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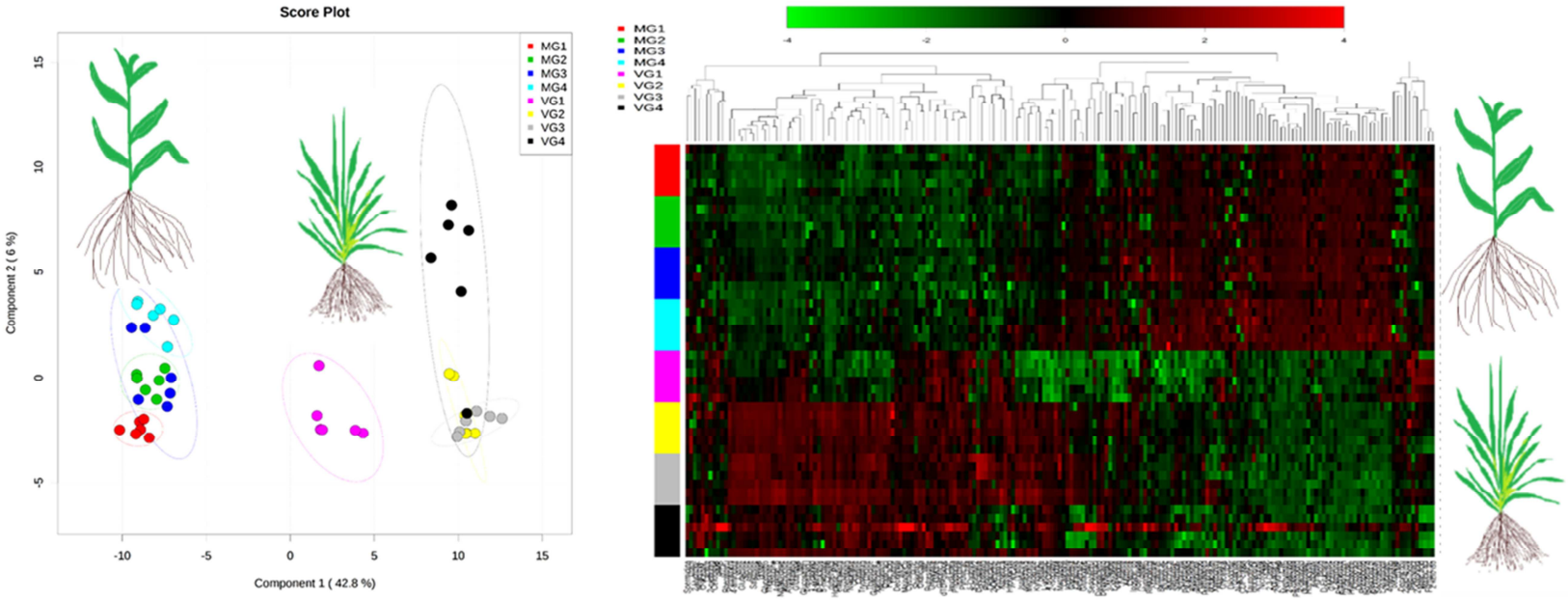

Comparative metabolic profiling of vetiver (*Chrysopogon zizanioides*) and maize (*Zea mays*) under Lead stress

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Abstract

Lead (Pb) contamination of residential soils in United States is attributed to use of Pb based paints prior to 1978 and their deterioration and accumulation on surface soils. Exposure to Pb due to ingestion and inhalation of Pb laden soil and dust causes neurological disorders, renal disorders, developmental and behavioral problems, particularly in children under the age of six. Vetiver grass is one of the leading choices for Pb remediation due to its ability to hyperaccumulate Pb, in addition to high biomass. In order to understand the effect of Pb on vetiver metabolic pathways, we compared the global metabolic changes in vetiver with that of maize, a Pb susceptible plant under Pb stress. Vetiver showed massive increase in levels of key metabolites in response to Pb, including amino acids, organic acids and coenzymes. Maize showed very modest increase in some of the same metabolites, and no change in others. The results provide the first indication of the difference in metabolic response of the hyperaccumulator, vetiver to lead stress as compared to maize.

Keywords: Phytoremediation, Lead, Vetiver, Maize, Metabolomics

1. Introduction:

Major sources of lead (Pb) in the environment include lead-based paint, leaded gasoline and Pb pipes for water distribution. Lead-based paint and leaded gasoline were banned in the U.S. by 1978 and 1996 respectively, resulting in lowering of Pb levels in the environment[1]. Recommendations of the Centers for Disease Control and prevention (CDC) lowered the acceptable blood Pb levels (BLL) from 10µg/dL to 5µg/dL in 2012, which brings more people under risk category[2]. Children are more prone to Pb poisoning, mostly through ingestion via hand-to-mouth activities and inhalation of Pb laden dust[3, 4]. In spite of phasing out Pb in gasoline and paint, CDC reported that there are four million contaminated households in the U.S. with high levels of Pb with children residing in them, and approximately half a million children below the age of five have BLLs above 5 µg/dL[2]. Several studies have shown that soil Pb is a major contributor to elevated BLLs in children[5, 6]. Phytoremediation is a promising and viable method to lower Pb levels in soils, as it is cost-effective and sustainable[7].

Lead is also toxic to plants, and affects a variety of functions such as seed germination, root growth, photosynthesis, cell division, transpiration etc.[8]. However, some plant species can withstand the deleterious effects of Pb through various tolerance mechanisms. Selective uptake, binding to the root surface or the cell wall, induction of chelating ligands such as phytochelatins, amino acids and organic acids etc. that help sequester Pb [8-10] are a few strategies used by plants to tolerate Pb. Antioxidative enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase and non-protein molecules also play important roles in Pb induced stress response[10-13]. Many

plants like *Sesbania drummondii*[7], *Brassica juncea*[8, 9] and *Thlaspi caerulescens*[14] have been studied for phytoremediation of Pb. *Chrysopogon zizanoides* (vetiver grass) was shown in our previous studies to accumulate high amounts of Pb in root and shoot[12]. High biomass, a large root system, high root to shoot metal translocation and tolerance to a wide range of environmental contaminants, makes it a suitable choice for phytoremediation[15-19].

Plants adapt to various environmental conditions by reconstituting their metabolic network and altering various physiological functions in order to survive and maintain homeostasis[20]. Metabolites play a major role in stress sensing, signaling and regulation of physiological functions. They are also the end result of altered gene expression due to stress conditions such as exposure to Pb, and reflect plant behavior in fighting the stress. A number of studies undertaken recently have indicated that metabolic adjustments are crucial for plant survival under stress. Metabolomics is an ideal approach to acquire a snapshot of the global state of metabolic changes under particular physiological conditions. Metabolomics has been adopted in recent studies to dissect various abiotic stress responses in plants[20]. For example, water stress was shown to result in the accumulation of several metabolites such as amino acids and sugars such as glucose and fructose and branch-chain amino acids, saccharopine, proline, and agmatine in *Arabidopsis*, which is associated with increase in ABA levels[21]. Comparison of metabolic response of grapevine to water and salt stress showed that water stressed plants accumulate higher levels of glucose, malate and proline than those of salt stressed plants[22]. Another study showed that when water stress was combined with heat stress, high levels of proline was replaced by high levels of sucrose[23]. Heat and cold stress

induced proline, glucose, fructose, galactinol and raffinose in Arabidopsis. Moreover, comparison of metabolic profiles of Arabidopsis plants exposed to heat and cold shock indicated that in both cases, proline, glucose, fructose, galactinol and raffinose are induced[24, 25].

We recently published a detailed metabolic profiling study of vetiver under Pb stress[10]. The study indicated a large induction in key metabolic pathways including amino acid metabolism, increased production of osmoprotectants and metal-chelating organic acids. Li et al.[26] reported changes in maize root metabolomic profile on exposure to 400 mg/L of Pb. The study revealed glycolysis, purine, pyrimidine, and phospholipids pathways were induced by Pb. However, the scope of the maize study was limited to the effect of Pb on root tissue alone, using a single Pb concentration. The main focus of the study was to develop a new quantitative metabolomic method, rather than elucidate the pathways involved in Pb tolerance. Although these two studies illustrated the molecular level responses of vetiver under Pb stress, the specific pathways responsible for Pb tolerance are still unclear. One approach to elucidate such potential pathways is to compare molecular-level responses of a Pb hyperaccumulating plant (such as vetiver) to those of a susceptible plant (such as maize). Such a study would provide clues to the specific adaptations in the metabolic pathways of tolerant plants to Pb stress.

Previous comparative metabolic profiling studies for plants under various abiotic stresses have provided important information on the strategies employed by tolerant plants in surviving under adverse environmental conditions. However, with the exception of a couple of studies on cadmium stress in varieties of Bermuda grass[27] and soybeans[28], such studies are lacking for plant tolerance to Pb. The results obtained in the

117 present study provide valuable insights into the molecular mechanism of Pb tolerance and
118 detoxification in hyperaccumulator plants.

119 **2. Materials and methods:**

120 **2.1 Experimental set up:**

121 Hydroponic experiments were set up as previously described by Andra et al.[12].
122 Vetiver plants were purchased from Florida Farms and Nurseries (FL, US) and were
123 grown in plastic pots with a commercial potting mix for 3 months and subsequently
124 transferred to hydroponic medium containing half strength Hoagland solution. After 4
125 weeks of acclimation, plants were moved to fresh half strength Hoagland solution
126 without KH_2PO_4 to prevent the formation of Pb phosphate complexes. Maize seeds were
127 germinated on wet filter paper and transferred to the hydroponic set up. The seedlings
128 were supported on rock wool, and suspended into the media using porous plastic baskets.
129 Maize seedlings were allowed to grow to 6-leaf stage before Pb treatment. Lead nitrate
130 (Acros organics) was used as Pb source added at concentrations of 400 mg/L, 800 mg/L
131 and 1200 mg/L. These concentrations represent USEPA standards for Pb in bare soil in
132 play areas (400 ppm) and non-play areas (1200 ppm)[29]. Six replicates were prepared for
133 each treatment, along with a set of controls without Pb. Deionized-water was added
134 periodically to maintain the water level in the hydroponic containers throughout the
135 experiment. The hydroponic setup was maintained in a greenhouse at 25°C with a
136 photoperiod of 16 h day/8h dark. The light intensity was kept at 250µmol m^{-2} s^{-1}, and the
137 relative humidity was 60 ± 2%. Plants samples were harvested after 10 days. After
138 washing of the roots with deionized water to remove the surface bound Pb, plants were
139 flash frozen in liquid nitrogen and stored at -80ºC until further analysis.

140 **2.2 Pb estimation in vetiver and maize**

141 For estimation of Pb in plant tissue, roots and leaves were separated and dried in an oven
142 at 60ºC. One gram of dried tissue was acid digested on a hot plate using nitric acid:
143 perchloric acid mixture following EPA 3050b[30] method. The samples were filtered and
144 diluted with deionized water. Pb concentration was analyzed using ICP-MS.
145

146 **2.3 Metabolites extraction**

147 Metabolites were extracted as previously described[31] with slight modifications[10]. Briefly,
148 100 mg of tissue was ground in liquid nitrogen. Six replicates were prepared for each
149 treatment and the control. Ampicillin (0.5 mg/ml) was added as internal standard prior to
150 extraction. Three volumes of extraction buffer (methanol: acetonitrile (50:50) with
151 0.125% formic acid) were added to each sample. The samples were sonicated for 15 min
152 at 40 kHz in an ice-water bath sonicator, and centrifuged at 20,000 g for 15 min at 4ºC.
153 The supernatant was passed through 0.2-µm PTFE syringe filters and transferred to 1.6
154 ml glass vials for LC-MS/MS analysis.
155

156 **2.4 LC-MS/MS conditions**

157 LC-MS/MS analysis was performed on an ABSciex Qtrap 5500 mass spectrophotometer
158 (AB Sciex, USA) equipped with a Turbo V electrospray ionization (ESI) source,
159 Shimadzu LC-20A system, and a PAL CTC auto sampler. LC-MS/MS conditions are
160 followed as described[10]. All the samples were kept at 4°C during analysis. A total of 325
161 metabolites were targeted in MRM mode.
162

2.5 Statistical analysis

A total of 325 metabolites encompassing all major metabolic pathways were targeted. Only metabolites that were detected in all the replicates of treatments and controls were used for statistical analysis. Relative concentrations of metabolites were normalized with internal standard and auto scaled using MetaboAnalyst software[32] and data was processed as described previously[10]. Mean values of six replicates were calculated and used for fold change calculation. Fold changes were calculated with reference to control. Significantly changed metabolites between control and treatments were identified using Student t-test. Partial least squares discriminate analysis (PLS-DA) and Hierarchical cluster analysis (HCA) models were used for root and shoot tissues separately for both plants using MetaboAnalyst software as described[31].

3. Results and discussion

3.1 Pb uptake in vetiver and maize

Vetiver showed much higher Pb accumulation in both root and shoot compared to maize in all the treatments (Table 1). Vetiver roots accumulated an average of 3,861, 5,132, and 18,562 mg of Pb per kg dry weight in 400, 800, 1200 mg/L treated plants, respectively. As expected, maize roots accumulated much lower concentrations, i.e., 39.6, 69.8, and 192.4 mg of Pb per kg dry weight in 400, 800, 1200 mg/L treatments, respectively. Vetiver shoot tissue accumulated 168.6, 426.6, and 93.1 mg of Pb per kg of dry weight in 400, 800, 1200 mg/L treatments, respectively. Maize shoot tissue accumulated 3.6, 8.7, and 1.9 mg of Pb per kg dry weight in 400, 800, 1200 mg/L treatments, respectively.

185 Vetiver is known to be a hyperaccumulator of Pb, as previously reported by our group[10],
186 and these results are very similar to the reported data.
187
188 **3.2 Metabolic changes in vetiver root and shoot tissue in response to Pb stress**
189 Plants often respond to heavy metal stress by synthesizing various metabolites, both to
190 maintain homeostasis as well as alter key metabolic pathways like sugar metabolism,
191 antioxidative pathways, signaling pathways etc. Vetiver and maize plants were subjected
192 to various concentrations of Pb, with Pb-free plants as controls. Relative concentrations
193 of 263 metabolites were compared among treatments and control. Six replicates were
194 used for each treatment and control. According to the PLS-DA analysis of vetiver root
195 metabolites, the response between control and treatments could be clearly differentiated,
196 with 32.8% and 20.2% variance explained by component 1 and component 2,
197 respectively (Fig. 1). Vetiver shoot exhibited a similar pattern with clear difference
198 between control and treatments with 44.1% and 6.2% explained by component 1 and 2,
199 respectively (Fig. 2). Heat maps of vetiver and maize root and shoot are presented in
200 Supplemental Information (SI) Figs. S-1 and S-2. The heat maps show the overall picture
201 of metabolite concentration changes among the treatments and the controls. A complete
202 list of metabolite changes in vetiver root and shoot is presented in Tables 2 and 3 and
203 maize root and shoot in Tables 4 and 5, respectively. Fold changes were calculated as a
204 ratio of relative metabolite concentration of the treatment to that of the control, presented
205 in SI (Tables S-1 and S-2). As shown in Tables 2-5, the patterns vary widely, with some
206 metabolites showing an increasing trend as the Pb concentration increased and some
207 showing a decreasing trend. Some other metabolites showed mixed patterns among

208 treatments. Student *t*-test was performed to evaluate the statistical significance of
209 treatment Pb concentration with respect to that of control. At least 109 root metabolites
210 and 133 shoot metabolites in all the treatments showed more than 2 fold increase
211 compared to control.
212
213 **3.3 Metabolic changes in Maize root and shoot tissue under Pb stress**
214 Maize is more susceptible to Pb, and also accumulates lower levels of Pb compared to
215 vetiver (Table 1). Under similar treatment conditions, 201 compounds in root and 247
216 compounds in shoot were compared between treatments and control with six replicates.
217 PLS-DA analysis of maize root and shoot metabolites are displayed in Figures 1 and 2,
218 respectively. In maize, 31.1%, and 8.6% of the variation between control and treatment
219 groups within root and 33.6% and 8.7% in shoot could be explained by component 1 and
220 component 2, respectively. As shown in Figures S-1 and S-2, heat maps for maize root
221 and shoot, respectively, depict concentration changes among treatments and in
222 comparison to the control. In root, at least 20 metabolites increased in concentration by
223 two fold among all treatments compared to control. In shoot, at least 37 compounds
224 showed two-fold increase in all the treatments compared to control.
225
226 **3.4 Comparison of metabolite changes in vetiver and maize**
227 Metabolite changes of vetiver and maize in both root and shoot tissues were
228 compared to decipher the differences in stress response in vetiver in comparison to maize
229 on Pb exposure. Only those metabolites that were detected in both plant species were
230 compared, ignoring the metabolites detected only in either vetiver or maize. PLS-DA

231 analysis of combined maize and vetiver root metabolites show that metabolites from the
232 two plants are grouped separately, with 42.8% and 6% explained by component 1 and
233 component 2, respectively, for the root tissues (Fig. 1). Similarly, shoot metabolites
234 showed variation between the two plants with 41.2% and 10.6% explained by
235 components 1 and 2, respectively (Fig. 2), with controls forming separate groups from
236 treatments in both plants. Hierarchical clustering analysis models are shown in figures S-
237 1 and S-2 for root and shoot tissues, respectively, which present the overall picture of
238 metabolite changes in the two plants. Color contrast indicating the changes in metabolite
239 concentrations shows a much higher increase in metabolite levels in vetiver treated with
240 Pb when compared to maize in both root and shoot tissue, indicating differences in
241 response to stress in these plants.

242 Under heavy metal stress, plants produce a variety of metabolites such as amino
243 acids proline and histidine; polypeptides such as glutathione and phytochelatins;
244 polyamines such as spermidine, putrescine etc. Phytochelatins and glutathione form
245 complexes with metals and help in their compartmentalization[33, 34]. Vetiver and maize
246 showed major differences in metabolite levels in root and shoot tissue as shown by
247 PLSDA analysis (Figs. 1 and 2), and heat maps (Figs. S-1 and S-2) indicating the
248 significance of metabolite concentrations in tolerance mechanisms. Among stress
249 induced amino acids, proline is well known to function as an osmolyte, radical scavenger,
250 macromolecule stabilizer and metal chelator under heavy metal stress[35, 36]. Vetiver
251 showed enormous increase of proline levels in both root and shoot tissue in treatments
252 compared to the control (Tables 2, 3, S-1 and S-2). In root, proline levels increased by 40,
253 59 and 16 fold in 400, 800 and 1200 mg/L treatments, respectively. However, maize

254 showed only a slight increase in proline content in root (< 2 fold), and a decline in proline
255 concentration in shoot tissue (Tables 4, 5, S-1 and S-2). Proline production is reported to
256 vary between root and shoot in different plants, and is also known to be metal specific[35].
257 Pb is reported to increase proline levels in wheat, sunflower, black gram and pigeon pea[36]
258 which supports current findings. Many earlier studies have also reported that proline
259 accumulation under stress is positively correlated with oxidative stress tolerance[37, 38, 39].
260 In addition to metals, several environmental stresses such as water deficit and high
261 salinity also induce proline levels[40].

262 Histidine is known to be associated with nickel-hyperaccumulating properties in
263 Alyssum species[41], in which nickel exposure resulted in high accumulation of free
264 histidine. Hyperaccumulating Brassica species also showed increased histidine levels
265 compared to non-accumulators[42]. Besides Ni, Zn and Cu also induced increased histidine
266 levels in another metal hyperaccumulator, *Thlaspi caerulescens*[43] and yeast[44]. Our data
267 show that vetiver and maize vary drastically in their histidine levels in both root and
268 shoot on Pb exposure (Tables 2, 3, 4 and 5). In vetiver, treated plants showed up to 17-
269 fold increase in histidine levels in root and up to 34-fold increase in shoot tissue
270 compared to control plants (Tables S-1 and S-2). Maize also showed increased histidine
271 levels in root by 3-fold, however shoot tissue showed decrease in histidine levels in Pb
272 treated plants compared to control plants (Tables 4, S-1).

273 In vetiver root and shoot, asparagine, which was reported to act as a ligand to
274 bind Pb in *in vitro* studies [44], increased about 3 fold (Tables S-1 and S-2) while in maize,
275 no significant increase was observed. Pb was reported to bind to phytochelatins, an
276 oligomer of glutathione, in vetiver in our earlier studies[12]. Current results show increase

277 in glutamine, a precursor of glutathione[45] and a known antioxidant. Reduced glutathione
278 increased up to 11-fold in root and up to 15-fold in shoot (Tables 2 and 3) in vetiver,
279 while maize root showed no significant increase (Tables 4 and 5), and maize shoot also
280 displayed decrease in glutamine concentration (Table 5).

281 Apart from the above, other amino acids such as tryptophan, isoleucine and valine
282 were also reported to increase in wheat under drought stress[46] and in tobacco under salt
283 stress[47]. Our data show that tryptophan in vetiver root increased by 22-fold, valine by 5.5
284 fold and isoleucine by 9-fold (Tables 2 and S-1). Shoot tissue also showed up to 35-fold
285 increase in tryptophan, up to 8-fold increase in valine and up to 6.5-fold increase in
286 isoleucine (Tables 3 and S-2), whereas in maize isoleucine showed only 2-fold increase
287 in root and 6-fold increase in shoot, tryptophan showed up to 4.9-fold increase in root and
288 no significant increase in shoot, valine showed a maximum of 2.8-fold increase in root
289 and decrease in shoot tissue (Tables 4, 5, S-1 and S-2). Vetiver also showed significant
290 increase in other amino acids such as threonine, methionine and isoleucine compared to
291 maize in both root and shoot. Threonine and methionine produce intermediates of
292 isoleucine biosynthesis, and their interdependency has significant role in stress
293 conditions[48], as reported in *Arabidopsis* and tomato under drought stress[49, 50].

294 In *Arabidopsis*, nicotinamide adenine dinucleotide (NAD) and nicotinamide
295 adenine dinucleotide phosphate (NADP) are reported to play rate-limiting roles in
296 generation and scavenging of reactive oxygen species (ROS) and protect guard cells from
297 ROS[51]. Nicotinamide is a precursor of NAD biosynthesis; our results show a very high
298 increase in nicotinamide content in both vetiver root and shoot under Pb stress. Root
299 tissue showed maximum of 287-fold increase in 400 mg/L treatment and shoot showed

133-fold increase in 800 mg/L treatment, however maize showed decreased content of nicotinamide in both root and shoot tissue. While root showed a slight increase in nicotinamide levels (0.96 fold in 400 mg/L, 2.05 fold in 800 mg/L and 0.73 fold in 1200 mg/L of lead), no increase in nicotinamide level (0.36-fold in 400 mg/L treatment, 0.29 fold 800 mg/L treatment and 0.16 in 1,200 mg/L treatment), in shoot was observed as a result of Pb exposure (Tables S-1 and S-2). In *Arabidopsis*, thiamine also plays an important role in abiotic stress tolerance and confers enhanced tolerance especially to oxidative stress[52]. Thiamine and thiamine pyrophosphate levels increased in *Arabidopsis* under various abiotic stress conditions, such as high light, cold, osmotic, salinity, and oxidative treatments[53]. Vetiver showed very high increase in both thiamine and thiamine pyrophosphate levels under Pb stress. Root tissue showed maximum of 51-fold increase for thiamine and 41-fold increase for thiamine pyrophosphate (Table S-1) and shoot tissue showed an astounding 390-fold increase of thiamine pyrophosphate (Table S-2) and up to 61-fold increase of thiamine. However, in the case of maize, the levels of thiamine and thiamine pyrophosphate were much lower, with 0.2 - 2 fold increase in levels in lead treated plants compared to the control (Tables S-1 and S-2).

Tricarboxylic acid (TCA) cycle intermediates are known to decrease under various abiotic stress conditions in plants[52, 54, 55]. Moreover, TCA cycle intermediates, especially α-ketoglutaric acid was reported to play a major role in maintaining redox homeostasis by reducing ROS[56]. Vetiver however, showed increase in many TCA intermediates like α-ketoglutaric acid, succinyl-CoA, fumaric acid, oxaloacetic acid, isocitric acid in both root and shoot, except decrease of fumaric acid in root (Tables 2, 3, S-1 and S-2). Other organic acids (malic acid, ascorbic acid, cis-aconitic acid, aspartic

323 acid) also increased in all tissues. However, changes in TCA intermediates in maize were
324 mixed and non-significant (P <0.05, Tables 4 and 5). Glycolysis, a commonly affected
325 pathway under abiotic stress showed mixed changes in metabolites such as fructose-6-
326 phosphate, fructose-1, 6-bisphosphate, glyceraldehyde-3-phosphate, glycerate-3-
327 phosphate and phosphoenol pyruvate in both vetiver and maize root and shoot tissues,
328 with no particular trend (Tables 2 - 5).

329 Soluble sugars perform various functions during abiotic stress, including
330 osmoprotection, maintaining turgor pressure, stabilizing cell membrane and scavenging
331 ROS[57, 58]. Vetiver showed increase in concentration of hexose in both root and shoot
332 tissues, between 4-11 fold in roots and 11-14 fold in shoot tissue. In maize, only a small
333 increase of 1.5-fold was observed in plants treated with 800 and 1200 mg/L of Pb (Tables
334 S-1 and S-2).

335 Several recent studies have attempted to decipher the similarities between plant
336 responses to various abiotic and biotic stresses. Increasing focus on understanding plant
337 response to a combination of stresses has revealed some interesting information[59].
338 Although these studies reveal some common plant responses, each type of stress, or
339 combination of stresses seems to elicit a unique set of responses, controlled by different
340 signaling pathways[60, 61, 62].

341 Very few studies have compared the metabolic profiles of hyperaccumulators to
342 non-accumulator plants, to decipher their differential response to metal stress. A previous
343 study with two genotypes of Bermuda grass treated with cadmium showed that amino
344 acids, organic acids and sugars accumulated more in the tolerant genotype compared to
345 the less tolerant one[27]. Our study compared two monocot species, a known

hyperaccumulator, and the other a high biomass grass, which has previously been used in studies for lead phytoremediation[14, 63], but is known to be a non-accumulator. Results indicate that while the same metabolic pathways are involved in stress response in both plants, the magnitude of the response exhibited by the two plants is very different. Vetiver showed enormous increase in levels of key metabolites in response to Pb, including amino acids, organic acids and coenzymes. In particular, amino acids appear to play a major role in metal stress tolerance in vetiver, including proline, histidine, asparagine, tryptophan, threonine, methionine, isoleucine and valine. The role of amino acids in stress tolerance is well known, as osmoprotectants, metal chelating agents and antioxidants. In addition, reduced glutathione increased, which not only binds metals, but also is a precursor to phytochelatins, which are small peptides involved in binding and sequestration of metals in vacuoles. Organic acids (malic acid, ascorbic acid, cis-aconitic acid) coenzymes or precursors of coenzymes such as nicotinamide, thiamine and thiamine pyrophosphate also increased in vetiver. In contrast, maize showed either minor increases (proline, histidine, branched chain amino acids, folate) or no increase in metabolites (Tables 4 and 5). These results provide a glimpse into the mechanism of lead stress response in vetiver, which induces the production of several magnitudes higher stress-mitigating metabolites than that of maize.

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Supplemental Information: Comparison of fold changes in concentrations of selected metabolites in vetiver and maize root (Table S-1) and vetiver and maize shoot (Table S-2) with respect to control. Heat maps of metabolite concentrations in vetiver and maize root (Figure S-1) and vetiver and maize shoot (Figure S-2). Vetiver and maize plants were treated with 400, 800 and 1200 mg/L of Pb for 10 days.

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Lead Conc. (mg/L)	Plant Tissue	Pb uptake (mg/kg dry wt.) Concentration ± Std. dev	
		Vetiver	Maize
0	Root	0	0
400	Root	$3.86 \times 10^3 \pm 124$	39.6 ± 9
800	Root	$9.83 \times 10^3 \pm 351$	69.8 ± 11
1200	Root	$1.85 \times 10^4 \pm 297$	192.4 ± 17
0	Shoot	0	0
400	Shoot	168.55 ± 32	3.60 ± 0.75
800	Shoot	426.56 ± 49	8.70 ± 3.2
1200	Shoot	93.08 ± 27	1.89 ±0.66

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578 **Table 1.** Pb uptake in vetiver and maize root and shoot tissues, measured using ICP-MS

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588 **Table 2:** Relative concentrations of metabolites in vetiver root. Metabolite concentrations measured using LC-MS/MS in vetiver root

589 samples exposed to 400, 800 and 1200 mg/L of Pb for 10 days (n=6)

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Metabolite name	Metabolite concentration*				T test		
	Control avg ± std.dev	400 avg± std.dev	800 avg± std.dev	1200 avg± std.dev	400 vs Control	800 vs Control	1200 vs Control
Acetyl-CoA	63.19 ± 6.86	566.25 ± 41.8	1894.68 ± 156.27	1149.98 ± 54.13	NS	P < 0.05	P < 0.05
alpha-Ketoglutaric acid	12.31 ± 1.71	211.09 ± 18.38	148.65 ± 12.95	263.66 ± 19.47	P < 0.05	P < 0.05	P < 0.05
Asparagine	12816.67 ± 2055.41	42305.67 ± 1991.33	41435.7 ± 1950.38	29177.87 ± 2048.29	P < 0.05	P < 0.05	NS
cis-aconitate	815.37 ± 181.8	5834.59 ± 409.59	9281.58 ± 651.57	1477.62 ± 69.56	P < 0.05	P < 0.05	NS
L-ascorbic acid	126.05 ± 15.52	1734.57 ± 143.07	1735.66 ± 143.16	3107.93 ± 146.29	P < 0.05	P < 0.05	P < 0.05
D-Fructose 1,6-bisphosphate	4.24 ± 0.54	5.12 ± 1.01	2675.5 ± 220.67	40.65 ± 7.73	NS	P < 0.05	NS
D-Fructose 1-phosphate	53.84 ± 5.28	154.59 ± 13.47	268.55 ± 23.39	150.22 ± 11.09	P < 0.05	P < 0.05	NS
Fumaric acid	1464.67 ± 234.89	488.15 ± 36.04	648.2 ± 47.85	216.55 ± 15.99	P < 0.05	P < 0.05	P < 0.05
Glucosamine-6-Phosphate	4.14 ± 0.53	1.92 ± 0.46	2.72 ± 0.64	2.88 ± 0.47	P < 0.05	NS	P < 0.05
Glutamate	1416.84 ± 227.22	3544.16 ± 248.8	5515.86 ± 387.22	1333.61 ± 62.78	P < 0.05	P < 0.05	NS
Glutamine	533 ± 118.84	5738.49 ± 402.85	9180.65 ± 644.49	2863.79 ± 134.8	P < 0.05	P < 0.05	P < 0.05
Glycerate 3-phosphate	104.99 ± 12.93	4.8 ± 0.95	3.07 ± 0.5	5.69 ± 0.64	P < 0.05	P < 0.05	P < 0.05
hexose	1750 ± 280.65	15195.61 ± 715.26	8501.35 ± 596.8	11189.66 ± 785.52	P < 0.05	P < 0.05	P < 0.05

Histidine	202.95 ± 23.7	2287.1 ± 188.64	3452.2 ± 242.35	1785.34 ± 84.04	P < 0.05	P < 0.05	P < 0.05
Isoleucine	581.34 ± 129.62	3429.05 ± 240.72	5325.83 ± 373.88	1845.37 ± 86.87	P < 0.05	P < 0.05	P < 0.05
Malic acid	21365 ± 3426.3	5743.49 ± 403.2	8318.86 ± 583.99	1381.8 ± 65.05	P < 0.05	NS	P < 0.05
Methionine	22.1 ± 1.98	138.29 ± 12.05	482.45 ± 35.62	100.83 ± 16.17	P < 0.05	P < 0.05	NS
Nicotinamide	36.14 ± 3.54	10380.69 ± 488.62	10120.71 ± 476.39	4095.44 ± 287.51	P < 0.05	P < 0.05	P < 0.05
Oxaloacetate	73 ± 7.93	142.4 ± 12.4	213.15 ± 18.56	669.12 ± 55.19	NS	NS	P < 0.05
Proline	357.75 ± 41.77	1439.35 ± 677.78	2125.6 ± 1000.27	1944.24 ± 361.13	P < 0.05	P < 0.05	NS
Succinyl-coA	197.9 ± 23.11	944.55 ± 77.91	805.52 ± 59.46	1149.89 ± 54.13	NS	NS	P < 0.05
Thiamine	10.86 ± 1.51	484.5 ± 35.77	562.33 ± 41.51	917.04 ± 75.64	P < 0.05	P < 0.05	P < 0.05
Thiamine-Pyrophosphate	40.5 ± 3.97	704.22 ± 51.98	1668.91 ± 137.65	1005.26 ± 82.92	NS	P < 0.05	P < 0.05
Threonine	207.7 ± 24.26	1456.02 ± 120.09	1536.61 ± 126.74	600.93 ± 44.36	P < 0.05	P < 0.05	P < 0.05
Trehalose	6.92 ± 0.78	59.12 ± 6.97	84.77 ± 10	14.07 ± 2.76	P < 0.05	P < 0.05	NS
Tryptophan	89.6 ± 9.73	2029.69 ± 167.41	1583.97 ± 130.65	807.91 ± 66.64	P < 0.05	P < 0.05	P < 0.05
Valine	1183 ± 189.72	3896.09 ± 273.51	6546.94 ± 459.6	2330.16 ± 109.68	P < 0.05	P < 0.05	NS

NS – non-significant

*Values represent normalized peak area

600 **Table 3:** Relative concentrations of metabolites in vetiver shoot. Metabolite concentrations measured using LC-MS/MS in vetiver
601 shoot samples exposed to 400, 800 and 1200 mg/L of Pb for 10 days (n=6)

Metabolite name	Metabolite concentration*				T test		
	Control avg ± std.dev	400 avg ± std.dev	800 avg ± std.dev	1200 avg ± std.dev	400 vs control	800 vs control	1200 vs control
Acetyl-CoA	159.86 ± 9.85	3421.66 ± 210.75	2651.66 ± 163.33	4156.06 ± 185.2	p< 0.05	p< 0.05	p< 0.05
Asparagine	9823.03 ± 604.7	925.02 ± 56.98	988.78 ± 60.91	715.61 ± 44.08	p< 0.05	p< 0.05	p< 0.05
Fumaric acid	1614.97 ± 99.42	5.24 ± 0.24	6.46 ± 0.29	5.81 ± 0.36	p< 0.05	p< 0.05	p< 0.05
Glutamate	847.7 ± 37.78	17493.92 ± 1709.12	15578.04 ± 1521.95	12537.68 ± 772.24	p< 0.05	p< 0.05	p< 0.05
Glutamine	1140.21 ± 50.81	5.46 ± 0.54	50.04 ± 4.89	4.71 ± 0.29	p< 0.05	p< 0.05	p< 0.05
Glycerol-3-phosphate	32.31 ± 1.99	27387.99 ± 2675.75	28522.76 ± 2786.62	28365.1 ± 1747.1	p< 0.05	p< 0.05	p< 0.05
Hexose Pool (fructose, glucose)	4884.74 ± 300.7	5410.85 ± 528.63	6268.05 ± 612.38	9777.61 ± 602.24	p< 0.05	p< 0.05	p< 0.05
Histidine	281.67 ± 17.35	4.74 ± 0.47	5.01 ± 0.49	4.58 ± 0.29	p< 0.05	p< 0.05	p< 0.05
Isocitrate	633.73 ± 28.24	7183.34 ± 442.45	5308.52 ± 326.97	7473.49 ± 460.32	p< 0.05	p< 0.05	p< 0.05
Isoleucine	1134.36 ± 50.55	347.11 ± 21.38	394.91 ± 24.33	64.17 ± 3.96	p< 0.05	p< 0.05	p< 0.05
Lysine	1191.87 ± 53.12	5172.8 ± 460.4	5891.08 ± 524.33	7519.15 ± 463.13	p< 0.05	p< 0.05	p< 0.05
Malic acid	20443.24 ± 1258.47	2386.16 ± 212.38	3078.68 ± 274.02	4156.06 ± 255.99	p< 0.05	p< 0.05	p< 0.05
Methionine	57.59 ± 3.55	533.37 ± 47.48	497.69 ± 44.3	568.45 ± 35.02	p< 0.05	p< 0.05	p< 0.05
Nicotinamide	17.91 ± 1.11	39.37 ± 2.43	133.86 ± 8.25	45.44 ± 2.8	NS	NS	NS
Oxaloacetic acid	94.03 ± 5.8	70.27 ± 4.33	67.58 ± 4.17	67.87 ± 6.05	p< 0.05	p< 0.05	p< 0.05
Succinyl-coA	239.67 ± 14.77	3.76 ± 0.46	3.21 ± 0.39	4.43 ± 0.44	p< 0.05	p< 0.05	p< 0.05

Thiamine	43.87 ± 2.71	4.83 ± 0.59	2.85 ± 0.35	2.31 ± 0.23	p< 0.05	p< 0.05	p< 0.05
Thiamine-Pyrophosphate	5.91 ± 0.58	4684.24 ± 568.78	4348.24 ± 527.98	6035.02 ± 589.61	p< 0.05	p< 0.05	p< 0.05
Valine	2977.59 ± 183.3	4.73 ± 0.58	7.7 ± 0.94	5.45 ± 0.67	p< 0.05	p< 0.05	p< 0.05

NS – non-significant

*Values represent normalized peak area

621 **Table 4:** Relative concentrations of metabolites in maize root. Metabolite concentrations measured using LC-MS/MS in maize root
622 samples exposed to 400, 800 and 1200 mg/L of Pb for 10 days (n=6)

Metabolite name	Metabolite concentration*				T test		
	Control Avg ± std.dev	400 Avg ± std.dev	800 Avg ± std.dev	1200 Avg ± std.dev	400 vs Control	800 vs Control	1200 vs Control
cis-aconitate	337.82 ± 20.81	378.9 ± 23.34	405.08 ± 24.95	579.27 ± 35.68	NS	NS	p<0.05
D-Glucose 1-phosphate	416.65 ± 25.67	491.51 ± 30.28	487.9 ± 30.06	865.46 ± 53.31	NS	NS	p<0.05
Fumaric acid	1062.79 ± 65.47	1134.98 ± 69.91	920.69 ± 56.71	1395.35 ± 85.95	NS	NS	p<0.05
Glutamine	119.68 ± 7.38	192.09 ± 11.84	411.12 ± 25.33	317.11 ± 19.54	NS	p<0.05	NS
Glyceraldehyde 3-phosphate	94.5 ± 5.83	94.82 ± 5.85	104.68 ± 6.45	193.91 ± 11.95	NS	NS	p<0.05
Glycerate 3-phosphate	108.29 ± 6.67	132.22 ± 8.15	202.54 ± 12.48	322.67 ± 19.88	NS	NS	p<0.05
Glycerol-3-phosphate	25.33 ± 1.56	30.31 ± 1.87	33.41 ± 2.06	41.27 ± 2.55	NS	NS	p<0.05
Hexose	2438.19 ± 108.65	856.01 ± 38.15	1811.61 ± 80.73	775.69 ± 47.78	p<0.05	NS	p<0.05
Histidine	28.77 ± 1.29	70.2 ± 3.13	93.83 ± 4.19	99.97 ± 6.16	p<0.05	p<0.05	NS
Lysine	135.06 ± 13.2	198.66 ± 19.41	412.23 ± 40.28	361.78 ± 22.29	NS	p<0.05	NS
Malic acid	8259.82 ± 806.97	8698.76 ± 849.85	4263.48 ± 416.54	2517.75 ± 155.08	NS	NS	p<0.05
Phosphoenolpyruvate (PEP)	1098.42 ± 67.66	1167.03 ± 71.89	1195.87 ± 73.66	1750.09 ± 107.8	NS	NS	p<0.05
Proline	112.03 ± 9.98	178.56 ± 15.9	176.3 ± 15.7	219.63 ± 13.53	p<0.05	NS	p<0.05
Succinic acid	108.44 ± 6.68	99.94 ± 6.16	112.2 ± 6.92	163.62 ± 10.08	NS	NS	p<0.05

Tryptophan	30.66 ± 1.89	151.26 ± 9.32	105.09 ± 6.48	139.23 ± 12.4	p<0.05	p<0.05	p<0.05
Tyrosine	240.06 ± 14.79	186.92 ± 11.52	229.89 ± 14.16	72.33 ± 6.44	NS	NS	p<0.05
Valine	1396.71 ± 86.03	2579.53 ± 158.89	3191.15 ± 196.56	4030.41 ± 358.72	p<0.05	p<0.05	p<0.05

NS – non-significant

*Values represent normalized peak area

637 **Table 5:** Relative concentrations of metabolites in maize shoot. Metabolite concentrations measured using LC-MS/MS in maize shoot

638 samples exposed to 400, 800 and 1200 mg/L of Pb for 10 days (n=6)

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Metabolite names	Metabolite concentration*				T test		
	Control Avg ± std.dev	400 Avg ± std.dev	800 Avg ± std.dev	1200 Avg ± std.dev	400 vs Control	800 vs Control	1200 vs Control
Asparagine	379.12 ± 23.36	522.04 ± 32.16	1345.97 ± 82.91	781.79 ± 48.16	NS	NS	p<0.05
Aspartic acid	138.37 ± 8.53	462.06 ± 28.46	853.66 ± 52.58	719.55 ± 44.32	p<0.05	p<0.05	p<0.05
cis-aconitate	140593.84 ± 8659.6	25249.26 ± 1555.18	28311.86 ± 1743.82	19855.11 ± 1222.94	p<0.05	p<0.05	p<0.05
D-Glucose 1-phosphate	319.09 ± 19.66	136.62 ± 8.42	266.87 ± 16.44	187.26 ± 11.54	p<0.05	NS	NS
Glutamine	1418.11 ± 138.55	118.26 ± 11.56	209.44 ± 12.91	459.97 ± 44.94	p<0.05	p<0.05	p<0.05
Hexose	4765.63 ± 293.53	2877.09 ± 177.21	7178.92 ± 442.18	6926.21 ± 426.61	NS	NS	NS
Histidine	1326.03 ± 81.68	219.39 ± 13.52	296.15 ± 18.25	243.09 ± 14.98	p<0.05	p<0.05	p<0.05
Isocitrate	67.99 ± 6.06	59.27 ± 5.28	557.47 ± 34.34	334.7 ± 29.79	NS	NS	p<0.05
Isoleucine	15615.9 ± 1389.86	2910.01 ± 259	4192.5 ± 258.23	4150.55 ± 369.41	p<0.05	p<0.05	p<0.05
Lysine	1307.73 ± 116.4	119.54 ± 10.64	204.96 ± 12.63	520.43 ± 46.32	p<0.05	p<0.05	p<0.05
Methionine	239.09 ± 21.28	31.14 ± 2.78	46.86 ± 2.89	43.64 ± 3.89	p<0.05	p<0.05	p<0.05
NAD+	1.38 ± 0.09	0.45 ± 0.03	0.38 ± 0.03	0.51 ± 0.04	p<0.05	p<0.05	p<0.05
NADH	1.87 ± 0.12	0.45 ± 0.03	0.38 ± 0.03	0.51 ± 0.04	p<0.05	p<0.05	p<0.05
NADP+	1.38 ± 0.09	0.45 ± 0.03	0.38 ± 0.03	0.51 ± 0.04	p<0.05	p<0.05	p<0.05

Oxalic acid	19.5 ± 1.21	3.07 ± 0.19	9.99 ± 0.89	4.24 ± 0.27	p<0.05	NS	p<0.05
Proline	1405.69 ± 125.11	227.88 ± 27.67	199.63 ± 19.51	546.64 ± 66.38	p<0.05	p<0.05	p<0.05
Pyrophosphate	339.7 ± 30.24	698.42 ± 84.81	619.35 ± 60.51	757.33 ± 91.96	p<0.05	p<0.05	p<0.05
Succinyl-CoA	1.38 ± 0.07	0.45 ± 0.06	0.38 ± 0.05	0.51 ± 0.07	p<0.05	p<0.05	p<0.05
Thiamine	201.41 ± 8.98	4.86 ± 0.59	19.65 ± 2.39	13.15 ± 1.6	NS	NS	NS
Thiamine phosphate	1.38 ± 0.07	0.45 ± 0.06	0.38 ± 0.05	0.51 ± 0.07	p<0.05	p<0.05	p<0.05
Threonine	690.09 ± 30.76	1662.86 ± 201.92	1408.59 ± 171.04	1423.54 ± 172.86	p<0.05	p<0.05	p<0.05
Tryptophan	696.58 ± 68.06	1733.04 ± 210.44	635.65 ± 77.19	309.26 ± 37.56	p<0.05	NS	p<0.05
Valine	32183.51 ± 1434.14	9547.53 ± 588.07	10166.32 ± 993.23	8818.44 ± 0	p<0.05	p<0.05	p<0.05

NS – non-significant

*Values represent normalized peak area

651

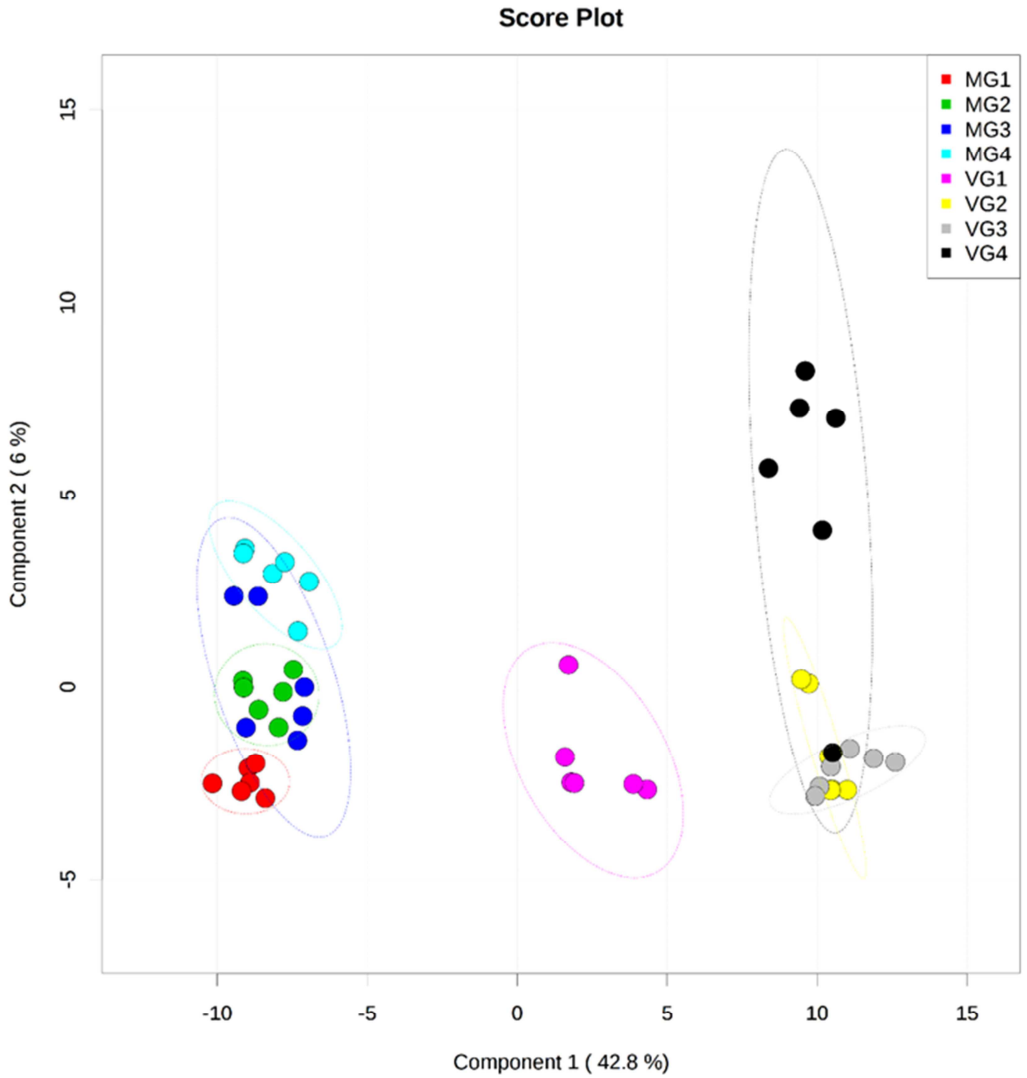
652 **Figure Legends:**

653 **Figure 1.** PLSDA analysis of metabolic profiles in roots of vetiver and maize exposed to lead for 10 days (n=6). M: maize V: vetiver.

654 G1: control; G2: 400mg/L; G3:800 mg/L; G4: 1200mg/L.

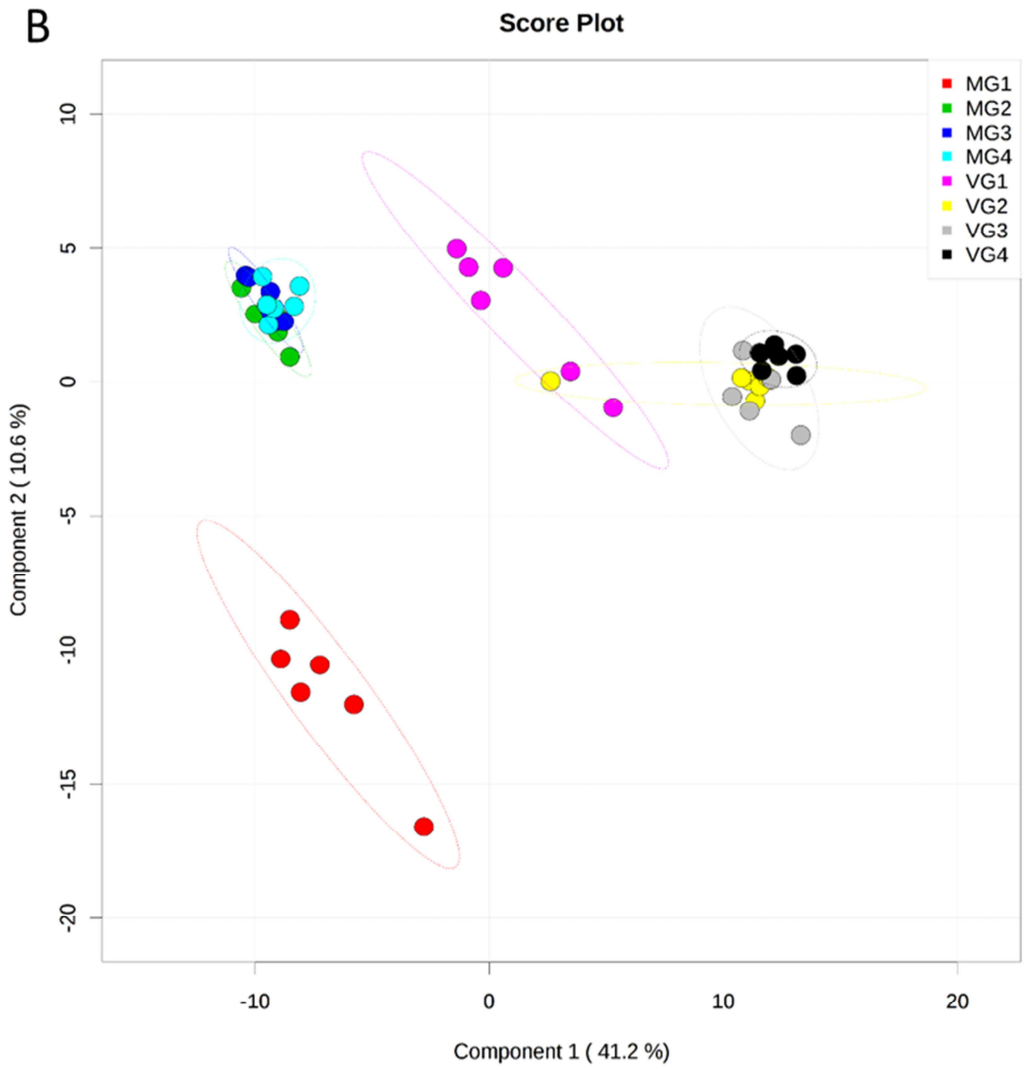
655 **Figure 2.** . PLSDA analysis of metabolic profiles in shoots of vetiver and maize exposed to lead for 10 days (n=6). M: maize V:

656 vetiver. G1: control; G2:400mg/L; G3:800mg/L; G4: 1200mg/L.


Score Plot
MG1
MG2
MG3
MG4
VG1
VG2
VG3
VG4
Component 2 (6 %)
15
10
5
0
-5
-10
-5
0
5
10
15
Component 1 (42.8 %)

657

Fig. 1.


B
Score Plot
MG1
MG2
MG3
MG4
VG1
VG2
VG3
VG4
Component 2 (10.6 %)
10
5
0
-5
-10
-15
-20
-10
0
10
20
Component 1 (41.2 %)

658

Fig. 2

Highlights

1. The study shows differences in metabolomics of vetiver and maize under lead stress
2. Massive increase in key metabolites was observed in response to lead.
3. Metabolites showing large increase include amino acids, organic acids and coenzymes
4. Maize showed modest increase in only a few of the same metabolites.