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SPECIAL FEATURE

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Interactions of the manganese hyperaccumulator *Phytolacca americana* L. with soil pH and phosphate

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Abstract Hyperaccumulators are plants that store exceptionally high concentrations of heavy metals or metalloids in their leaves. *Phytolacca americana* is one of the few species known to hyperaccumulate manganese (Mn); however, it is a common weedy species and has no specific association with high-Mn soils. Neither the mechanism by which *P. americana* hyperaccumulates Mn nor the ecological significance of this trait are well understood. It has recently been suggested that *P. americana* secretes acids into the rhizosphere as a means of acquiring phosphate, which might coincidentally increase Mn uptake. To determine whether *P. americana* acidifies the surrounding soil, plants were grown in rhizoboxes providing access to living roots. A thin layer of agar containing bromocresol green pH indicator dye was placed on the roots to observe color changes indicating acidification. Comparative studies showed that *P. americana* acidifies the rhizosphere significantly more than the non-accumulating plant *Acalypha rhomboidea*. A second experiment studied whether adjustment of soil pH and phosphate affect foliar Mn concentrations of *P. americana*. Concentrations of Mn in leaves were highest when plants were grown in acidified soils but were significantly lower in soils that were alkaline and/or enriched with phosphate. These results suggest that Mn hyperaccumulation may be a side effect of rhizosphere acidification as a phosphorus-acquisition mechanism, rather than an adaptation in its own right. The findings provide fundamental information about hyperaccumulator physiology and evolution, and may be relevant to attempts to utilize *P. americana* for phytoremediation.

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Introduction

The term hyperaccumulation refers to uptake and storage of heavy metals or metalloids in plant leaves at exceptionally high concentrations, without detrimental effects on the plant. The defining threshold of metal concentration for a hyperaccumulator varies depending upon the metal in question, but conceptual guidelines require that the metal concentration is 100–1000 times higher than that of average plants growing on normal (non-metalliferous) soil, and at least ten times higher than the typical range of plants on metalliferous soils (van der Ent et al. 2013). Based on these guidelines, minimum criteria for various metals/metalloids (measured in micrograms of metal per gram of dry leaf mass) are 100 μg g^{-1} for Cd, Se, and Tl; 300 μg g^{-1} for Co, Cr, and Cu; 1000 μg g^{-1} for As, Ni, and Pb; 3000 μg g^{-1} for Zn; and 10,000 μg g^{-1} for Mn (van der Ent et al. 2013).

Of approximately 300,000 known vascular plant species, only about 700 have been documented as hyperaccumulators (Reeves et al. 2017), the majority of which hyperaccumulate nickel. Most known hyperaccumulators are specifically endemic to soils that are enriched in the metal they accumulate; however, about 10–15% are facultative hyperaccumulators, which occur on both metalliferous and non-metalliferous substrates (Pollard et al. 2002). Studies of facultative hyperaccumulators have indicated that physiological ability to hyperaccumulate usually occurs throughout the species, even in populations growing on soil with metal concentrations too low to allow plants to reach the hyperaccumulation threshold concentration (Pollard et al. 2014).

Hyperaccumulation of manganese (Mn) occurs in only 10–20 species, making it an especially rare phenomenon (Pollard et al. 2009; Fernando et al. 2013; van

der Ent et al. 2013). While Mn is an essential plant nutrient, most plants experience manganese toxicity at tissue concentrations of 200–3500 μg Mn g^{-1} dry leaf mass (Krämer 2010). For a plant to be considered a manganese hyperaccumulator, it must have a foliar manganese concentration, on a dry-weight basis, of at least 1% or 10,000 μg g^{-1} (Baker and Brooks 1989). *Phytolacca americana* L. (pokeweed) has been documented as a facultative hyperaccumulator of Mn. It is a broadleaf ruderal species indigenous to the southeastern US, where it occurs abundantly on disturbed locations and roadsides, and is invasive in many other regions of the world (Weber 2017). It is known to hyperaccumulate in natural populations at two locations: the Xiangtan Mn mine in Hunan Province, China (Xue et al. 2010) and the Roberts Mn mine in Cherokee County, South Carolina, USA (Pollard 2016). On non-metalliferous soils it does not reach the threshold to be recognized as a hyperaccumulator, but does have anomalously high Mn concentrations compared to other plants in the community (Pollard et al. 2009).

The importance of rhizosphere processes in hyperaccumulation is not well understood, and has been studied in only a few hyperaccumulator species (Alford et al. 2010). In a review of Mn accumulation in plants, Lambers et al. (2015) proposed that many species take up Mn as a side-effect of phosphorus-acquisition mechanisms in their roots. Specifically, they hypothesized that *P. americana* releases protons into the rhizosphere in order to increase the solubility of phosphate, resulting in coincidental mobilization of Mn. This suggestion was based on circumstantial evidence such as a high internal concentration of oxalate anions in *P. americana* roots (Dou et al. 2009), but has not to our knowledge been tested experimentally. The only published study of Mn in the rhizosphere of *P. americana* emphasizes adsorption and binding onto root cell walls as a means of Mn immobilization and tolerance (Xu et al. 2015) but provides no information on mechanisms of uptake and transport leading to Mn hyperaccumulation in leaves.

To investigate the Mn accumulation mechanism proposed by Lambers et al. (2015), we conducted experiments designed to test three specific hypotheses. First, if proton secretion enhances Mn uptake, there should be measurable pH reduction in the rhizosphere of *P. americana*, and it should be stronger than in other species that grow in the same community but accumulate less Mn. Second, if low pH increases Mn uptake, then application of exogenous acids to the rhizosphere should result in higher foliar Mn concentrations. And third, if Mn uptake is an indirect result of an inducible phosphorus uptake mechanism, then addition of phosphate to the rhizosphere could decrease proton secretion and consequently suppress foliar Mn concentrations. The first hypothesis was addressed using a rhizobox technique and pH-sensitive dyes. The second and third hypotheses were explored using factorial addition of acids and soluble phosphorus to plants growing in soil.

Materials and methods

Field sampling

Plant samples were collected from two sites. The Roberts Mine in Cherokee County, South Carolina, USA (35.118732°N, 81.446487°W) is a small, shallow, surface mine from which manganiferous schist is extracted for use as an additive in brick-making. Previous research has indicated that soils surrounding the mine have moderately high Mn concentrations (approximately 7500 μg g^{-1}) and that leaves of *P. americana* plants possess high Mn concentrations, in some cases exceeding the 10,000 μg g^{-1} criterion for hyperaccumulation (Pollard 2016). The second site was a disturbed area with weedy vegetation on the campus of Furman University in Greenville, South Carolina, USA (34.9218°N, 82.4362°W). This area was described in Pollard et al. (2009) and has clay loam soil with Mn concentrations in the typical range for uncontaminated soil.

For rhizobox studies, live seedlings of *P. americana* were collected from both sites. In addition, seedlings of *Acalypha rhomboidea* Raf. (Euphorbiaceae) were collected from the Furman University site. This species was chosen for comparison because it grows in the same community but has significantly lower foliar Mn concentrations than *P. americana* (~ 250 vs. ~ 2000 μg g^{-1}, according to Pollard et al. 2009). Seedlings at the 3–5 leaf stage were collected by loosening the soil with a trowel and gently freeing the roots. Seedlings were placed in water for transport to the laboratory, and maintained in hydroponic cultivation until needed for experiments. The hydroponic medium was a modification of 10% Hoagland solution (Hoagland and Arnon 1950), containing 0.5 mM KNO_3, 0.1 mM KH_2PO_4, 0.4 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 20 μM Fe-EDDHA, 10 μM H_3BO_3, 2.0 μM $MnSO_4$, 0.2 μM$CuSO_4$, 0.2 μM $ZnSO_4$, and 0.1 μM $NaMoO_4$. Seedling roots were inserted through holes in a polystyrene foam sheet lined with polyethylene sheeting on its surface so that their roots were submerged in solution while their shoots rested above the foam floating at the solution's surface. Hydroponic containers were placed in an environmental chamber (Model EF7, Conviron, Winnipeg, Canada) set for a 16-h day at 30 °C and an 8-h night at 22 °C, and continuously aerated.

In addition to seedling collections, ripe fruits were collected from ten plants around the periphery of the Roberts Mine. Seeds were removed from fruits, rinsed with water to remove fruit pulp, air-dried, soaked in concentrated (18 M) sulfuric acid for 15 min, and again rinsed thoroughly with water. Acid treatment has been shown to enhance seed germination (Edwards et al. 1988). These seeds were used in studies of Mn uptake on amended soils. Soil samples from the upper 15 cm of the soil profile were also collected at both field sites for laboratory analysis of pH and Mn concentration.

Effects of *Phytolacca americana* on rhizosphere pH

Rhizoboxes are growth chambers for roots with transparent windows that allow non-destructive measurement and observation of root growth (Neuman et al. 2009). Guidelines from Marschner and Römheld (1983) as well as Whiting et al. (2000) were used in the design of an inexpensive rhizobox system for examining effects of *P. americana* on the pH of its rhizosphere. Rhizoboxes were made using polystyrene circular petri dishes 13 cm in diameter and 1.5 cm deep. A notch was made in the lip of each half of the dish to create a hole for the plant stem when the dish was closed. After seedlings had established in the hydroponic system and were showing new root growth, they were moved into individual rhizoboxes, with their shoot system protruding through the hole. Seedling roots were embedded in fine sand with no additional nutrients. Dishes were sealed around their edges with paraffin film and covered with aluminum foil to exclude light. The rhizoboxes were laid at 45° angles in the environmental chamber with the lids facing downward so that roots would grow across the lid surface. The environmental chamber was kept at the conditions described above for 10 days.

Rhizosphere pH was assessed using bromocresol green indicator dye in agar (Marschner and Römheld 1983; Haussling et al. 1985). A boiled 1% agar solution was combined with 0.012% bromocresol green dye, poured into empty 15-cm glass petri dishes to a depth of 3 mm and allowed to cool. Once a rhizobox was opened to reveal the roots, the surface was misted heavily with deionized water to ensure the agar would adhere to it. A disk of cooled agar was carefully lifted out of the casting dish, laid across the root surface, and left for 24 h to change color.

To determine the corresponding pH of the indicator dye, the same sand was used to fill the cells in a 12-well culture dish. Three millilitre of McIlvaine's citrate–phosphate buffer, ranging from pH 2.0 to 6.0 in increments of 0.5, were dispensed into nine of the wells. Bromocresol-green-dye-infused agar was placed across the surface of the wells after being cut to shape. This provided a color-coded key to determine approximate pH levels of the sand in the rhizosphere around plant roots (Fig. 1).

After 24 h, photographs of each rhizobox and the key were taken under fluorescent lighting with a digital camera (Rebel XSi DSLR, Canon, New York, NY, USA) attached to a photo stand. Identical settings and lighting were employed for each image.

Effects of soil phosphorus and pH on foliar manganese concentration

To determine the effects of soil pH and phosphorus on Mn uptake, plants were grown in a factorial design of pH and phosphate treatments. The soil was a 1:1 mixture of mine tailings from the Roberts Mn Mine and general-purpose potting soil (Jolly Gardener Pro-Line 44N Growing Mix, Oldcastle Lawn and Garden, Poland Spring, ME). The mixture was manually homogenized.

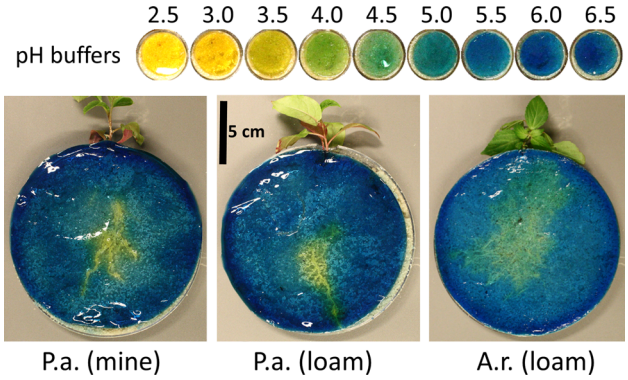

Fig. 1 Representative rhizoboxes showing rhizosphere color changes in 3 mm-thick agar slabs containing bromocresol green pH dye, after 24 h on substrate. *P.a. (mine)*, *Phytolacca americana* collected from a manganese mine; *P.a. (loam)*, *P. americana* from uncontaminated clay loam soil; *A.r. (loam)*, *Acalypha rhomboidea* from the same clay loam soil. These are merely examples; the full experiment included 27 rhizoboxes. The pH buffers are dishes of sand moistened with citrate–phosphate buffer adjusted to the specified pH values, to act as a visual key

There were three pH treatments—acidic, water, and basic—created by adding 1 M HCl solution, deionized water, or 1 M NaOH solution, respectively, at 2% of soil volume. Soil for each pH treatment was divided into two portions, one of which received additional phosphate (+P) and the other of which did not (−P). For the +P treatment, 1 g of sodium phosphate ($NaH_2PO_4 \cdot H_2O$) per kg of soil was dissolved in water and homogenized into the soil, a "luxurious" rate of application based on standard recommendations (Lindsay 1979; Hendry and Grime 1993; Kuo 1996). Aliquots of each soil were collected for pH and Mn analyses. The soils were lightly packed into divided flats (each pot compartment approximately $4 \times 4 \times 7.5$ cm), which were placed in trays for water retention. Each tray of 72 pots contained a single treatment combination, to avoid contamination.

Preparation of *P. americana* seeds from the Roberts Mine has been described above. Three to four seeds were added to each pot and lightly covered with a peat-based seed starting mix. Throughout the experiment, pots were watered as needed by addition of deionized water to the base of the tray. Until seeds germinated, each tray was covered with a clear, plastic lid to maintain high humidity. Trays were kept in an environmental chamber (Model E15, Conviron, Winnipeg, Canada) set for a diurnal cycle of 8 h night (20 °C, no light), 3 h morning (23 °C, 50% light), 10 h day (26 °C, full light), 3 h evening (23 °C, 50% light).

After 3 months of growth, all surviving plants were numbered, and eight plants were selected randomly (using a random-number table) from each treatment except the acid/−P treatment in which only six plants grew. The two largest leaves were taken from each plant.

Leaves were washed in a solution containing 1% sodium dodecyl sulfate (SDS) as a detergent and 5 mM ethylenediaminetetraacetic acid (EDTA) as a chelating agent in deionized water, and then rinsed with deionized water, in order to remove surface metal contamination (Faucon et al. 2007). Samples were then dried at 66 °C for at least 24 h before metal analysis as described below.

Chemical analyses

Approximately 0.01 g of each leaf sample was weighed on an analytical balance, placed in a borosilicate glass test tube, incinerated in a muffle furnace (Heatech, LabLine Inc., Melrose Park, IL, USA) at 500 °C for 12 h, dissolved in 1 mL of 12 M HCl (TraceMetal Grade, Fisher Chemical Co., Pittsburg, PA, USA), diluted with 4 mL H_2O, and swirled with a vortex mixer. Solutions were then analyzed for Mn using flame atomic absorption spectrophotometry (Model 551, Instrumentation Laboratory Inc., Wilmington, MA, USA).

Both field-collected soils and laboratory-manipulated soils were tested for pH and Mn concentration. Measurements of pH were made using a digital pH meter on a 1:1 slurry of freshly collected soil and deionized water. To determine Mn concentration, soil samples (approx. 0.5 g) were dried for 24 h at 66 °C, weighed on an analytical balance, digested with 16 M HNO_3 in a block heater set at 110 °C for 3 h, cooled, and re-digested with 12 M HCl in the block heater at 80 °C for 1.5 h. Digests were then filtered, made up to 25 mL with deionized water, and analyzed using the same atomic absorption spectrophotometry procedures used for leaves.

Data analysis

Rhizobox photographs were analyzed using ImageJ software (version 1.51p "Fiji"; National Institutes of Health, USA). Under acidic conditions, the agar changed color from blue to yellow, as seen in the buffer standards (Fig. 1). The pixels in an 8-bit RGB image are defined by a combination of red, blue, and green intensity values, each of which may range from 0 to 255. In this color-space, yellow is formed by a combination of red and green primary colors, with relative absence of blue. Using the "color threshold" function of ImageJ, we determined empirically that the threshold setting which best differentiated between the most acidic buffer standards (pH 2.5–3.0) and those with higher pH was a combination of red intensity 130–250, green intensity 130–250, and blue intensity 0–130. Images of agar on rhizoboxes were thresholded with these settings, and then two measurements were made on the portion of the image that met threshold criteria. The area of the thresholded region (in pixels) was measured as a percentage of total rhizobox area, to determine the extent of the acidified region. Also, the average intensity of the red signal within the thresholded area was measured (using the ImageJ "color histogram" function) to indicate strength of acidification.

Data from rhizobox image analyses were subjected to one-way analysis of variance (ANOVA) to compare rhizosphere acidification between three groups: *P. americana* from the Roberts Mine, *P. americana* from Furman University, and *A. rhomboidea* from Furman University. Two-way ANOVA was performed on foliar Mn data to determine if there were significant differences in mean Mn concentrations of *P. americana* among factorial combinations of pH treatments and phosphate treatments. In all statistical procedures, data were log-transformed to correct for inequality of treatment variances as indicated by a Brown–Forsythe test. Statistical analyses were conducted using R (version 3.2.2: The R Foundation for Statistical Computing, Vienna, Austria).

Results

Analysis of field-collected soils

The clay loam soil on the campus of Furman University where both *P. americana* and *A. rhomboidea* seedlings were collected had a mean pH of 4.80 ± 0.22 (SE) and a mean Mn concentration of 130.8 μg g^{-1} ± 13.8. Mine spoil at the Roberts Manganese Mine had mean pH of 5.37 ± 0.03 and a mean Mn concentration of 9204.0 μg g^{-1} ± 1798.8, with the high standard error reflecting heterogeneity across the mine. However, the sparsely vegetated area where *P. americana* seedlings were growing was near the low end of this range, with a mean Mn concentration of 2116.1 μg g^{-1} ± 259.1 (SE based on four replicate subsamples).

Effects of *Phytolacca americana* on rhizosphere pH

In rhizobox experiments, both *P. americana* and *A. rhomboidea* acidified the rhizosphere as indicated by color change of the bromocresol green pH dye from blue to green or yellow in the root zone (Fig. 1). The rhizosphere of *P. americana*, however, often appeared as an intense yellow in contrast to that of *A. rhomboidea*, which in most cases only changed to a light green. There was considerable variation from one rhizobox to another within a given species, both in intensity of color change and in the area that it covered.

Results of digital image analysis are shown in Fig. 2. The mean area of color that met threshold color criteria was largest in *P. americana* from the Roberts Mine, about eight times larger than in either *P. americana* or *A. rhomboidea* from clay loam soil on the Furman University campus. Although the difference in means appears large, there was also a very large standard error for the mine plants, and analysis of variance on log-transformed data suggested that differences among the

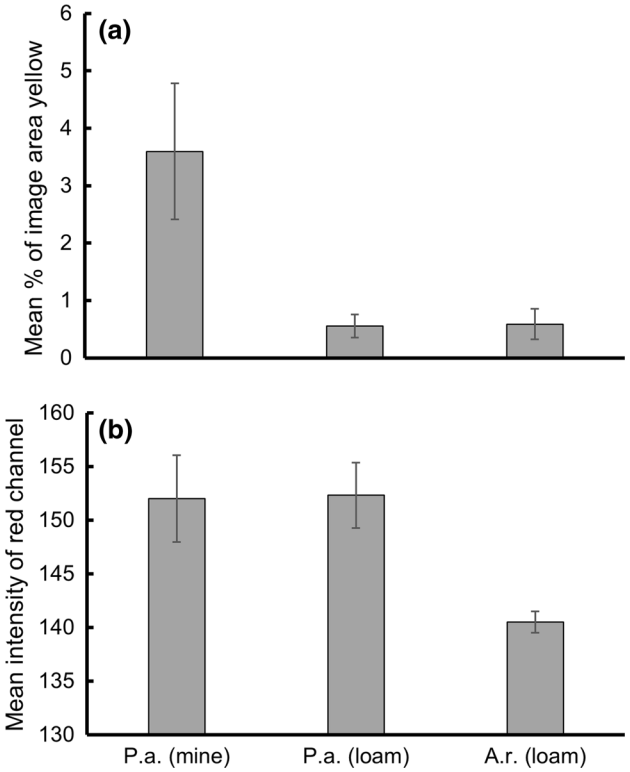

Fig. 2 Quantitative estimates of color change in bromocresol green pH indicator dye applied to rhizoboxes. Digital photographs were analyzed with ImageJ software, and yellow areas corresponding to regions of low pH were selected. **a** Mean percentage of rhizobox area in the yellow region. **b** Mean intensity of the red channel (8-bit RGB image, with values ranging from 0 to 255) within the selected region, indicating intensity of color change. *P.a. (mine)*, *Phytolacca americana* collected from a manganese mine ($N = 5$); *P.a. (loam)*, *P. americana* from uncontaminated clay loam soil ($N = 10$); *A.r. (loam)*, *Acalypha rhomboidea* from the same clay loam soil ($N = 12$). Vertical lines represent standard error of means

three groups were not quite statistically significant ($F_{2,24} = 3.35$, $P = 0.052$). The average intensity of color change from the initial blue agar in the rhizosphere did show a highly significant difference ($F_{2,24} = 8.96$, $P = 0.0012$). Multiple comparisons using the Tukey HSD test indicated that *A. rhomboidea* had a significantly less intense color change than *P. americana*, but that there were no significant differences between the two populations of *P. americana*.

Effects of soil phosphorus and pH on foliar manganese concentration

The homogenized mixture of mine tailings and potting soil in which plants were grown had a Mn concentration of 7400 μg g^{-1} and a pH of 5.61. Addition of HCl lowered soil pH to 4.34, whereas addition of NaOH raised it to 7.62.

Mean foliar Mn concentrations were highest in plants grown on acidified soil and declined as soil pH increased, and were consistently lower in plants grown on soils with added phosphate (Fig. 3). Two-way ANOVA revealed that pH effects were highly significant ($F_{2,40} = 35.10$, $P < 0.0001$), as were the effects of phosphate treatments ($F_{1,40} = 67.03$, $P < 0.0001$). There were not, however, any statistically significant interaction effects between pH and phosphate ($F_{2,40} = 2.01$, $P = 0.148$).

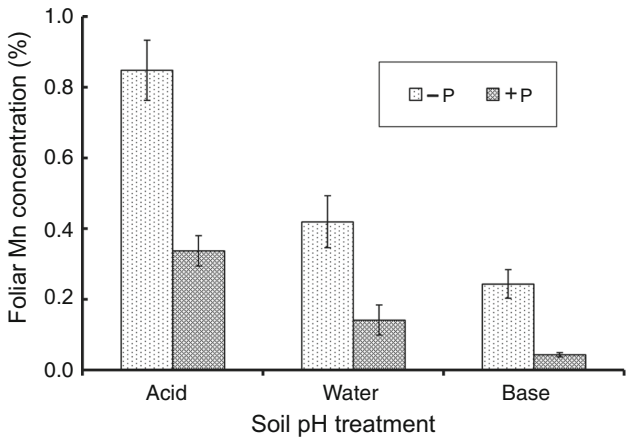

Fig. 3 Mean foliar manganese concentration (%) of *Phytolacca americana* grown in high manganese soil, subjected to three soil pH amendments (acid = 1 M HCl; water = deionized water; base = 1 M NaOH) with and without addition of sodium phosphate (1 g per kg). Data are from 46 plants and 92 leaves. Vertical lines represent standard error of means

Discussion

These results strongly support the hypothesis that Mn hyperaccumulation in *P. americana* results, at least in part, from secretion of acidic root exudates (Lambers et al. 2015). A previous study has shown that *P. americana* leaves have much higher Mn concentrations than those of *A. rhomboidea*, even when both species are growing in the same community on non-metalliferous soils (Pollard et al. 2009). The significant differences in intensity of acidification detected in our rhizobox experiments, and the enhanced Mn uptake we observed in acidified soils, indicate that rhizosphere acidification could be a mechanism underlying this pattern.

In facultative hyperaccumulators, physiological traits responsible for metal hyperaccumulation may be present throughout the species, or only in populations growing on metalliferous soils (Pollard et al. 2014). We did not detect differences in intensity of rhizosphere acidification between mine and non-mine populations of *P. americana*, indicating that ability to hyperaccumulate is a species-wide property, in agreement with Xue et al. (2005). There was a large difference between populations in the size of the acidified region in our rhizoboxes; however, this difference was marginally insignificant in a statistical sense. We regard intensity of acidification as a more reliable measure, because differences in the amount

of root growth in an environment like a rhizobox could be artifacts of plant age, vigor, or transplantation success, unrelated to issues of metal uptake. Nonetheless, the difference may be worthy of further investigation with more quantitatively accurate tools such as pH microelectrodes.

Several hypotheses have been proposed to explain the selective advantages that might lead to the evolution of hyperaccumulation, including the possibility that hyperaccumulation may contribute to metal tolerance, drought tolerance, competitive ability, or defense against herbivores (Boyd and Martens 1992; Boyd 2004). However, these adaptive explanations apply to species that have evolved primarily or exclusively on metalliferous soil. It is difficult to see how they could drive evolution of species-wide hyperaccumulator physiology in facultative hyperaccumulators, especially those in which the majority of populations occur on non-metalliferous soils. Pollard et al. (2014) suggested that facultative hyperaccumulation might evolve because (a) ability to hyperaccumulate was phylogenetically conserved in cases where facultative hyperaccumulators descended from obligate hyperaccumulators, or (b) because of some incremental advantage of high (but sub-hyperaccumulation) metal concentrations on normal soil, or (c) as a side effect of some other physiological process such as nutrient acquisition. The last of these possibilities was termed "inadvertent uptake" by Boyd and Martens (1992).

Lambers et al. (2015) specifically hypothesized that *P. americana* acidifies its rhizosphere by direct secretion of protons, because it belongs to the non-cluster-rooting and non-mycorrhizal family Phytolaccacceae (Gerdemann 1968; Janos 1980), which suggests that these plant roots must have a method to acquire phosphate other than the carboxylate-releasing strategy of cluster roots and the symbiotic fungal relationship of mycorrhizal plants. It is well documented that some plants use acid secretion as a means of acquiring nutrients, especially phosphorus (Lopez-Bucio et al. 2000). As a consequence of accumulating phosphate, in the case of *P. americana*, plants might also accumulate Mn in extremely high concentrations (Lambers et al. 2015), an example of the inadvertent uptake models of Boyd and Martens (1992) and Pollard et al. (2014). *P. americana* plants could potentially withstand a high internal concentration of Mn^{2+} because they possess high concentrations of oxalate which could bind the metal ions (Dou et al. 2009). Lambers et al. (2015) also predicted that *P. americana* secretes protons inducibly, in response to low phosphate. This would explain why foliar Mn concentrations were highest in soil without added phosphate and why the roots secreted acids in the low-phosphate sand of the rhizoboxes. Modifying the rhizobox protocol to include nutrient additions could be an avenue to investigate inducibility.

There are alternative explanations for suppression of Mn uptake by phosphate addition. One possibility is that Mn^{2+} and PO_4^{3-} might form an insoluble precipitate (e.g. $MnHPO_4$), decreasing availability of Mn to plants. Hydroponic experiments have indicated that precipitation is not an important aspect of P-induced Mn deficiency in barley (Pedas et al. 2011), but the applicability of those findings to the high-Mn soils employed in our experiments is unclear. Another possibility is that P directly interferes with Mn uptake by alterations in membrane transporter activity (Pedas et al. 2011). Again, this has not been investigated directly in *P. americana*, but might be a productive subject for future research. It should also be noted that pH is only one factor affecting Mn bioavailability and others, especially redox potential, may also be important (Fernando and Lynch 2015).

The inadvertent uptake hypothesis (Boyd and Martens 1992) postulates that there is no direct adaptive advantage of hyperaccumulation; however, it is fundamentally problematic to prove a negative hypothesis. Pollard et al. (2014) proposed two other explanations for the evolution of facultative hyperaccumulation. The phylogenetic conservation hypothesis argues that ability to take up high metal concentrations might be inherited from an ancestral obligate hyperaccumulator. That seems unlikely in this case, because there are no other known hyperaccumulators closely related to *P. americana* (earlier reports of Mn hyperaccumulation in *P. acinosa* were probably misidentifications of *P. americana*—Xue et al. 2010). The Phytolaccaceae is a small family in the order Caryophyllales, which contains relatively few hyperaccumulators, according to the Global Hyperaccumulator Database (http://www.hyperaccumulators.org—see Reeves et al. 2017). However, there are undoubtedly many hyperaccumulators not yet discovered. It is also possible that the concentrations of Mn found in *P. americana* on normal soil, which are high but fall short of hyperaccumulation (Pollard et al. 2009), might have some direct advantage. Other studies have demonstrated that metals at concentrations below hyperaccumulation may provide defense against herbivores (Coleman et al. 2005). A similar role has not been investigated for Mn.

Although the concept of inadvertent uptake as an underlying explanation for hyperaccumulation was proposed over 25 years ago, this is one of the first attempts to analyze it experimentally. The ability of *P. americana* to accumulate metals, along with its fast growth and high biomass, have stimulated significant interest in utilizing it for phytoremediation of Mn and Cd (Liu et al. 2015). The findings reported here provide new insight into the mechanisms of Mn hyperaccumulation in *P. americana*, as well as its ecological and evolutionary context.

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