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Increased Lead Accumulation in a Single Gene Mutant of Pea (*Pisum sativum* L.)

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Lead (Pb) is ranked number two on the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) priority list of hazardous substances, and is considered a major hazardous chemical found on 47% of the United States Environmental Protection Agency (USEPA) national priorities list sites (Hettiarachchi and Pierzynski, 2004). Lead poses a significant risk to humans, especially children. In the US alone it has been estimated that Pb poisoning affects more than 800,000 children between the ages of one and five (Pirkle et al., 1998). Soil is a main pathway of human Pb exposure (Madhavan et al., 1989; Mielke and Reagan, 1998). Pb is also one of the more persistent metals, and is estimated to have a soil retention time of 150–5,000 years (Shaw, 1990). Engineering-based technologies such as leaching with acids, excavation, and electro-physical separation for abating Pb in soils are not only costly but also harmful to the soil's physical and chemical properties. Over the last 20 years there has been increasing interest in plant-based bioremediation or phytoremediation. Phytoremediation encompasses several strategies including phytostabilization and phytoextraction (Cunningham et al., 1995; Salt et al., 1998, Huang and Chen, 2003). Phytostabilization is the use of plants and soil amendments to reduce the intrinsic hazard of Pb-contaminated soil by reducing Pb bioavailability in the soil. Phytoextraction is the use of plants to remove Pb from contaminated soils (Huang et al., 1997a, 1997b; Lasat, 2002). Plant roots absorb Pb from soil and transport it to the shoots. Through the continued cultivation of selected plant species on Pb-contaminated soil and the harvest of shoots, the soil could be decontaminated. Since plant cultivation and harvest are less expensive than the engineering-based practices, phytoextraction represents an attractive alternative for the cleanup of Pb-contaminated soils.

The success of Pb phytoextraction depends on two key factors: Pb bioavailability in soils, and the plant's ability to absorb Pb from soils to roots and translocate it from roots to shoots. A plant capable of accumulating Pb at a concentration of 1,000 mg kg^{-1} or higher in shoots is considered to be a Pb hyperaccumulator (Baker and Brooks, 1989; Brooks, 1998; Lasat, 2002). Approximately 400 plant species were reported to hyperaccumulate metals (Baker and Brooks, 1989; Brooks, 1998), however, there is no known Pb hyperaccumulator (Lasat, 2002). A possible reason for the lack of naturally occurring Pb hyperaccumulators could be that Pb occurs largely in insoluble precipitates such as phosphates, carbonates, and hydroxyl-oxides, which are unavailable to plant roots (Hettiarachchi and Pierzynski, 2004). Thus, plants may not have developed effective mechanisms for Pb absorption.

Using *Arabidopsis* thaliana as a model system, Chen et al. (1997) screened ethyl methanesulfonate (EMS) mutated populations for Pb hyperaccumulators. Results showed that selected mutants not only accumulated Pb but also other metals such as Ca, Fe, Mg, Mn, and Zn. It was postulated that a mutant, after losing its selectivity for a particular metal, might accumulate other metals (Chen et al., 1997). In a study of Fe accumulation in the 'bronze' mutant, E107 (*brz brz*), of pea (*Pisum sativum* L.), Welch and LaRue (1990) found that extremely high concentrations of Fe as well as high levels of Mn, Cu, Ca, and Mg were accumulated in shoots. The increased Fe accumulation

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resulted in its precipitation in the form of electron-dense particles in basal leaves characterized by brown necrotic spots, thus the name bronze mutant. E107 was a single gene mutant isolated from EMS-treated seeds of the cultivar Sparkle. It has a defect in the regulation of Fe absorption, which enhances proton and Fe(III) reductant extrusion due to the constitutively high Fe(III) reductase activity in the roots (Welch and LaRue, 1990; Grusak et al., 1990). Whether the bronze mutant also accumulates Pb in shoots has not been studied.

The objective of this study was to culture the pea E107 mutant and its wild type Sparkle in both hydroponic and Pb-contaminated soil systems to determine if the defect in Fe(III) reductase could also result in Pb accumulation in shoots.

Materials and Methods

Seeds of Sparkle and E107 were soaked in water for five hours and sown in a commercial potting mix (Metro mix 360, Scotts Sierra Horticultural Products Co., Marysville, OH) for germination. Seedlings were watered with a nutrient solution described below for two weeks. Uniform seedlings were selected, placed singly in polyethylene cups (5.0 cm diameter), and covered with black polyethylene beads. Four seedling cups were placed in holes bored through the polyethylene lid of an 8-liter bucket containing an aerated nutrient solution. The composition of the nutrient solution was, in mg L^{-1}, K, 78 (KNO_3, KH_2PO_4, and KCl), Ca, 20 [$Ca(NO_3)_2$], Mg, 4.9 ($MgSO_4$), NH_4, 1.8 (NH_4NO_3), NO_3, 192 [$Ca(NO_3)_2$, NH_4NO_3, and KNO_3], P, 0.3 (KH_2PO_4), Cl, 1.8 (KCl), B, 0.1 (H_3BO_4), Mn, 0.1 ($MnSO_4$),), Fe, 1.1 (Fe-HEDTA); and in µg L^{-1}, Zn, 33 ($ZnSO_4$), Cu, 13 ($CuSO_4$), and Mo, 9.6 (Na_2MoO_4). A continuous flow (80–100 ml h^{-1}) of the solution from reservoirs (25-liter plastic containers) into 8-liter buckets was established using microprocessor-controlled multi-channel cartridge pumps (Cole-Parmer Instrument Co., Vernon Hills, IL). Each bucket had an inlet and an outlet for a continuous nutrient solution flow, thus maintaining an 8-liter volume of solution for the duration of the experiment. The initial pH was maintained between 4.5 and 5.0, and concentrations of nutrient elements were monitored weekly by randomly sampling the effluent. After seedlings were grown in the nutrient solution for two weeks, Pb, as Pb $(NO_4)_2$, was added into the reservoirs resulting in a Pb concentration of 4.1 mg L^{-1}. There were five replicate buckets for each cultivar. Plants were harvested after 14 days of the Pb exposure. Shoots of the four plants from each bucket were dried and combined, subsamples of ground plant material (400 mg) were digested in a mixture of $HNO_3/HClO_4$, and the digested samples were analyzed for Pb, Al, Ca, Cu, Fe, Mg, Mn, and Zn using inductively coupled argon-plasma emission spectrometry (ICP) (Huang and Cunningham, 1996).

Sparkle and E107 were also tested in a Pb-contaminated soil collected from a contaminated site in New Jersey. Chemical and physical properties of the soil are presented in Table 1. Two uniform seedlings were planted in each 15 cm diameter plastic container holding 600g of the Pb-contaminated soil. 12 replicate pots were prepared for each cultivar. After 4 weeks of growth 12 pots were divided in half; EDTA was applied to the six of the 12 pots at a rate of 1 g EDTA per kg of Pb-contaminated soil. The remaining six pots of each cultivar were used as controls and irrigated with water only. Two weeks later the shoots of the plants were harvested, and the concentrations of the elements indicated above were determined.

Both the hydroponic and soil experiments were conducted in an air-controlled greenhouse with a temperature range of 24 to 28°C. Plants were grown under a maximum photosynthetic photon flux density (PPFD) of 1,800 µmol m^{-2} s^{-1}. The plants in the Pb-contaminated soil were watered with 200 ml per pot of the hydroponic solution void of Pb two to three times weekly. Differences between Sparkle and E107 in metal accumulation were examined using Student's *t*-test (Snedecor and Cochran, 1982).

Results and Discussion

Sparkle and E107 showed comparable growth vigor during the first two weeks in the hydroponic system. Starting from the fourth week small bronze spots appeared on old leaves of E107, indicating Fe accumulation. Analysis of shoot tissue suggested that the concentration of Fe was 9,700 mg kg^{-1} in E107 compared to 141 mg kg^{-1} in Sparkle (Table 2).

The concentration of Pb in shoots of E107 was 175-fold higher than that of the nutrient solution, while Pb concentration in Sparkle shoots was only 27-fold higher than

Table 1 Physical and chemical characteristics of Pb-contaminated soil used in this study

Characteristic	Value[1]	Metal	mg kg^{-1}
Sand (%)	66.5 ± 4.0	Ca	2,154 ± 12
Silt (%)	19.5 ± 0.5	Cu	13.6 ± 1.2
Clay (%)	14.0 ± 0.4	Fe	358 ± 4.5
Soil texture	Sandy loam	Mg	265 ± 4.4
Organic matter content (%)	3.8 ± 0.4	Mn	7.5 ± 1.6
Soil pH (1:1 soil/water ratio)	5.4 ± 0.2	Pb	2,205 ± 20
CEC (mequiv/100g)	37.5 ± 4.0	Zn	80 ± 5.8

[1] Values are mean ± SE (*n* = 3)

Table 2 Concentrations of Ca, Cu, Fe, Mg, Mn, and Zn in shoots of pea mutant E107 and wild type Sparkle grown in solution culture

Plant	Ca	Cu	Fe	Mg	Mn	Zn
	mg kg^{-1}					
E107	22,600	16.6	9,700	16,000	900	82.3
Sparkle	1,500	10.4	141	4,650	88	43.2
t-test [a]	**	*	**	**	**	**

[a] *t*-test: * and ** indicate that means are significantly different at the 5% and 1% levels, respectively. Values are mean for each treatment (n = 5)

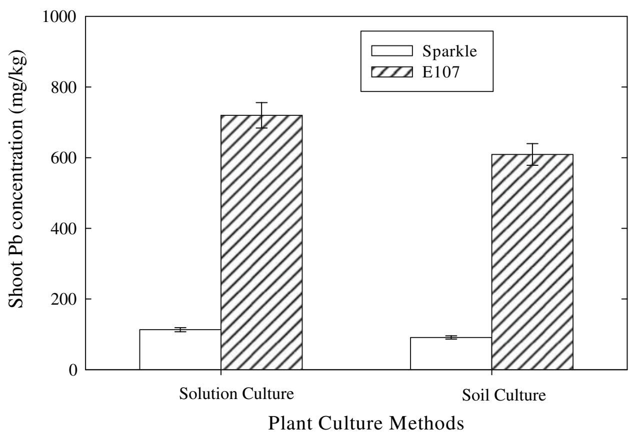

Fig. 1 Lead accumulation in shoots of the pea wild type Sparkle and mutant E107 grown in solution culture with a solution Pb level of 4.1 mg L^{-1} and soil culture with total soil Pb of 2,205 mg kg^{-1} and soil solution Pb of 3.5 mg L^{-1}

in the solution (Fig. 1). Shoot Pb concentrations were more than sixfold higher in E107 than in Sparkle (Fig. 1). The results demonstrated that E107 had greater ability to accumulate Pb in the shoots than the wild type Sparkle.

As far as is known this is the first report showing that a Fe hyperaccumulator also accumulates high concentration of Pb, one of the most stable elements in the soil. Besides the increased accumulation of Pb and Fe in shoots E107 also accumulated significantly higher concentrations of Ca, Cu, Mg, Mn and Zn in shoots than Sparkle (Table 2). The data were in agreement with the results obtained by Welch and LaRue (1990) except for Zn. In their report Zn was not accumulated in hydroponic cultures, but accumulated in soil culture by E107. The hydroponic culture of this study showed E107 accumulated significantly higher concentration of Zn than Sparkle.

Both Sparkle and E107 were then tested in Pb-contaminated soil. The total soil Pb was 2,205 mg kg^{-1}, while soil solution Pb, recovered by centrifugation, was 3.5 mg L^{-1}. Plants grown in the soil showed little morphological difference regardless of cultivar until near the time of harvest when minor bronze spots appeared on the old leaves of E107. Similar to the hydroponic culture, Pb concentration

Table 3 Concentrations of Al, Ca, Cu, Fe, Mg, Mn, and Zn in shoots of pea mutant E107 and wild type Sparkle grown in a Pb-contaminated soil

Plant	Al	Ca	Cu	Fe	Mg	Mn	Zn
	mg kg^{-1}						
E107	950	17,500	25.5	3,980	8,200	1,200	76.3
Sparkle	71	11,300	16.8	453	3,800	49	38.5
t-test [a]	**	**	*	**	**	**	**

[a] *t*-test: * and ** indicate that means are significantly different at the 5% and 1% levels, respectively. Values are mean of the treatment (n = 6)

in shoots of E107 was 174-fold higher than that in the soil solution, while the Pb level in shoots of Sparkle was 26-fold higher than the Pb level in the soil solution (Fig. 1). Shoot Pb concentration in E107 was more than six times higher than that of Sparkle. These results demonstrate that E107 is capable of accumulating much higher levels of Pb in shoots than its wild type Sparkle in soil culture as well. Analysis of other metals in shoots of the two cultivars again showed that E107 accumulated significantly higher concentrations of Al, Ca, Cu, Fe, Mg, Mn, and Zn than Sparkle (Table 3).

The ability of E107 to accumulate Pb from Pb-contaminated soil is particularly significant. As indicated previously, Pb in soil is largely in non-soluble forms (Hettiarachchi and Pierzynski, 2004), and few plant species have been identified that accumulate Pb in shoots. The Pb accumulation may indicate that the increased activity of Fe(III) reductase in E107 acidifies the root rhizosphere, which helps in the release of Pb and other metals from the soil. Consequently, more metals are absorbed by roots, transported to and accumulated in shoots. Guinel and LaRue (1993) demonstrated that E107 was able to extrude 2.5 times more protons than Sparkle in root systems, and the proton extrusion caused Al accumulation as well.

The treatment of Pb contaminated soil with EDTA resulted in all leaves of Sparkle and E107 wilting one week after application. Thus, plants were harvested at that time. The change in morphological appearance was apparently related to metal toxicity, particularly Pb, as Pb in Sparkle and E107 reached more than 8,000 mg kg^{-1} (Table 4). Additionally, there were no significant differences between the two cultivars in accumulation of other metals. The EDTA-aided Pb accumulation could be viewed as EDTA accelerating Pb and other metal release from the soil, and multimetal accumulation occurring in both cultivars. As a consequence, the action of Fe(III) reductase in E107 was sheltered by EDTA application.

Thus far, significant progress has been made in phytoextraction of As, Cd, Hg, Ni, and Zn (Arthur et al., 2005; Pilon-Smits, 2005). Phytoextraction of Pb, however, has been less successful except for chelate-enhanced extrac-

Table 4 Concentrations of Pb, Al, Ca, Cu, Fe, Mg, Mn, and Zn in shoots of pea mutant E107 and wild type Sparkle grown in a Pb-contaminated soil treated with 1g kg^{-1} of EDTA

Plant	Pb	Al	Ca	Cu	Fe	Mg	Mn	Zn
	mg kg^{-1}							
E107	9,330	1,120	34,500	36.2	4,880	7,990	2,200	184
Sparkle	8,950	1,270	32,800	25.5	5,230	7,650	2,310	143
t-test[a]	ns	ns	ns	ns	ns	ns	ns	ns

[a] *t*-test: ns indicates non-significance at either the 5% or 1% levels. Values are the mean of each treatment ($n = 6$)

tion. Adding chelating agents to the soil may have negative effects on the ecosystem if excess chelating agents are left in the soil following phytoextraction (Romkens et al., 2002). Many chelating agents such as EDTA are not biodegradable under natural conditions and will persist in the environment. The main obstacle for non-chelate-aided phytoextraction of Pb has been the lack of Pb hyperaccumulators. The rarity of naturally occurring Pb-hyperaccumulators could be due to the circumstance that Pb in soil is largely in non-soluble forms, and plants that inhabit Pb contaminated soil may not evolve mechanisms for Pb accumulation. This study, however, demonstrated that a Fe hyperaccumulator pea mutant accumulated a significant quantity of Pb from Pb contaminated soil. It is possible that plants capable of accumulating Pb in shoots at 1,000 mg kg^{-1} or higher could be isolated among artificially induced metal accumulating mutants.

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