting



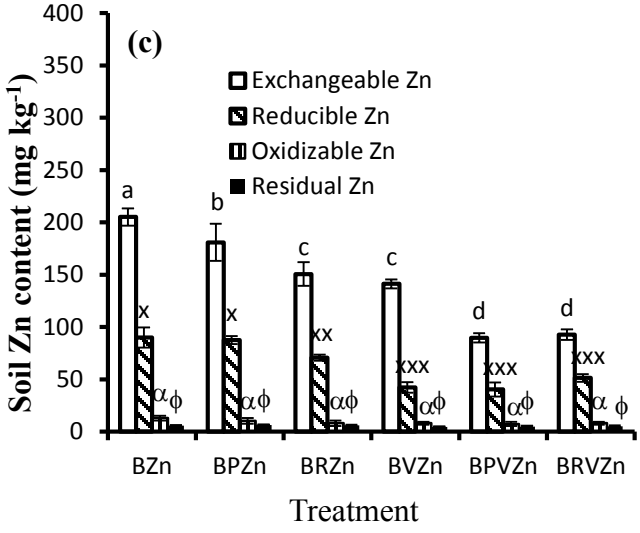


Figure 2. (a) Comparison of *B. juncea* plant height in uncontaminated soil (Bo) with height under Zn contamination in sole planted (BZn), bacteria inoculated (BPZn & BRZn), mixed planted (BVZn), inoculated & mixed planted (BPVZn & BRVZn) *B. juncea*, (b) Zn removed per mass of soil from the total soil Zn concentration of 448 mg kg^{-1}, and (c) amount of Zn in different fractions of remediated soil under BZn, BPZn, BRZn, BVZn, BPVZn and BRVZn plants. Bars are mean values at 6 weeks after planting and error bars show standard errors (n=3). Different letters/symbols indicate significant ($p<0.05$) differences between treatments.

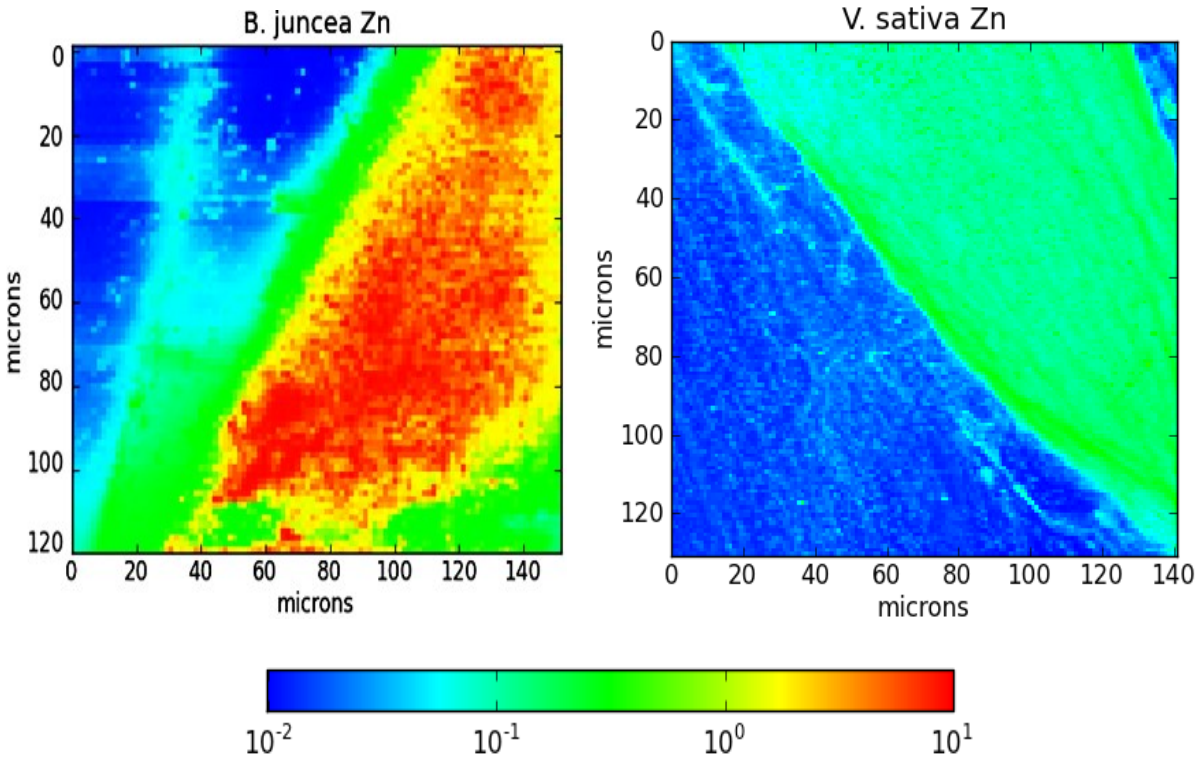
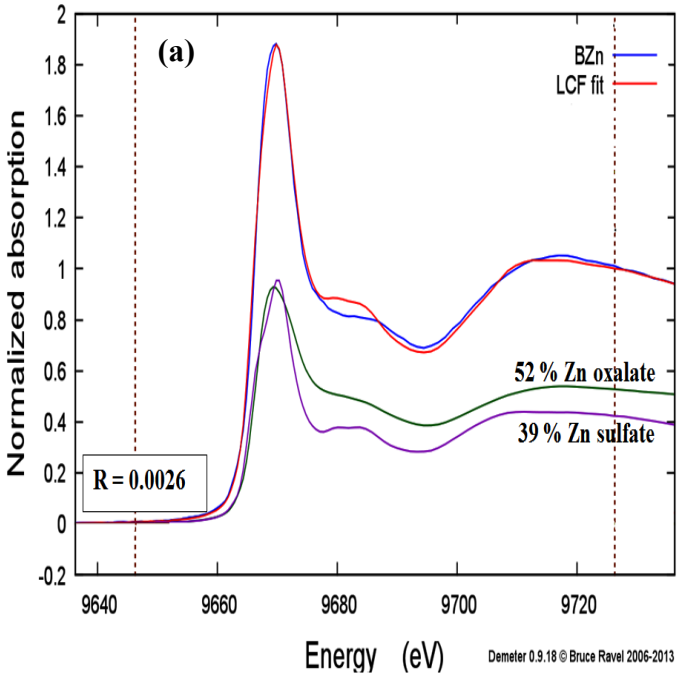
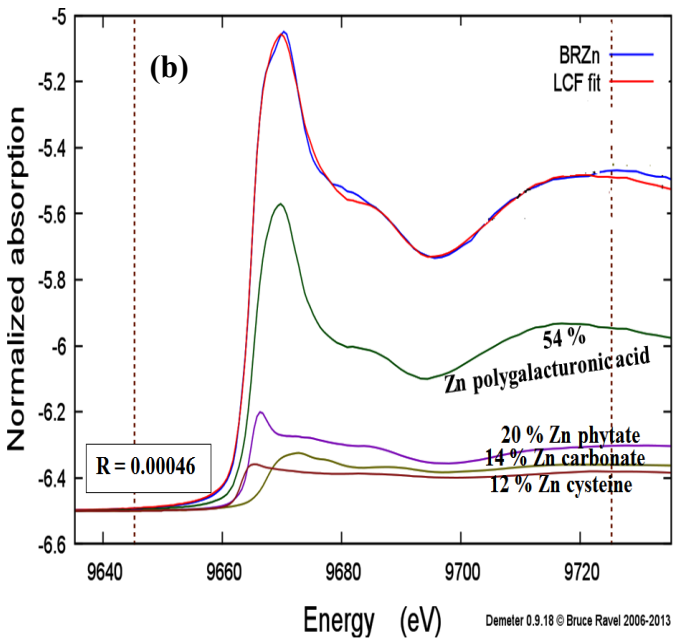


Figure 3. Synchrotron based μXRF images of Zn distribution in the primary root of *B. juncea* and *V. sativa* plants from the BZn and VZn treatments. Zn counts are normalized to incoming beam intensity and the beam detector was at the same distance from the sample for the acquisition of the maps. Colour bars ($\log_{10}$ scale) indicate Zn counts in plant roots from lowest (blue) to highest (red).


(a)
BZn
LCF fit
52 % Zn oxalate
39 % Zn sulfate
R = 0.0026
Normalized absorption
Energy (eV)
Demeter 0.9.18 © Bruce Ravel 2006-2013


(b)
BRZn
LCF fit
54 %
Zn polygalacturonic acid
20 % Zn phytate
14 % Zn carbonate
12 % Zn cysteine
R = 0.00046
Normalized absorption
Energy (eV)
Demeter 0.9.18 © Bruce Ravel 2006-2013

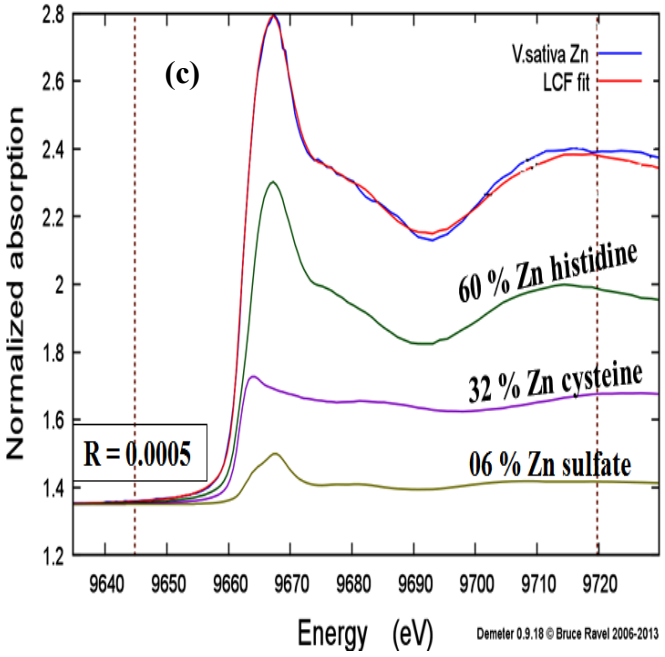

(c)
V.sativa Zn
LCF fit
60 % Zn histidine
32 % Zn cysteine
06 % Zn sulfate
R = 0.0005
Normalized absorption
Energy (eV)
Demeter 0.9.18 © Bruce Ravel 2006-2013

Figure 4. Normalized XANES (blue), LCF fits (red), R factor and % Zn compound composition for (a) *B. juncea* (BZn), (b) *B. juncea* inoculated with *R. leguminosarum* (BRZn) and (c) *V. sativa* (VZn) plant roots. $R = \Sigma i\ (\text{experimental-fit})^2 / \Sigma i\ (\text{experimental})^2$. The lower the *R* value the better the fit.

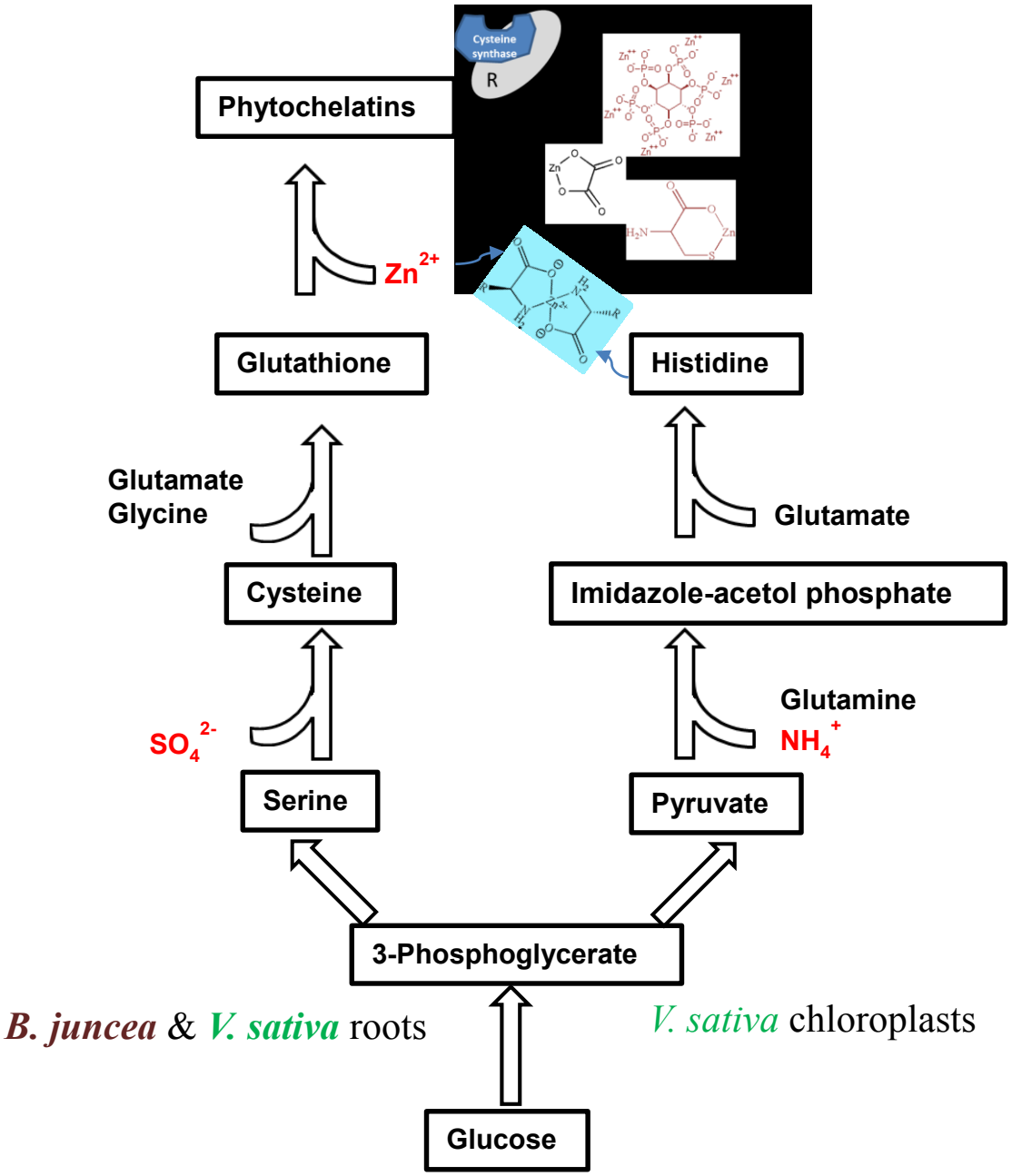

Cysteine synthase
R
Phytochelatins
Zn2+
Glutathione
Histidine
Glutamate
Glycine
Glutamate
Cysteine
Imidazole-acetol phosphate
Glutamine
SO4 2-
NH4 +
Serine
Pyruvate
3-Phosphoglycerate
B. juncea & V. sativa roots
V. sativa chloroplasts
Glucose

Figure 5. Biochemical model of histidine synthesis in *V. sativa* and phytochelatins production in the rhizosphere of *B. juncea* inoculated with *R. leguminosarum* and mixed planted with *V. sativa* in soil contaminated with Zn sulphate. Histidine synthesized through nitrogen metabolism enhanced Zn detoxification and helped confer Zn toxicity tolerance to *V. sativa*. *R. leguminosarum* enhanced cysteine secretion in *B. juncea* through sulfur metabolism. The synergetic effects of the phytochelatins conferred Zn tolerance and enhanced soil Zn remediation.

Mixed planting with a leguminous plant outperforms bacteria in promoting growth of a metal remediating plant through histidine synthesis

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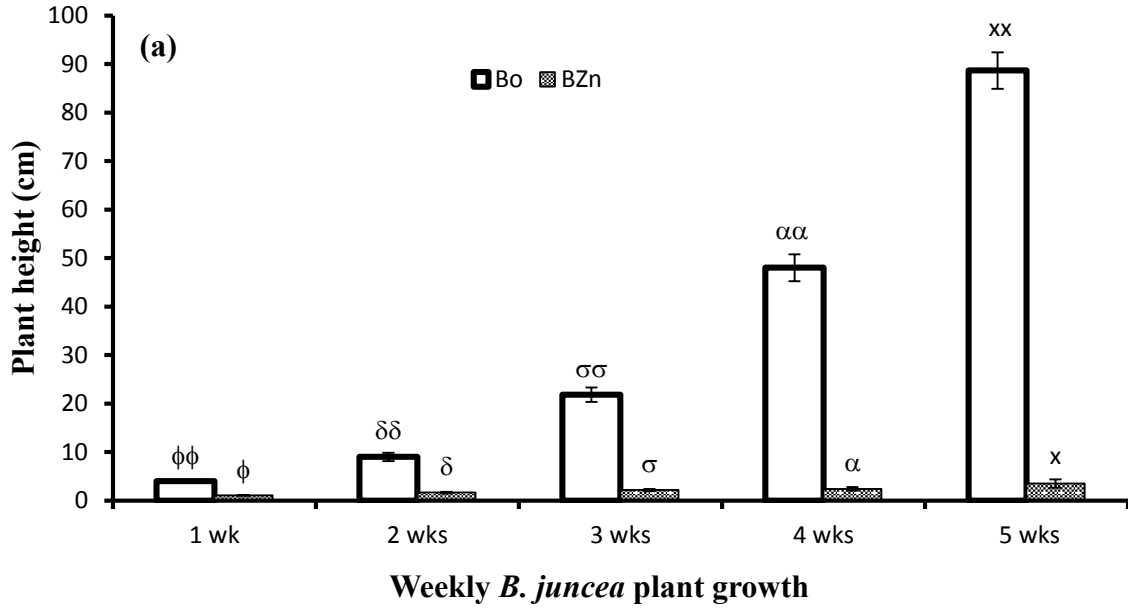
Table S1. Freshly prepared or purchased Zn standards used for XANES analysis

Zn standard	Characteristics
Zn oxalate	7.0 mM $Zn(NO_3)_2$ + 70 mM sodium oxalate, pH 7.0
Zn phosphate	7.0 mM $Zn(NO_3)_2$ + 70 mM sodium phosphate, pH 7.0
Zn histidine	7.0 mM $Zn(NO_3)_2$ + 80 mM histidine, pH 7.0
Zn cysteine	7.0 mM $Zn(NO_3)_2$ + 70 mM cysteine, pH 7.0
Zn phytate	7.0 mM $Zn(NO_3)_2$ + 70 mM phytic acid solution, pH 7.0
Zn polygalacturonate	7.0 mM $Zn(NO_3)_2$ + 70 mM polygalacturonic acid solution, pH 7.0
Zn formate	7.0 mM $Zn(NO_3)_2$ + 70 mM formic acid solution, pH 7.0
Zn sulfate, Zn nitrate, Zn citrate, Zn acetate and Zn carbonate	Purchased from Sigma Aldrich



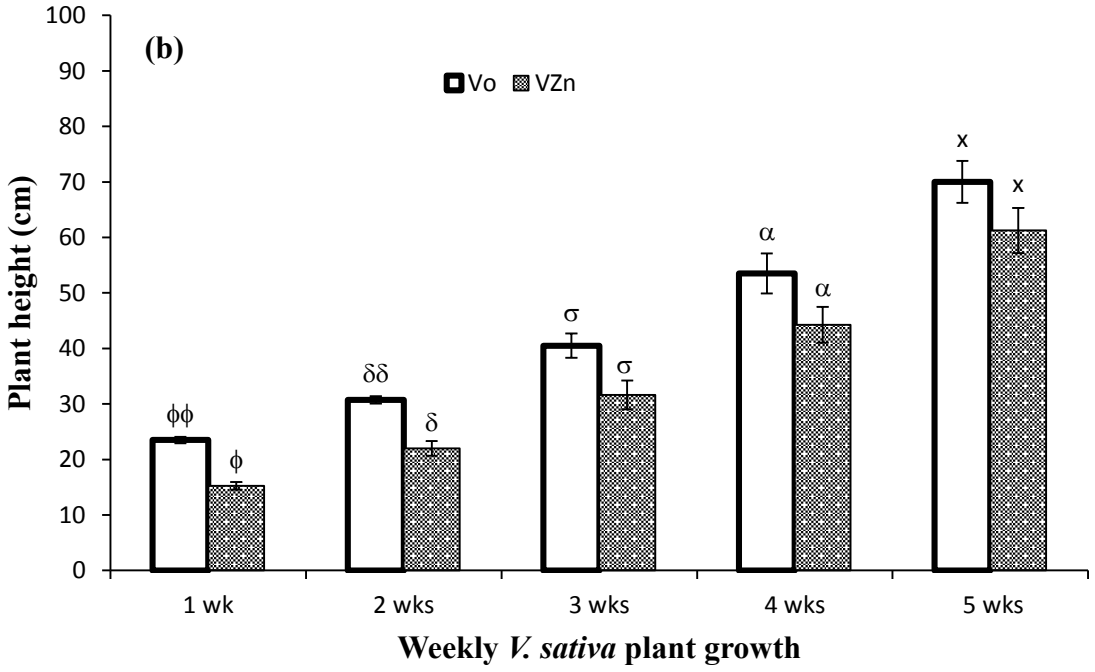


Figure S1. Weekly plant height of *Brassica juncea* (a) and *Vicia sativa* (b) in uncontaminated soil (Bo and Vo) and in Zn contaminated soil (BZn and VZn). Bars are mean plant heights from the 3 experimental pots and error bars show standard errors. Weekly means were subjected to T-test pairwise comparison. Different symbols indicate significant ($p<0.05$) differences in weekly plant height between treatments (n=3).

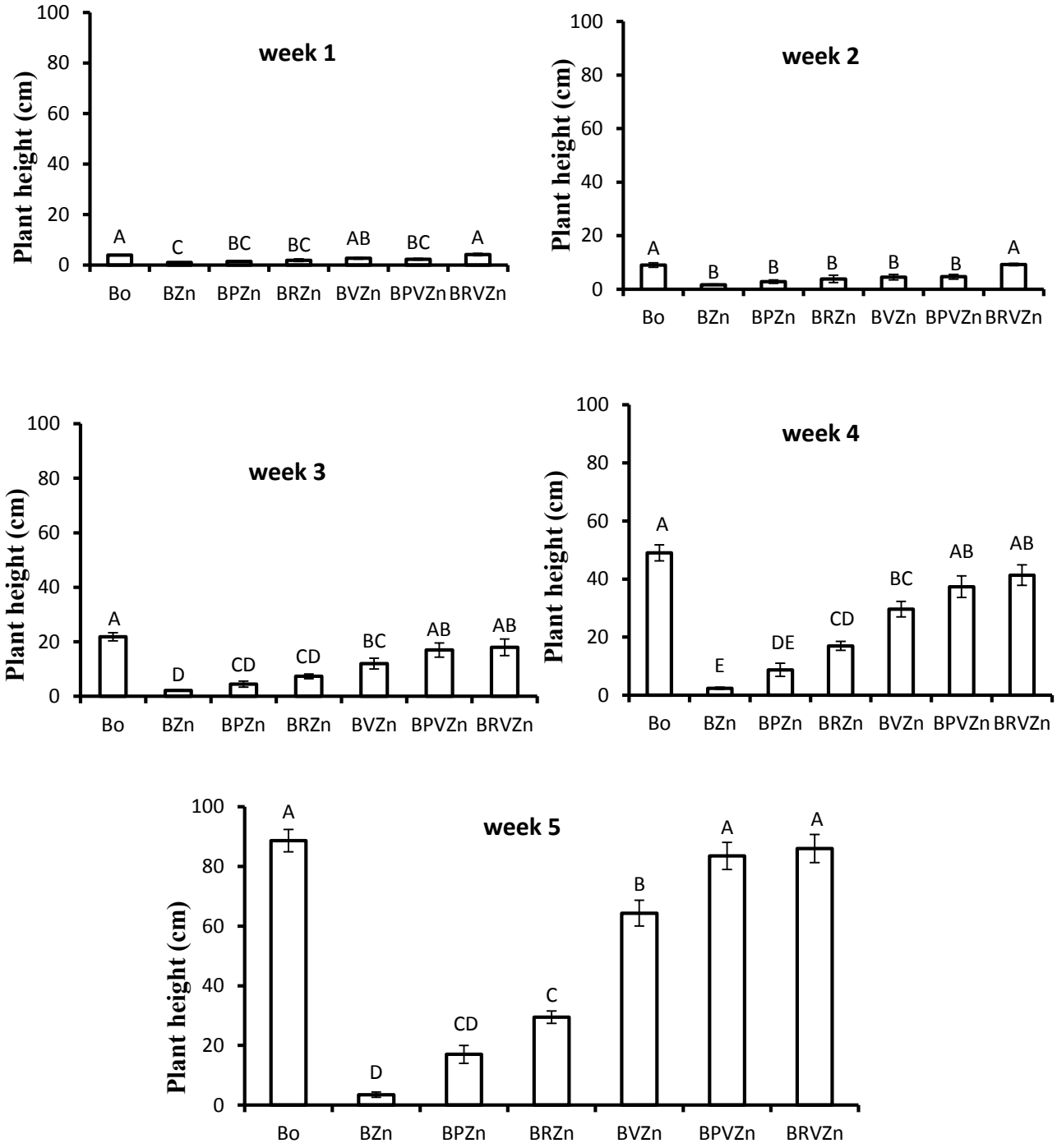


Figure S2. Weekly comparison of *B. juncea* plant height in uncontaminated soil (Bo) with height under Zn contamination in sole planted (BZn), bacteria inoculated (BPZn & BRZn), mixed planted (BVZn), inoculated & mixed planted (BPVZn & BRVZn) *B. juncea*. Bars are mean plant heights from the 3 experimental pots and error bars show standard errors. Means were subjected to 1-way ANOVA followed by Tukey's HSD multiple comparison. Different symbols indicate significant ($p<0.05$) differences in weekly plant height between treatments (n=3).

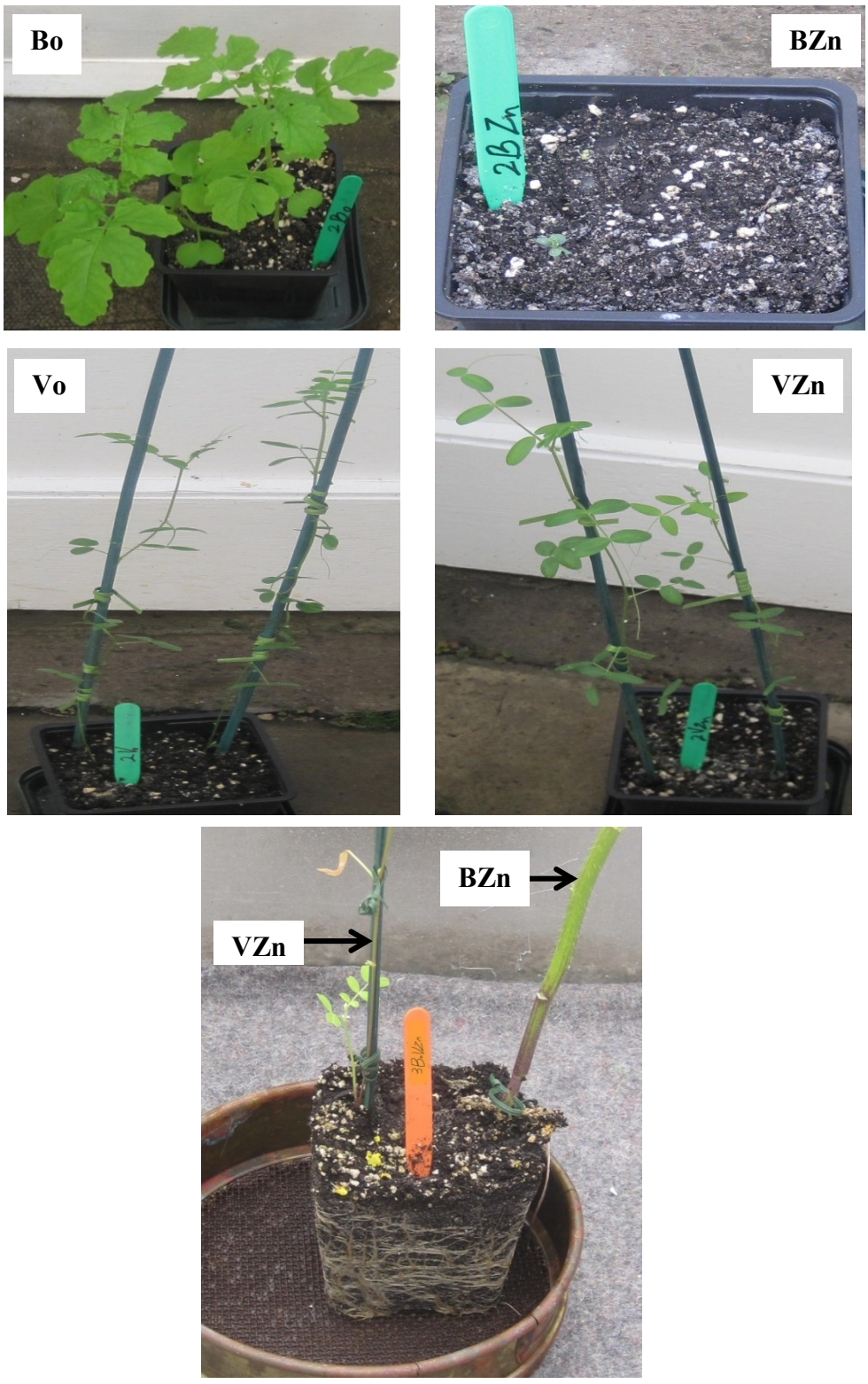


Figure S3. *Brassica juncea* and *Vicia sativa* in uncontaminated soil (Bo and Vo) and in Zn contaminated soil (BZn and VZn) at 20 days after planting, and the mixed planting system under Zn contamination at 35 days after seeds planting

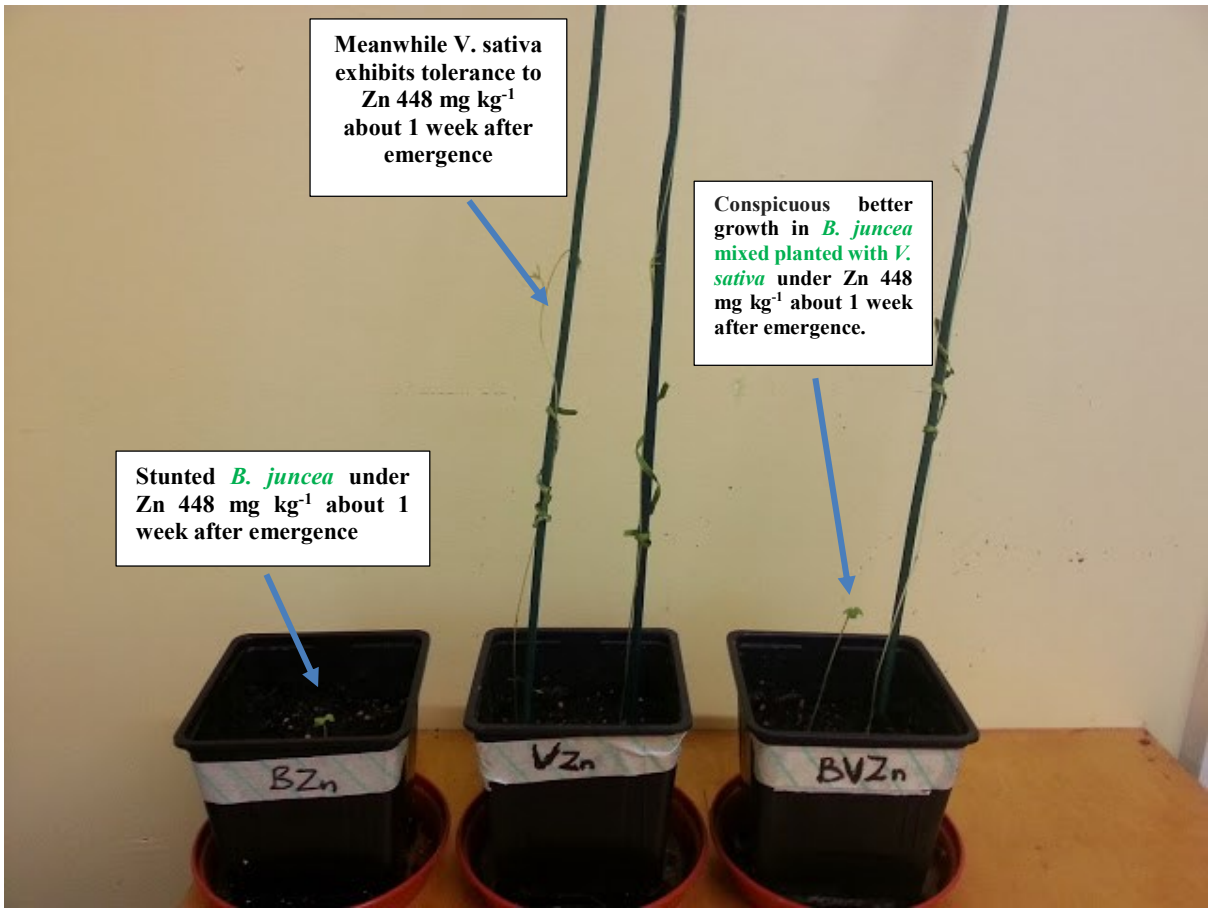


Figure S4. Post experimental analysis of the tolerance and *B. juncea* growth promotion ability of *V. sativa* under Zn 448 mg kg^{-1}. Please not that experiment was conducted in the laboratory with fluorescent light as the source of energy for photosynthesis.