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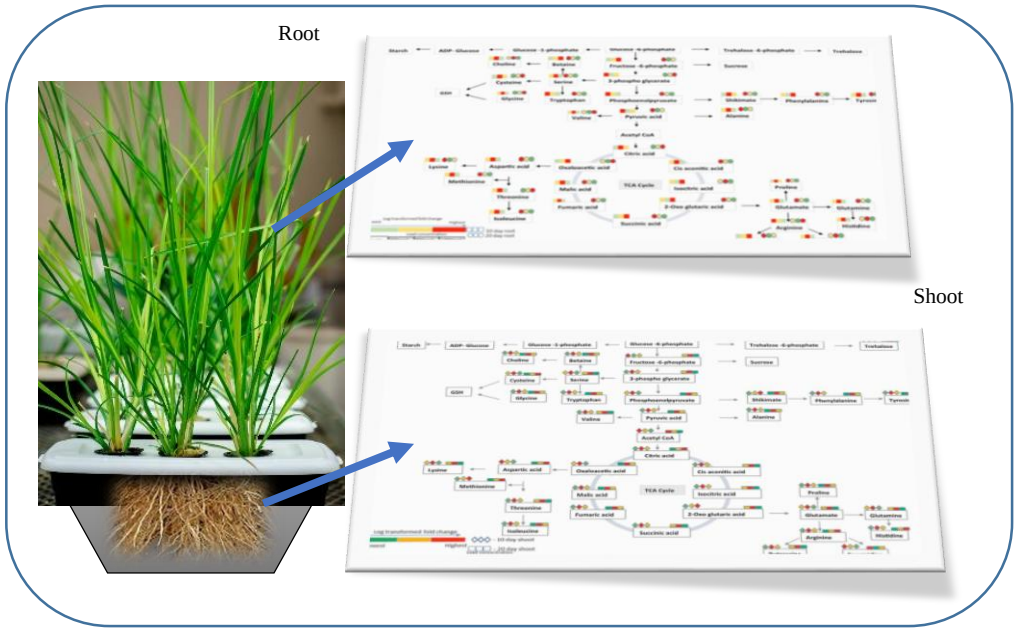
Root
Shoot

Identification of biochemical pathways associated with lead tolerance and detoxification in *Chrysopogon zizanioides* L. Nash (vetiver) by metabolic profiling

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Abstract

Lead (Pb) is a major urban pollutant, due to deteriorating lead-based paint in houses built before 1978. Phytoremediation is an inexpensive and effective technique for remediation of Pb-contaminated homes. Vetiver (*Chrysopogon zizanioides*), a non-invasive, fast-growing grass with high biomass, can tolerate and accumulate large quantities of Pb in their tissues. Lead is known to induce phytochelatins and antioxidative enzymes in vetiver; however, the overall impact of Pb stress on metabolic pathways of vetiver is unknown. In the current study, vetiver plants were treated with different concentrations of Pb in a hydroponic setup. Metabolites were extracted and analyzed using LC/MS/MS. Multivariate analysis of metabolites in both root and shoot tissue showed tremendous induction in key metabolic pathways including sugar metabolism, amino acid metabolism, increase in production of osmoprotectants like betaine and polyols and metal-chelating organic acids. The data obtained provides a comprehensive insight into the overall stress response mechanisms in vetiver.

Keywords: Phytoremediation, Lead, Vetiver, Metabolomics, LC-MS/MS

1. Introduction:

Lead (Pb) has been used since the ancient times; the earliest known article made of Pb dates back to 4000 B.C.[1] Lead was extensively used in batteries, pipes, solders, ammunition, paints, ceramic products etc[2]. Increased awareness of Pb toxicity has limited its use as leaded gasoline and in paints. In the United States, Pb-based paint was used to paint houses built before 1978[2]. Unless remediated, deteriorating paint in older homes is the main source of Pb contamination in residential soils. United States Environmental Protection Agency (U.S.EPA) has set the maximum acceptable limit of Pb in the soil as 400 mg/kg[2]. Nevertheless, extremely high concentrations of Pb have been reported in various residential and public places in a number of cities across the United States[3-5]. Exposure to Pb can cause a broad range of adverse health effects such as reproductive abnormalities, fetal death, neurological and behavioral disorders[6-8]. Moreover, studies show that children are more prone to Pb poisoning due to accidental ingestion and inhalation of Pb dust from the residential and play areas, and their gastrointestinal tract is more absorptive in comparison to adults and puts them at higher risk [11, 12]. Until 2012, 10 µg/dL of blood Pb levels (BLL) were considered acceptable, however new Centers for Disease Control and prevention (CDC) recommendations have reduced the number to 5 µg/dL, which brings more people under risk category [11].

Remediation of Pb in soil and water bodies is particularly challenging because Pb is strongly adsorbed to soil, and is not readily available for plant uptake[12]. Use of phytoremediation for Pb removal from residential soils is promising compared to the conventional methods which are expensive and environmentally destructive[13]. An ideal plant for phytoremediation should have high biomass, adaptability to various environmental conditions, fast growth, and hyperaccumulation capability. To date, several Pb hyperaccumulator plants have been identified including Indian mustard (*Brassica juncea*) (absorbs 3600mg/kg of Pb from soil[14], rattle bush (*Sesbania drummondi*) (absorbs 1500 mg/L of Pb in hydroponic conditions)[15] and sea purslane (*Sesuvium portulacastrum*) (absorbs 1409 mg/L of Pb in hydroponic conditions)[16].

Vetiver is a tall (1–2 m), fast-growing, perennial grass, with a massive root system (3–4 m). It is non-invasive and indigenous to more than 70 countries in the tropics and subtropics[17]. Thus, vetiver has all the ideal characteristics as a facultative Pb hyperaccumulator[18]. Due to its

tolerance and hyperaccumulation of various contaminants, it is emerging as a primary choice for phytoremediation[19, 20].

Little is known about the biochemical mechanisms responsible for high Pb tolerance in vetiver. Plants might synthesize various metabolites and signaling molecules to acclimatize and cope with the presence of heavy metals in their tissue. Antioxidants[21], osmoprotectants, plant hormones, organic acids, phenolic compounds, and signal transduction molecules are known to be produced in excess under metal stress conditions[22, 23]. In previous studies, we reported the induction of Pb-binding phytochelatins in response to Pb stress in vetiver[18]. We also reported enhanced antioxidant enzyme activity induced by Pb in vetiver tissues[23]. The recent technical advances in "omics" technology provide more robust and comprehensive approaches to dissect plant responses to abiotic stresses. Metabolomics, which measures hundreds of endogenous metabolites, is particularly attractive because it is not hampered by the absence of genome sequence information. Comparative metabolomic profiling is useful for understanding the physiological adaptations to certain stress conditions in plants.

The objective of the present study was to perform a detailed examination of the metabolic changes in vetiver due to exposure to Pb, and understand the major metabolic pathways involved in Pb tolerance in vetiver. The data obtained provides a comprehensive insight into the overall stress response mechanisms in vetiver, which is valuable for future biotechnological improvement in metal hyperaccumulating plants, especially when other genetic information are not yet available.

2. Materials and methods

2.1 Experimental set up:

Hydroponic experiments were setup as previously described by Andra et al. (2009)[17]. Briefly, vetiver plants were purchased from Florida Farms and Nurseries (FL, US). Plants were initially grown in 2-gallon plastic pots using commercial potting mix. Plants were acclimatized in soil for 3 months and then transferred to hydroponic medium containing Hoagland solution after

washing off the soil completely. Hoagland's solution for Pb treatment was prepared as previously described[17] without KH_2PO_4 to prevent lead phosphate complexes. Lead nitrate (Acros Organics, USA) was used as the Pb source, added at concentrations of 400 mg/L, 800 mg/L and 1200 mg/L, with control plants grown without Pb. These concentrations were chosen to represent USEPA standards for Pb in bare soil in play areas (400 ppm) and non-play areas (1200 ppm)[24]. Plant availability of soil Pb depends on soil physicochemical properties, and can be expected to be lower compared to the hydroponic media. We chose the above concentrations because (1) our earlier survey of residential soil in San Antonio and Baltimore uncovered some very high Pb levels in residential yards, with estimated plant-available Pb approaching 2000 ppm in some areas[25] (2) we have consistently used these concentrations to study the impact of Pb on vetiver growth, antioxidative responses and phytochelatin induction in vetiver[17, 20, 23, 31], and wanted to understand the metabolic profile using similar concentrations. Plant samples were harvested after 10 and 20 day periods. Six replicates were prepared for each treatment and each time point. Deionized water was used to prepare all the solutions and maintain the solution level in hydroponic tanks throughout the experiment. The hydroponic setup was maintained in a greenhouse at 25°C with a photoperiod of 16 h. The light intensity was kept at 250µmol m^{-2} s^{-1}, and the relative humidity was 60 ± 2%. Plant samples from various time points (0, 10, and 20 days) were washed with deionized water to remove traces of Pb and flash frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.2 Lead uptake in vetiver

Plant samples were dried in oven overnight at 85ºC, and 0.5 g of dried plant root and shoot tissue was digested separately with 4 mL HNO_3 and 2 mL of $HClO_4$. The plant-acid mixture was allowed to pre-digest overnight at room temperature, followed by heating on a hot plate at 125ºC. After 3 hours, the temperature was raised to 200ºC for 2 hours to allow the perchloric acid to further oxidize the plant material. The samples were allowed to cool and filtered. The filtrate was diluted with deionized water. Lead concentration was analyzed using atomic absorption spectrophotometer (AAS 3100, Perkin Elmer, USA) in flame mode (air-acetylene).

2.3 Metabolites extraction

Metabolites were extracted as previously described[26] with slight modification. Briefly, 100 mg of root and shoot samples were ground in liquid nitrogen separately. Six biological replicates were used in each group. Ampicillin (0.5 mg/mL) was added as internal standard prior to extraction. Three volumes of extraction buffer (methanol: acetonitrile (50:50) with 0.125% formic acid) were added to each sample. The samples were then sonicated for 15 min at 40 kHz in an ice-water bath. The samples were centrifuged at maximum speed (20,000 g) for 15 min at 4ºC. The supernatant was passed through 0.2 µm PTFE syringe filters and transferred to 1.6 mL glass vials for LC-MS/MS analysis.

2.4 LC-MS/MS conditions

LC-MS/MS analysis was performed on an ABSciex Qtrap 5500 mass spectrophotometer (Sciex, USA) equipped with a Turbo V electrospray ionization (ESI) source, Shimadzu LC-20A system, and a PAL CTC auto sampler. Twenty µM of 4-nitrophenol was added as the chromatographic standard prior to injection. Ten microliter of extract was injected onto a 250 mm × 2.1 mm, 5µm Luna NH_2 amino propyl column (Phenomenex, CA) held at 25°C for chromatographic separation. The mobile phase was solvent A: 95% water with 20mM ammonium acetate and 20mM NH_4OH (pH 9.4), solvent B: 100% acetonitrile [26, 27]. Separation was achieved using the following gradient: 0 min-95% B, 4 min-30% B, 19min-2% B, 22 min-2% B, 23 min - 95% B, 28 min-end. The flow rate was 300µl/min. All the samples were kept at 4°C during analysis. A total of 325 metabolites were targeted in multiple reaction monitoring (MRM) mode [27, 29]. Two injections were performed, one for negative mode (ESI-) and the other one for positive mode (ESI+). The dwell time was set at 5 ms. The compound-specific MS/MS parameters were optimized using the purified standards.

2.5 Data processing and statistical analysis

Peaks were manually reviewed and peak area of each metabolite was intergraded through Multiquant v3.0 (Sciex, USA). Metabolite peak areas were normalized with the peak area of the internal standard (ampicillin), chromatographic standard (4-nitrophenol) and fresh weight of tissues prior to statistical analysis. Statistical analysis was performed using MetaboAnalyst 2.0[30].

Missing values were imputed by KNN (k-nearest neighbor) method; data values were filtered by inter quartile range and were normalized by auto scaling. Partial least squares Discriminant Analysis (PLS-DA) was chosen for multivariate analysis. VIP score > 1.5 was considered as significance. One-way ANOVA followed by Tukey HSD test was performed to find the significance among different treatments using R software.

3. Results and Discussion:

3.1 Lead uptake by vetiver

Vetiver plants accumulated higher amounts of Pb in root tissue compared to shoot. Gradual increase in Pb accumulation was observed in roots as the concentration of the Pb increased from 400 mg/L to 1,200 mg/L in both time periods (Table 1). Roots accumulated more Pb at the 20-day period at 1200 mg/L concentration compared to 10-day period. Similarly, in shoot tissue, Pb accumulation increased from 400 mg/L to 800 mg/L at both time periods. However, at 1,200 mg/L concentration, Pb accumulation in shoots decreased in both 10 day and 20 day-old plants (Table 1). Lead uptake levels were very similar to the results from earlier studies reported from our lab [17, 31]. Impact of Pb on vetiver biomass is shown in Table 2. Lead had a marked impact on plant biomass, showing increased growth inhibition both with increase in Pb concentration and treatment period.

3.2 Metabolomic profiling of vetiver root

Overall, 325 metabolites were targeted in a single run covering the primary metabolic pathways in plants. Multivariate analysis was performed to identify the discriminating metabolites in vetiver in response to Pb stress; after which the metabolic network involved in Pb tolerance in vetiver was analyzed. To visualize the overall metabolic changes in both root and shoot tissue at different time periods and different treatments, PLSDA analyses of approximately 270 metabolites covering major metabolic pathways were performed (Figure 1, 2). Control tissues showed clear variance from metal treated tissues in both vetiver root and shoot. Different metal treatments showed component 1, 40.6% variance and component 2, 5.9% variance in 10-day root

(Fig. 1a). In 20-day root tissue (Fig. 1b), metal treatments were closely grouped with component 1 (29.2%) and component 2 (22.1%). In the case of shoot, control was distantly grouped compared with metal treatments (Fig. 2a, 2b), Treatments displayed are grouped in close proximity with variance component 1 and 52.1%, 5.8% and component 2 39.7% and 11.3% in 10 days and 20 days shoot respectively (Fig. 2a, 2b). Several metabolites in both 10 and 20-day root samples showed statistically significant differences among different concentrations of metal treatments. Tukey HSD test was used to find the significance between treatments (Tables S-1a, b).

Sugar metabolic pathways are the commonly affected pathways in various types of abiotic stresses, as soluble sugars function as signals in stress-induced pathways[32-34] as osmoprotectants during abiotic stress maintaining turgor pressure and stabilizing and protecting cell membrane in plants [33] and also as ROS (Reactive Oxygen Species) scavengers[35]. Primary carbohydrate metabolism is altered due to hydrolysis of sugar polymers induced by plant defense responses[36]. In our experiment, vetiver showed increase in concentration of soluble sugars such as hexose in treatments compared to control in the 400 mg/L Pb treatments, but showed a decreasing trend in both 10 day and 20 day root as the concentration of Pb increased (Table S-1). Abiotic stress affects the carbon assimilation process, by affecting the enzymes of Calvin cycle and activation of antioxidant enzymes and lipid oxidation[32]. Calvin cycle intermediates glyceraldehyde-3-phosphate and 3-phosphoglycerate are negatively impacted by stress in vetiver root and shoot tissue (Tables S-1 and S-2). In addition, metabolites of the TCA cycle decreased in all the treatments compared to control, among treatments in 20 day root tissue, but showed an increasing trend among the treatments in 10 day roots (Fig. 3).

Plants respond to various environmental stresses by altering structural and functional processes at the molecular level, including various secondary metabolites and amino acid pathways[37]. Accumulation of amino acids is commonly observed under various abiotic stress conditions[37]. Proline is a well-known stress related amino acid and its accumulation is attributed to functional roles such as osmolytic functions, ROS scavenging and acting as a molecular chaperone. Vetiver showed increasing concentrations of proline as the concentration of Pb increased in 10-day root, however decreased from 400 mg/L to 1200 mg/L Pb in 20-day root. Decreasing trend in

metabolite levels in 20 days root is likely to be caused by prolonged exposure to high concentrations of Pb. Proline is produced from glutamate, which in turn showed a decreasing trend as Pb concentration increased in root (Fig. 3). γ-Amino butyric acid (GABA) is a non-protein amino acid, a common metabolite that accumulates under conditions of stress[37]. Vetiver root showed increased concentration of GABA after 10 days as the Pb concentration increased and showed decreasing trend in the 20-day root. Metabolites such as alanine, betaine, serine, glutamate, spermidine, and threonine showed increasing trend in 10 days root tissue as the metal concentration increased from 0 mg/L to 800 mg/L, and decreased at 1,200 mg/L. However, all the above metabolites except spermidine showed increase in concentration in 400 mg/L compared to control, but showed a decreasing trend from 400 mg/L to 1,200 mg/L in 20-day root tissue.

3.3 Metabolomic profiling of vetiver shoot

As in the case of root tissue, metabolites in both 10 and 20 day shoot samples showed significant differences among treatments of various Pb concentrations. Tukey HSD test was used to find the significance (Table S-2a, b). Lead treatment affected the same metabolic pathways as in the case of the root tissue, in addition to photosynthesis. As expected, the majorly affected pathways include photosynthesis, carbohydrate metabolism, and amino acid metabolism. Abiotic stress affects the carbon assimilation process, by impacting the Calvin cycle, antioxidant and lipid oxidation enzymes[32, 33]. Vetiver shoot showed significant decrease (> 10-fold), in glycerate-3-phosphate indicating impact on Calvin cycle (Table S-2). TCA cycle metabolites exhibited mixed patterns, amino acids showed overall increase compared to control in both 10 and 20 day shoot, but showed an overall decreasing trend from 400 -1200 mg/L (Table S-2). Soluble sugar levels decreased in shoot; however, trehalose showed an increasing trend. Hexose sugars increased in 10 day shoot in treatments compared to controls, but decreased in 20-day shoot tissue (Table S-2).

Relative concentrations of metabolites in both 10 and 20 days plants treated with different Pb concentrations were compared with that of respective control plants and fold changes were log transformed and compared in the form of a heat map and depicted in relation to pathways (Fig. 3, 4). In our earlier studies, vetiver plant was shown to tolerate high amounts of Pb and

phytochelatins play an important role in tolerating Pb[17, 23]. Amino acids, glycine and cysteine which are constituents of phytochelatins as well as, glutathione concentrations showed increasing trend in both root and shoot as the Pb concentration increased in 10 and 20 day root (Fig. 3). In general, 10 days root showed increasing trend of metabolites directly or indirectly related to metal stress such as proline, cysteine, glycine, glutamate, and histidine. Whereas 20 days root showed a mixed pattern, mostly increasing from 400 to 800 mg/L and decreasing in 1200 mg/L treatment, most likely due to toxicity of high Pb concentration.

3.4 Metabolic flux induced by abiotic stress

Several metabolic profiling studies have been carried out to illustrate various abiotic stress responses, such as drought, salinity, cold and heat in plants over the last decade. However, only a handful of very recent studies are available on heavy metal stress response in plants. Cadmium-treated *Silene cucubalus* cell cultures showed increase in glucose, malic acid and acetate, and a decline in glutamate and branched chain amino acids[38]. In the halophyte *Suaeda salsa*, treated with 20 μg/L Pb, increased accumulation of isocaproate, glucose and fructose, and decreased malate, citrate and sucrose were detected[39]. Since isocaproate is derived from leucine under anaerobic conditions, the authors speculated increase in anaerobic respiration under Pb stress. Decrease in levels of malate and citrate indicated decreased TCA cycle and disturbance in energy metabolism[39]. On the other hand, when *S. salsa* plants were exposed to 100 μg/L zinc, while there were several similarities in the metabolic profile with that of Pb treated plants, there were certain differences, such as increased phosphocholine and betaine, and decreased choline. Betaine is an osmolyte derived from choline, which is likely to be an adaptation to osmotic stress.

A study with two genotypes of bermuda grass; the tolerant WB242 and the less tolerant WB144, treated with 1.5 mM cadmium for 14 days reported that metabolites including amino acids (norvaline, glycine, proline, serine, threonine, glutamic acid and gulonic acid), organic acids (glyceric acid, oxoglutaric acid, citric acid and malic acid) and sugars (xylulose, galactose and talose) accumulated more in the tolerant genotype[40]. The authors attributed the enhanced tolerance of genotype WB242 to the increased accumulation of the above metabolites.

A recent quantitative metabolomic study of maize roots exposed to 400 mg/L Pb for a 7 day period showed an increase in organic acids and amino acids such as citric, malic and oxalic acids, proline, and polyamine, which are likely to be involved in metal chelation[41]. In addition, ascorbic acid, urate, and glutathione were accumulated which are likely to help in combating oxidative stress[41].

Schneider et al.[42] investigated the mechanism of Zn hyperaccumulation in leaf epidermal and mesophyll tissues of *Noccaea caerulescens*. They reported that epidermal cells showed high concentrations of malate and citrate, which could play a role in Zn complexation. Daş et al.[43] studied *Nicotiana tabaccum* plants with medium (10 μM) and high (100 μM) concentrations of Zn, and reported that while medium Zn concentration could be responsible for activating a GABA-shunt, which helps initiate various protective mechanisms in plants; high Zn concentrations result in the activation of programmed cell death. Zhao et al.[44] have recently performed metabolic profiling studies on nano-copper toxicity in cucumber plants, and reported that exposure to nano-Cu resulted in several interesting metabolic changes, such as up-regulation of amino acids, ascorbic acid and phenolic compounds to sequester Cu, reduce ROS-related stress and improve antioxidant response.

The current study is the first metabolomic profiling of Pb induced stress in vetiver plants, and was done in a comprehensive manner, using environmentally relevant concentrations of Pb. Although it is well known that a major fraction of soil Pb remains tightly bound, and is not plant-available, the experiments were designed to study the response of vetiver to Pb concentrations similar to our earlier studies[17, 20, 23, 31]. Tremendously high levels of several metabolites were induced, including sugars (hexoses, sucrose, trehalose) in root and shoot tissues (Tables S-1 and S-2). Similarly, very high levels of amino acids (proline, histidine, alanine, glutamate and glutamine) were induced in both root and shoot tissues (Tables S-1 and S-2). In addition, very high levels of betaine were induced in root and shoot. Organic acids (malic acid, ascorbic acid, cis-aconitic acid, aspartic acid) also increased in all tissues. Interestingly, there was a large increase in AMP levels, possibly indicating decline in energy metabolism. Other metabolites that showed large induction include dihyroorotic acid (a precursor of pyrimidine metabolism) and 4-aminobutyrate (GABA, a derivative of polyamine putrescine), reported to be induced widely

during both abiotic and biotic stress responses. GABA is involved in stress signaling, and a shift in C:N ratio, indicating enhanced oxidative stress response, or a shift in proteomic profile due to increased expression of stress-associated proteins[45-48]. The cyclic polyol, myo-inositol also showed a large induction. Myo-inositol reportedly accumulates in halotolerant species due to salt stress[47]. In addition, glycerophosphocholine, phosphoryl choline and choline levels increased, possibly due to phospholipid degradation as a result of the negative impact of ROS on membranes[47]. Decrease in the levels of several metabolites were seen in 20 day tissues at the highest Pb treatments, possibly due to the extreme toxicity of Pb. A major metabolite that declined in shoot tissue is glycerate-3-phosphate, which indicates effect on photosynthesis.

While a large induction in the metabolites in the above pathways was observed, a comparison of vetiver with a non-accumulating plant would further illustrate the differential response of vetiver with susceptible plants. Further investigations are ongoing in our lab to fully elucidate the differences between metabolic profiles of the hyperaccumulator vetiver and a non-accumulator plant.

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Supplemental Information: Relative concentrations of metabolites in vetiver root and shoot tissues treated with Pb 10 and 20 days, with fold changes.

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465

466 Table 1. Lead uptake in vetiver root and shoot tissue after 10 and 20 days (n =3). Metal
467 accumulation was analyzed using ICP-MS.

Time period (days)	**Tissue**	**Concentration of Pb used in treatments (mg/L)**	**Pb uptake (mg/kg dry wt.) ± Std.dev**
10	Root	0	0
10	Root	400	4.49×10^{3} ± 221
10	Root	800	1.16×10^{4} ± 314
10	Root	1200	2.01×10^{4} ± 213
20	Root	0	0
20	Root	400	3.79×10^{3} ± 128
20	Root	800	1.12×10^{4} ± 320
20	Root	1200	3.24×10^{4} ± 234
10	Shoot	0	0
10	Shoot	400	176 ± 32
10	Shoot	800	526 ± 113
10	Shoot	1200	332 ± 45
20	Shoot	0	0
20	Shoot	400	421 ± 47
20	Shoot	800	1.45×10^{3}1,450.12 ± 145
20	Shoot	1200	475 ± 34

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Table 2: Biomass changes in Vetiver treated with various concentrations of lead after 10 and 20 days. + = increase in weight compared to time 0, - = loss of weight compared to time 0. (n=6)

Lead concentration (mg/L)	Time period (days)	Avg.biomass change (gm)± standard deviation
0	10	+0.89± 0.15
	20	+2.33 ± 0.23
400	10	-1.61± 0.40
	20	-4.70 ±0.33
800	10	-5.87± 0.81
	20	-11.36 ± 1.31
1200	10	-9.02± 0.91
	20	-14.12 ± 1.91

Figure Legends:

Figure 1: PLS-DA plot showing variance among treatment groups of vetiver roots in response to Pb. a: 10 day; b: 20 day. G1: control group: G2: 400 mg/L Pb; G3: 800 mg/L: G4: 1200 mg/L. (n=6).

Figure 2: PLS-DA plot showing variance among treatment groups of vetiver shoot in response to Pb. a: 10days; b: 20days. G1: control group: G2: 400 mg/L Pb: G3: 800 mg/L: G4: 1200 mg/L. (n=6).

Fig 3: Pathway representation and heat map of metabolite changes in root tissue. Fold change in metabolites are calculated with respect to that of control, log transformed the fold change values, compared among treatments and assigned color based on the value from green to red. Green represents lowest, yellow represents median, and red represents the highest. Squares represent 10 day and circles represent 20 day samples.

Fig 4: Pathway representation and heat map of metabolite changes in shoot tissue. Fold change in metabolites are calculated with respect to that of control, log transformed the fold change values, compared among treatments and assigned color based on the value from green to red. Green represents lowest, yellow represents median, and red represents the highest. Diamond shapes represent 10 day and rectangles represent 20 day samples.

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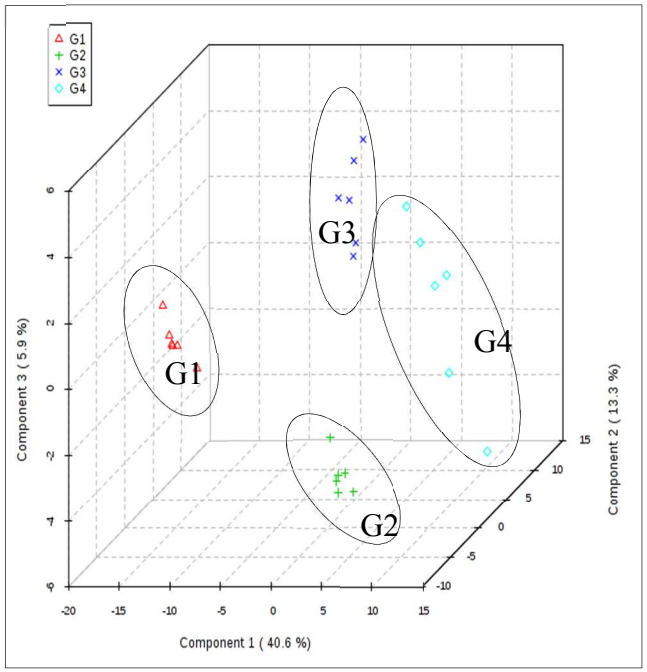
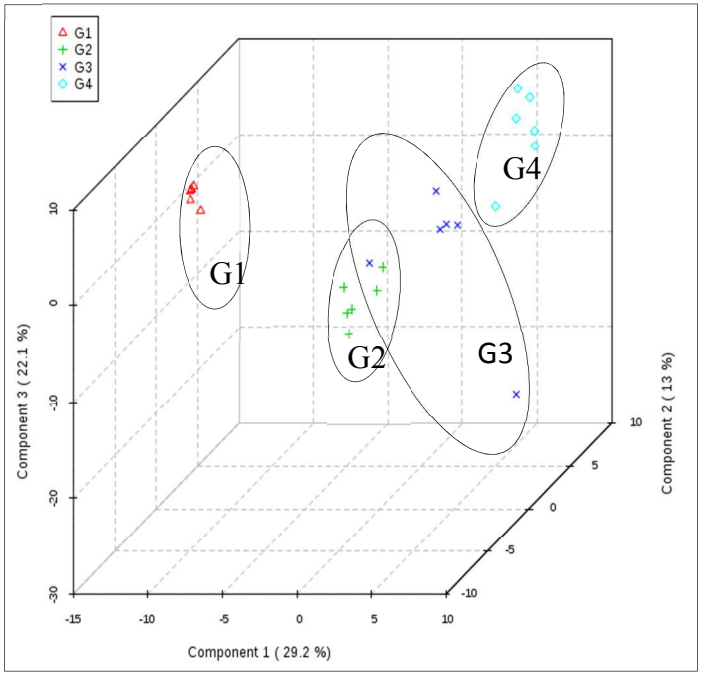
521 **Figure 1:**

a.



b.



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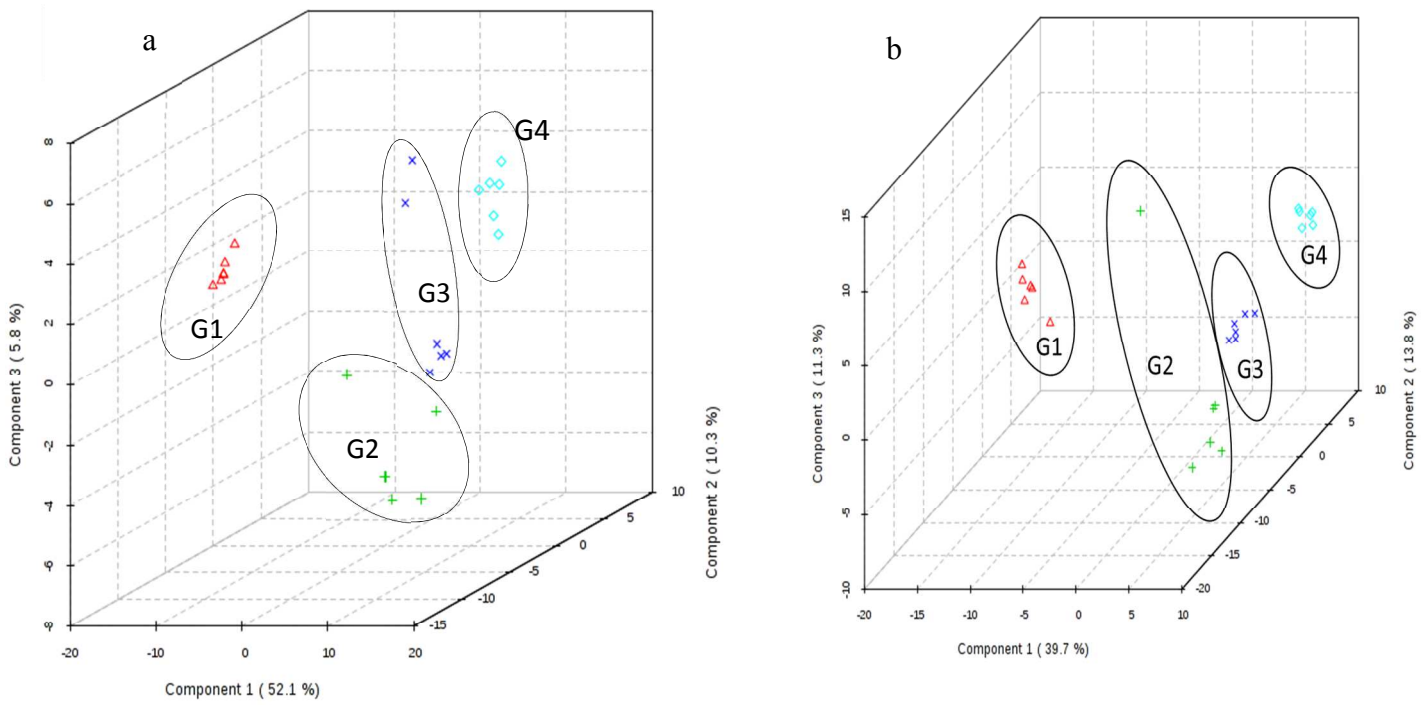
526 **Figure 2**:



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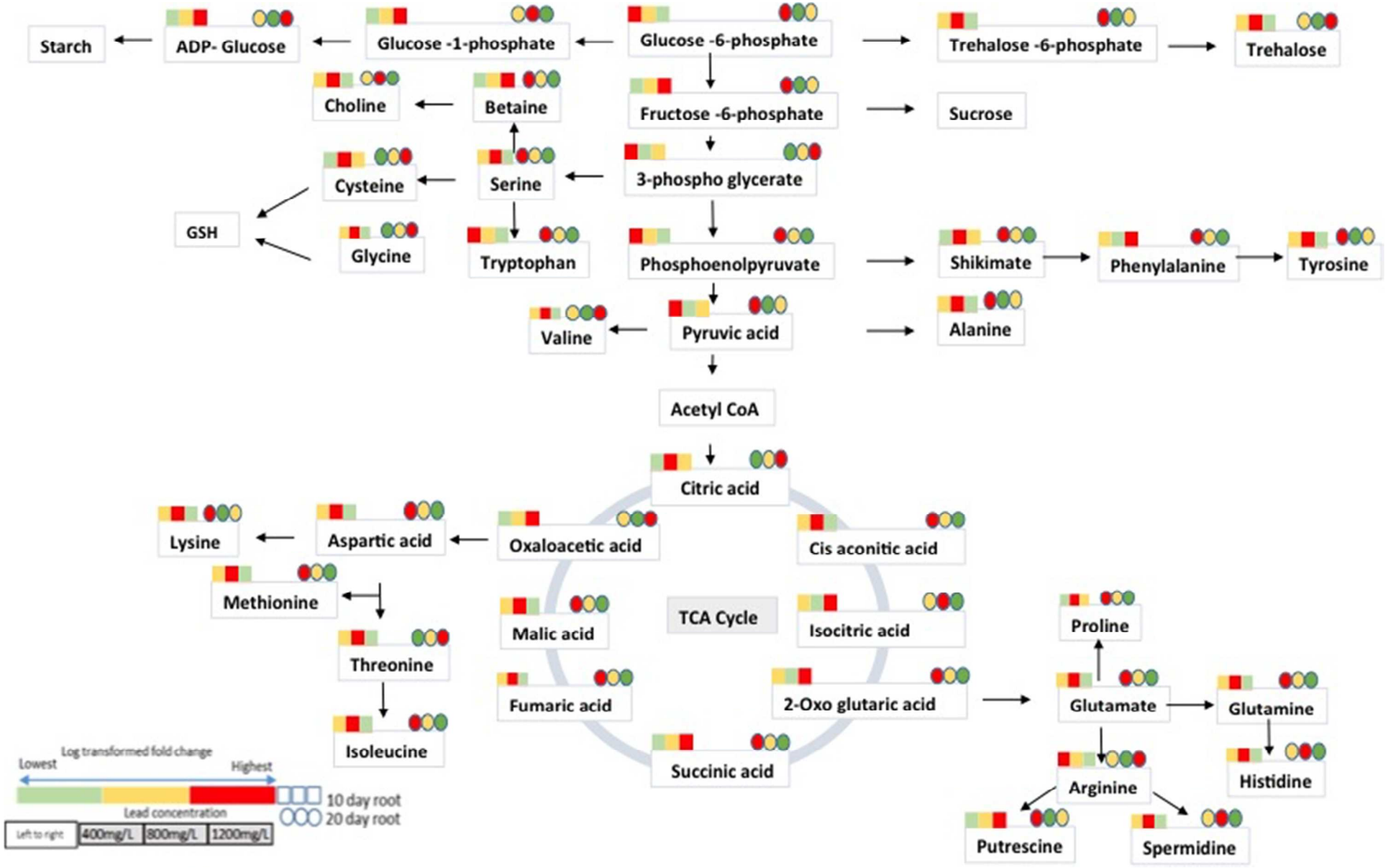
Starch
ADP- Glucose
Glucose -1-phosphate
Glucose -6-phosphate
Trehalose -6-phosphate
Trehalose
Choline
Betaine
Fructose -6-phosphate
Sucrose
Cysteine
Serine
3-phospho glycerate
GSH
Glycine
Tryptophan
Phosphoenolpyruvate
Shikimate
Phenylalanine
Tyrosine
Valine
Pyruvic acid
Alanine
Acetyl CoA
Citric acid
Lysine
Aspartic acid
Oxaloacetic acid
Cis aconitic acid
Methionine
Malic acid
TCA Cycle
Isocitric acid
Proline
Threonine
Fumaric acid
2-Oxo glutaric acid
Glutamate
Glutamine
Isoleucine
Succinic acid
Arginine
Histidine
Putrescine
Spermidine
Log transformed fold change
Lowest
Highest
Lead concentration
Left to right
400mg/L
800mg/L
1200mg/L
10 day root
20 day root

529 **Figure 3:**

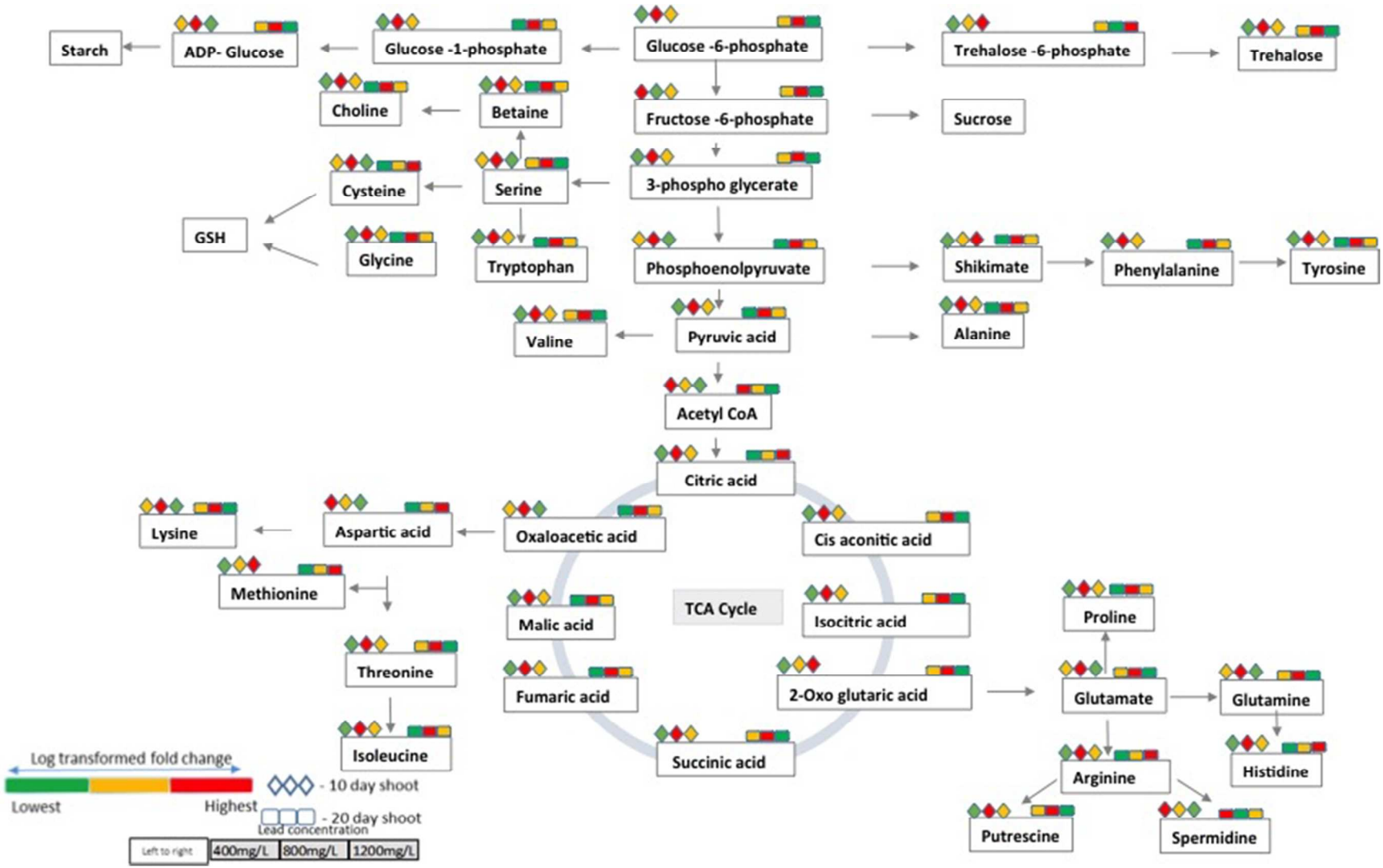
Starch
ADP- Glucose
Glucose -1-phosphate
Glucose -6-phosphate
Trehalose -6-phosphate
Trehalose
Choline
Betaine
Fructose -6-phosphate
Sucrose
Cysteine
Serine
3-phospho glycerate
GSH
Glycine
Tryptophan
Phosphoenolpyruvate
Shikimate
Phenylalanine
Tyrosine
Valine
Pyruvic acid
Alanine
Acetyl CoA
Citric acid
Lysine
Aspartic acid
Oxaloacetic acid
Cis aconitic acid
Methionine
Malic acid
TCA Cycle
Isocitric acid
Proline
Threonine
Fumaric acid
2-Oxo glutaric acid
Glutamate
Glutamine
Isoleucine
Succinic acid
Arginine
Histidine
Putrescine
Spermidine
Log transformed fold change
Lowest
Highest
- 10 day shoot
- 20 day shoot
Lead concentration
Left to right
400mg/L
800mg/L
1200mg/L

Figure 4: