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Mycorrhiza and heavy metal resistant bacteria enhance growth, nutrient uptake and alter metabolic profile of sorghum grown in marginal soil

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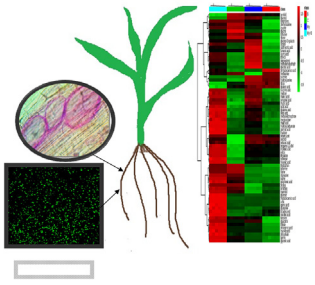
HIGHLIGHTS

- Heavy metal resistant bacteria increased sorghum biomass grown in marginal soil.
- Arbuscular mycorrhiza enhanced uptake of most elements by sorghum.
- Dynamic changes in host metabolic pathways regulated by mycorrhiza and PGPB.

GRAPHICAL ABSTRACT



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ABSTRACT

The main challenge for plants growing in nutrient poor, contaminated soil is biomass reduction, nutrient deficiency and presence of heavy metals. Our aim is to overcome these challenges using different microbial combinations in mining-impacted soil and focus on their physiological and biochemical impacts on a model plant system, which has multiple applications. In the current study, sorghum BTx623 seedlings grown in mining-impacted soil in greenhouse were subjected to plant growth promoting bacteria (PGPB or B) alone, PGPB with arbuscular mycorrhizal fungi (My), My alone and control group with no treatment. Root biomass and uptake of most of the elements showed significant increase in all treatment groups in comparison with control. Mycorrhiza group showed the best effect followed by My + B and B groups for uptake of majority of the elements by roots. On the contrary, biomass of both shoot and root was more influenced by B treatment than My + B and My treatments. Metabolomics identified compounds whose levels changed in roots of treatment groups significantly in comparison to control. Upregulation of stearic acid, sorbitol, sebacic acid and ferulic acid correlated positively with biomass and uptake of almost all elements. Two biochemical pathways, fatty acid biosynthesis and galactose metabolism, were regulated in all treatment groups. Three common pathways were upregulated only in My and My + B groups. Our results suggest that PGPB enhanced metabolic activities which

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resulted in increase in element uptake and sorghum root biomass whether accompanied with mycorrhiza or used solely.

1. Introduction

The utilization of marginal lands are increasingly being considered as crucial for the production of the second generation of bioenergy crops, which can provide a viable alternative to the use of prime agricultural land (Kang et al., 2013). However, plants grown in these soils have reduced biomass and nutrient deficiency (Brown and Chaney, 2016). Deficiency of nitrogen, phosphorus, and other macro- and micronutrients in soil results in significant loss of plant productivity and affects the quality and quantity of biomass (Shanker et al., 2005). Several studies have reported on the role of microbial interactions in improving soil rhizosphere, availability of macro and micronutrients and increase in plant tolerance (Li et al., 2014; Augé et al., 2014).

Sorghum is a C4 plant with a wide range of adaptations and resistance to several adverse biotic and abiotic factors (Serna-Saldívar et al., 2012). Sorghum is a major staple food crop in many developing countries in the semi-arid tropics of Africa, Asia and Central America and is now a biofuel source in countries such as the U.S., with 30% grain use for ethanol production (Serna-Saldívar et al., 2012). In addition, sorghum growing in heavy metal contaminated soil showed phytoextraction ability (Zhuang et al., 2009). Significant increase in heavy metal tolerance, mycorrhizal colonization, shoot length and total biomass was reported when sorghum was inoculated with plant growth promoting bacteria (PGPB) (Duponnois et al., 2006). Also, the use of PGPB in heavy metal contaminated and nutrient poor soil increased plant growth and metal tolerance of maize (Li and Ramakrishna, 2011).

Earlier studies have demonstrated that the use of a combination of mycorrhiza and PGPB confer several positive effects on plants. These include an increase of mycorrhizal symbiosis, decrease in plant disease symptoms (Jäderlund et al., 2008) and increase in plant biomass (Rajesh Kannan et al., 2011). To the best of our knowledge, the relation between microbial interactions and sorghum biomass, nutrient uptake and metabolic changes has not been investigated. Metabolomics can provide a glimpse of dynamic changes in metabolic pathways in the host plant regulated by mycorrhiza and PGPB. In order to study the influence of microbial interactions on sorghum growth, nutrient uptake and metabolic profile, sorghum roots were subjected to three treatments: mycorrhizal mix, mycorrhiza + PGPB, and PGPB alone. The changes in biomass, element uptake and metabolites were investigated in comparison with untreated group (control).

2. Material and methods

2.1. Plant growth conditions and treatments

The effect of PGPB and mycorrhiza on sorghum biomass and nutrient uptake was studied in marginal soils collected from Lake Linden (Upper Peninsula, Michigan). Two day old germinated seedlings of sorghum BTx623 (USDA) were planted in the greenhouse (30 °C and 65% humidity). Each pot was filled with 600 g of pasteurized soil. Sorghum seedlings subjected to different treatments were classified as the following groups: C (stamp sand with dead inoculum), B (*Pseudomonas* sp. TLC 6-6.5-4), My: mycorrhizal mix, and My + B (*Pseudomonas* sp. TLC 6-6.5-4 + mycorrhizal mix). The PGPB used in this study was isolated from Torch Lake core rich in copper and was found to be resistant to multiple heavy metals and promoted maize growth (Li and Ramakrishna, 2011). The seeds were incubated for an hour for each inoculum before germination and sprayed on the soil surface after germination. Group C represents plants inoculated with 9 g of pasteurized mycorrhiza inoculum powder dissolved in 120 ml of 0.85% NaCl solution. *Pseudomonas* sp. TLC 6-6.5-4 was transformed with rhizosphere stable plasmid pPROBE-GTkan (Miller et al., 2000) according to the transformation protocol described by Krzyzanowska et al. (2012). *Pseudomonas* sp. TLC 6-6.5-4 with *gfp* was grown at 30 °C for 48 h in LB broth supplemented with kanamycin. The bacterial pellet was collected by centrifugation at 6000 rpm for 10 min. Group B represents plants inoculated with 120 ml of *Pseudomonas* sp. TLC 6-6.5-4 (harboring *gfp*) bacterial suspension (10^8 cfu/ml) which was sprayed on the soil surface. Group My represents plants inoculated with 9 g of mycorrhizal mix containing *Glomus intraradices*, *Glomus mosseae*, *Glomus aggregatum*, and *Glomus etunicatum* (100,000 propagules/lb) (Valentine Country Inc., ND, USA), dissolved in 0.85% NaCl. Group My + B represents plants treated with both PGPB and mycorrhiza.

2.2. Plant analyses

Sorghum plants were harvested after 90 days for various analyses. Total chlorophyll content was estimated by atLEAF digital chlorophyll meter. Root and shoot fresh weight was recorded. These samples were dried at 70 °C for 24 h for measuring dry weight biomass. Plant biomass and nutrient uptake was carried out as described below (Motsara and Roy, 2008) and extraction of metabolites was performed according to Lisec et al. (2006). Uptake of the following elements: P, K, Ca, Mg, S, Zn, Mn, Cu, Fe and Al, in roots and shoots was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) to evaluate the effect of different treatments. Biomass and element uptake were evaluated using three replicates for each treatment. Metabolites extraction was performed using a protocol modified from Fiehn (2006) and analyzed by ALEX-CIS GC-TOF MS (West Coast Metabolomics Center, University of California, Davis) with three replicates for each group. Rtx5Sil-MS column (Restek Corporation, USA, 30 m length x 0.25 mm internal diameter with 0.25 μm film made of 95% dimethyl/5% diphenylpolysiloxane) was used with ribitol as an internal standard. (Fiehn et al., 2008). Metabolite data was acquired at 50–330 °C with 1 ml min^{-1} and 0.5 μL injection volume. Helium was used as the mobile phase.

2.3. Localization of mycorrhiza and PGPB

The growth of *Pseudomonas* sp. TLC 6-6.5-4 harboring the rhizosphere stable plasmid pPROBE-GTkan tagged with GFP was evaluated in the presence and absence of kanamycin to estimate the added PGPB only and rhizospheric bacteria plus PGPB, respectively. Mycorrhiza images were taken with a 40 DIC (0.65–100/0.17) trinocular microscope (Nikon, Optiphot, Japan) using digital camera Nikon E 8800 with 10× optical zoom. Successful GFP tagging was identified by visualizing labeled *Pseudomonas* sp. TLC 6-6.5-4 in LB agar with transilluminator (Dark Reader, Clare Chemical Research)

light (Fig. 1a). Additionally, *Pseudomonas* sp. TLC 6-6.5-4 labeled with GFP was examined using confocal microscope (Olympus FluoView FV1000, Imaging Core Facility, Chemistry Department, Michigan Tech University) with excitation at 490 nm and emission at 510 nm (Fig. 1b). The mycorrhiza was localized using root sections stained with trypan blue (0.05%) or lactofuchsin (Vierheilig et al., 2001; Vierheilig et al., 2005). The total microbial count was determined for labeled and non-labeled bacterial community at the end of the experiment (90 days). Rhizosphere soil was collected by shaking maize roots in a plastic bag (~0.5–1 cm around roots). Soil suspension was prepared with 1 g of collected soil and 9 ml of sterile saline (0.85% NaCl). The suspension was serially diluted until reaching 10^{-8} and plated on LB agar media to count rhizospheric bacteria. Part of the LB agar was supplemented with kanamycin to track labeled PGPB growth when used alone or with mycorrhiza. The pH and electric conductivity was determined in a 1:5 (w/v) soil/water extract. EC was measured using HM Digital EC-3 Electrical Conductivity meter. Microbial dehydrogenase activity in soil was estimated using 2, 3, 5-triphenyltetrazolium chloride test (Singh and Kumar 2008).

2.4. Statistical analyses

Analysis of Variance (ANOVA) was performed on biomass and element uptake data using SPSS 17. Multivariate analyses was performed with Partial Least Squares-Discriminant Analysis (PLS-DA) using MetaboAnalyst on normalized metabolites (Xia et al., 2012) to determine significant metabolites in each group relative to control. Fold change was calculated from the change of metabolites produced in comparison to control (treatment/control). Significant compounds were subjected to integrating enrichment analysis and pathway topology analysis to determine their involvement in metabolic pathways.

3. Results

3.1. Mycorrhizal colonization of roots and changes in rhizosphere microbial activity

Soil subjected to pre-experiment analysis showed 2.9 mg/kg Cu and Zn and 4.9 mg/kg Fe. The level of Al was 185 mg/kg, which is considered non-toxic at pH $\geq$ 7. Heavy metals in the Lake Linden soil were below the levels considered to be toxic to plants (<20 mg/kg) (Singh et al., 2011). Sorghum roots were successfully colonized by arbuscular mycorrhiza, as indicated by the presence of intraradical vesicles in cortical root cells whether used alone (Fig. 1c) or with PGPB (Fig. 1d). Rhizosphere soil characteristics including pH and electric conductivity (EC) showed significant changes in different groups (Table 1). While rhizosphere soil in B group showed significantly lower pH in comparison to the C group, EC of B and My + B groups was significantly higher than My and C groups. The soil rhizosphere dehydrogenase increased in all three treatment groups but the increase was significant only in My + B group. The total bacterial cell count was significantly different between control and B group but not when mycorrhiza was added, in My and My + B groups. The growth of labeled PGPB showed no significant difference between B and My + B groups (data not shown). The microbial activity increased, as indicated by a significant increase in rhizospheric dehydrogenase activity in all of the three groups relative to control. The chlorophyll content of sorghum plants increased in all three treatment groups compared to control but the increase was not statistically significant (Table 1).

3.2. Microbial interactions enhanced sorghum biomass

Shoot and root biomass increased significantly for all the treatments in comparison to control. The average shoot fresh

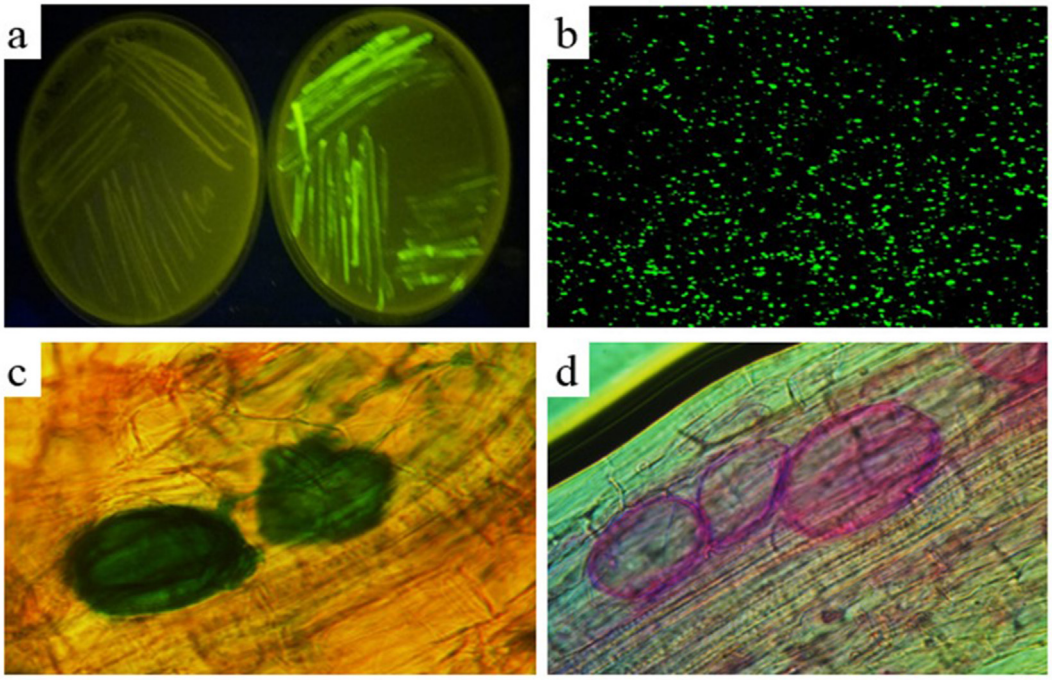

Fig. 1. Visualization and detection of microorganisms responsible for enhancing plant biomass and element uptake. a) *Pseudomonas* sp. TLC 6-6.5-4 grown for 48 h on LB agar visualized with a transilluminator. The petri dish on the left has wild type bacteria whereas the one on the right side has GFP tagged bacteria). b) GFP tagged *Pseudomonas* sp. TLC 6-6.5-4 photographed with 60× confocal microscope with excitation wavelength of 490 nm and emission wavelength of 510 nm. Sorghum roots stained with c) trypan blue and d) fuchsin. The images showed root cortical cells with stained intraradical vesicles following 90 days of treatment with (c) mycorrhiza or (d) PGPB + mycorrhiza.

Table 1
Electric conductivity (EC), pH, dehydrogenase activity, plant chlorophyll and soil bacterial growth following 90 days of treatment.

Treatments	pH	EC (dS/m)	Dehydrogenase (μg/g)	Bacterial growth (cfu)	Chlorophyll (μg/cm^2)
Control	7.34 ± 0.04a	123.3 ± 17b	46.3 ± 5.3b	2500 ± 408b	16.36 ± 2.33a
My	7.47 ± 0.06a	136.7 ± 9.43b	55.7 ± 3.4b	3866.7 ± 449a,b	17.33 ± 0.82a
My + B	7.37 ± 0.04a	783.3 ± 65.5a	134.3 ± 46.5a	4233.3 ± 713a,b	18.96 ± 0.69a
B	7.06 ± 0.03b	640 ± 71.2a	57.3 ± 4.9b	4500 ± 637a	19.23 ± 1.76a

C: control; B: PGPB; My: mycorrhiza; My + B: mycorrhiza with PGPB. All the values are mean of three replicates ± standard deviation (SD). Analysis of variance (one-way ANOVA) followed by Tukey's test. Values in rows in each column indexed by different letters indicate statistical significance ($p < 0.05$).

weight was 10-fold for B, 9-fold for My + B and 6.5-fold for My groups and shoot dry weight was 12-fold for B, 7.5-fold for My and 6-fold for My + B groups in comparison to control (Fig. 2a). While fresh root weight was 47-fold for B, 23-fold for My and 18-fold for My + B groups, dry root weight was 28-fold, 15.5-fold and 15-fold for B, My and My + B groups, respectively (Fig. 2b). Similar trend was noticed in shoot and root biomass increase where B group showed the highest increase followed by My and My + B groups. In addition, the fold increase in biomass was higher in roots compared to shoots.

3.3. Differential uptake of macronutrients

A significant increase was noticed in the levels of primary macronutrients (P, K) in roots where phosphorus levels ranged from 2-fold in B to 5.6-fold in My (Fig. 3b), while P in shoot increased significantly maintaining a consistent level (about 1.4-fold) in all of the groups compared to control (Fig. 4b). Root potassium levels showed a similar trend as P, ranging from 2-fold in B to 5-fold in My (Fig. 3a), while shoot K was 2-fold for the three treatment groups in comparison with control (Fig. 4c). The fold increase as well as the normalized levels of primary macronutrients in treated groups were much higher in roots than in shoots.

The levels of secondary macronutrients, Ca, Mg and S increased significantly in root and shoot of all the groups but showed different trends. Root Ca levels ranged from 3-fold in My + B group to 5.5-fold in My group compared (Fig. 3a) to shoot Ca levels which ranged from 6-fold in B group to 9-fold in My group (Fig. 4c). Root Mg was higher in My + B group (5-fold) than My (3-fold) and B (2-fold) groups (Fig. 3a). However, shoot Mg levels ranged from 6-fold in My + B group to 9-fold in B group (Fig. 4c). Root S levels ranged from 4-fold in B and My + B groups to 6-fold in My group whereas shoot S levels ranged from 3-fold in B and My groups to 4-fold in My + B group in comparison with control (Fig. 4b). Overall, secondary macronutrients showed higher normalized levels in roots than shoots but unlike primary nutrients, the fold increase (except S) in treated groups was higher in shoots than in roots.

3.4. Microelements and heavy metal uptake

The uptake of micronutrients, Mn, Fe, Cu and Zn increased significantly in both shoots and roots of treated groups. In roots, Mn levels were 3-fold higher in B and My groups and 10-fold higher in My + B group (Fig. 3b), while their levels in shoots were constant (about 9-fold) in all of the groups (Fig. 4a) compared to control. Root Fe levels were 3-fold higher in My and B groups and 5-fold higher in My + B (Fig. 3a), while shoot Fe increased from 4-fold in B group to 6-fold in My group (Fig. 4a) compared to the control group. Copper levels were about 3-fold higher in B and My + B groups, and 4-fold higher in My group in roots (Fig. 3b) whereas their shoot levels increased from 4-fold in B group to 9-fold in My group (Fig. 4a). Root Zn increased from 5-fold in B group to 6.5-fold in My group (Fig. 3c), while shoot Zn increased from 3-fold in B group to about 5-fold in My + B group (Fig. 4a). Micronutrients showed higher normalized levels in roots than shoots like macronutrients, but there was no predominant pattern of fold increase in roots or shoots. Similarly, root Al showed significant increase from 2.5-fold in B group to 5-fold in My + B group (Fig. 3a), while shoot Al varied from 2-fold in B group to 2.5-fold in My + B and My groups compared with control (Fig. 4a).

3.5. Effect of microbial treatments on sorghum root metabolites

Sorghum root metabolic profiling detected 282 metabolites, out of which 106 compounds were identified using KEGG database (http://www.genome.jp/kegg/pathway.html). Seventy top metabolites are shown in Fig. S1. The principal component analysis (PCA) scores plot showed a clear separation between the metabolic profiles of the three treatments: B, My and My + B, and the control (Fig. S2). The results showed 38 metabolites commonly upregulated in all of the treatment groups compared to the control. A total of 48 metabolites increased in B group with 43 overlapping with

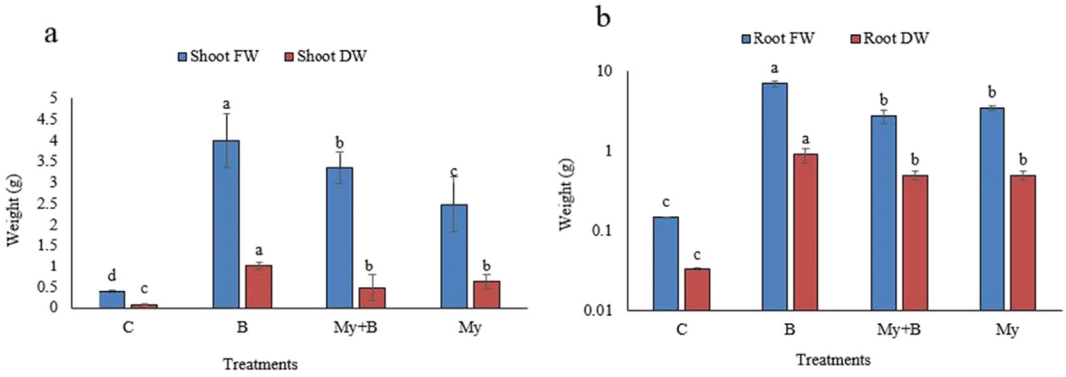

Fig. 2. Sorghum biomass increased after 90 days of treatment in (a) shoot and (b) root. C: control, B: PGPB, My: mycorrhiza, and My + B: mycorrhiza with PGPB. FW: fresh weight, DW: dry weight. Data represent mean ± SD ($n = 3$). Different letters indicate significant difference among the groups according to ANOVA ($p \leq 0.05$) followed by Tukey's test.

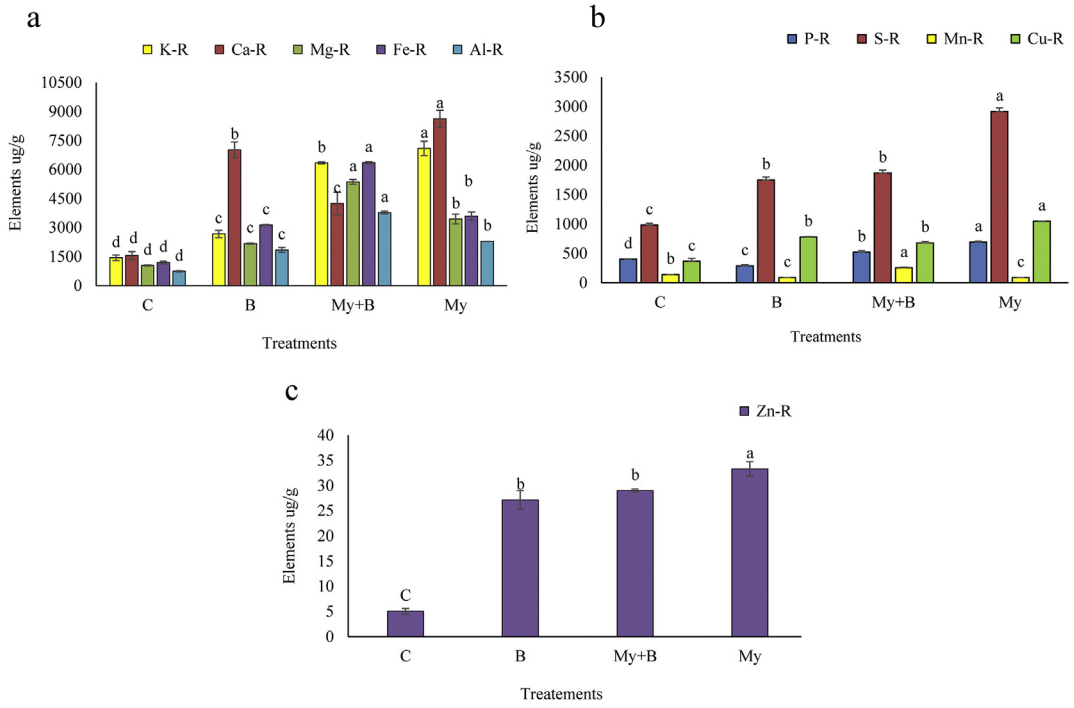

Fig. 3. Uptake of elements by sorghum roots following microbial treatments. (a) K, Ca, Mg, Fe and Al, (b) P, Mn, S and Cu, (c) Zn. Labeling, data representation, number of replicates and statistical analysis were performed as described for Fig. 2.

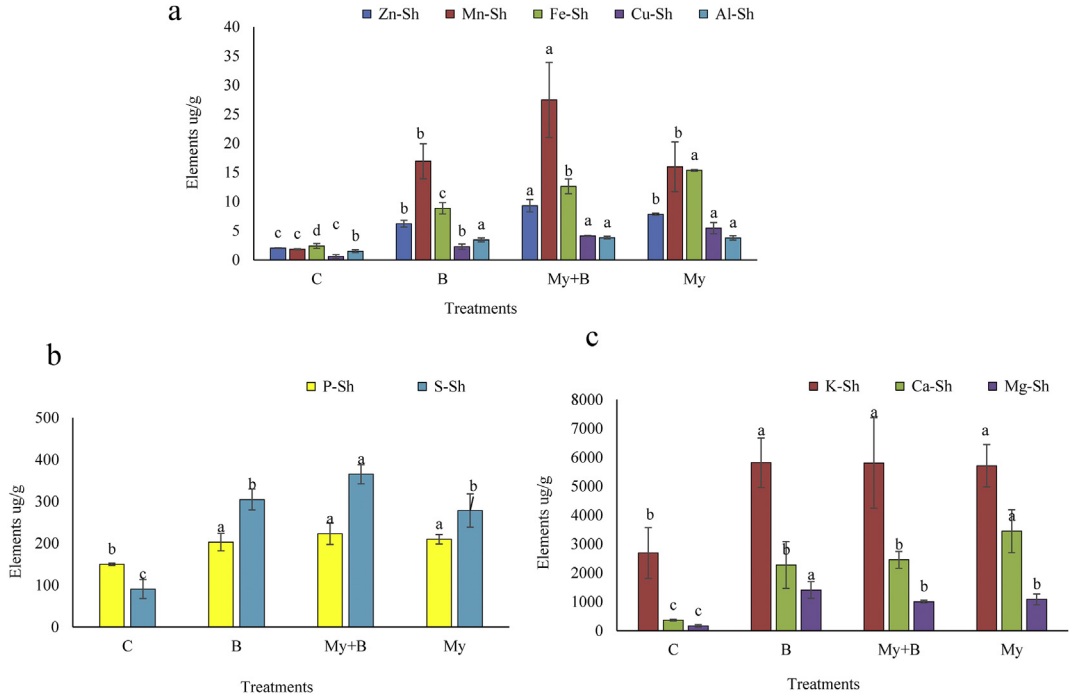

Fig. 4. Uptake of different elements by sorghum shoots following microbial treatments. (a) Zn, Mn, Fe, Cu and Al, (b) P and S, and (c) K, Ca and Mg. Labeling, data representation, number of replicates and statistical analysis were performed as described for Fig. 2.

My + B group. The remaining four metabolites (glutaric acid, dodecanoic acid, isopropanol ester, beta-sitosterol and 1-monostearin) were also upregulated in My group whereas methionine was upregulated only in B group. Mycorrhizal treatment showed up-regulation of 64 metabolites with 55 of them overlapping between My and My + B groups. Out of the remaining nine, five were upregulated exclusively in My group (glycerol-3-galactoside, erythrose, adenine, 5-methoxytryptamine and 4-hydroxybenzoate) and four were common with B group (glutaric acid, dodecanoic acid-isopropanol ester, beta-sitosterol and 1-monostearin). The number of metabolites commonly down-regulated in all of the three groups were 19. B group showed

down regulation of 58 metabolites with 24 of them overlapping between B and My + B groups and 17 were exclusive to B group. Mycorrhiza showed down-regulation of 44 metabolites with 20 of them overlapping with My + B group and 7 of them exclusive to My group. A total of 17 metabolites were common between B and My groups. The mixed group My + B showed down regulation of 29 metabolites with 15 of them downregulated only in this group.

3.6. Common upregulated metabolites in the three groups correlate positively with biomass and uptake of elements

Thirty eight common metabolites were induced in all of the three treatment groups. These metabolites showed positive correlation with sorghum biomass or element uptake or both (Table 2). Biomass correlated positively with eight metabolites (stearic acid, sorbitol, sebacic acid, pentadecanoic acid, hydroxylamine, cerotinic acid, arachidic acid and 3,4-dihydroxycinnamic acid). Four other metabolites (sucrose, aconitic acid, 4-aminobutyric acid and 3-aminoisobutyric acid), were positively correlated with 3 out of 4 attributes (root and shoot dry and wet weight) of sorghum biomass (Table 2). Root and shoot element uptake correlated positively with four metabolites (stearic acid, sebacic acid di(2-octyl) ester, mucic acid and ferulic acid). Further, shoot element uptake correlated positively with an additional six metabolites (xylitol, trehalose, sorbitol, shikimic acid, 1-monopalmitin, and propane-1,3-diol). Uptake of heavy metals (Zn, Al, Fe and Cu) by roots correlated positively with seven upregulated metabolites including the four common metabolites described above for root and shoot element uptake as well as sinapinic acid, propane-1,3-diol, and conduritol-beta-epoxide. Shoot heavy metal (Zn, Al, Fe, and Cu) uptake correlated positively with 19 metabolites. The most striking observation is the positive correlation with the levels of four metabolites, stearic acid, sorbitol and sebacic acid with uptake of almost all elements and root and shoot biomass.

Microbial treatments regulated metabolic pathways by significant upregulation of 3–6 compounds in each pathway ($p \leq 0.05$ using the over-representation algorithm). B group upregulated metabolites are part of two pathways: galactose metabolism and fatty acid biosynthesis (Fig. 5a). In comparison, My group showed upregulation of metabolites involved in three additional metabolic pathways: glyoxylate and dicarboxylate metabolism, alanine, aspartate and glutamate metabolism, and citrate cycle (TCA cycle) (Fig. 5b). In My + B group, where most of the positive effect was observed on sorghum shoot element uptake, upregulated metabolites were part of seven pathways. In addition to the five pathways upregulated in My group, arginine and proline metabolism, and starch and sucrose metabolism were upregulated (Fig. 5c). Galactose metabolism and fatty acid biosynthesis were upregulated in all three groups whereas alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, and citrate cycle (TCA cycle) were common between My and My + B groups.

3.7. Common downregulated metabolites show negative correlation with biomass and uptake of elements

A total of 17 downregulated metabolites common among the three treatment groups showed a negative correlation with biomass and element uptake (Table S1). The biomass increase correlated negatively with all the downregulated metabolites except pentitol, which showed significant correlation with fresh shoot weight only. However, lysine and glycerol-3-galactoside did not show significant correlation with fresh shoot weight. The uptake of elements by shoots and roots (except Mn) showed significant negative correlation with asparagine, allantoic acid, and alanine. Further, 1,2-cyclohexanedione showed significant negative correlation with all elements except Mn in shoot. The correlation analysis indicated that downregulation of most metabolites showed negative correlation or no effect on the uptake of elements and increase in biomass.

3.8. Downregulation of PGPB group metabolites show positive effect on uptake of elements

Methionine, glutaric acid, dodecanoic acid-isopropanol ester, beta-sitosterol and 1-monostearin were solely upregulated in B group in comparison to control. These five upregulated metabolites showed no correlation with sorghum biomass. Methionine showed negative correlation with uptake of majority of elements by root and Ca, Fe and Cu by shoot (Table S2). Glutaric acid, dodecanoic acid-isopropanol ester and 1-monostearin showed negative

Table 2
Correlation coefficients between sorghum biomass or element uptake and root metabolites upregulated in all treatment groups.

	xylitol	trehalose	threitol	sucrose	stigmasterol	stearic acid	sorbitol	sinapinic acid	shikimic acid	sebacic acid	raffinose	propane-1,3-diol	pentadecanoic acid	oleic acid	mucic acid	methyltetrahydropthen anthrenone	methanolphosphate	maleic acid	lauric acid	lactic acid	isocitric acid	hydroxylamine	hexonic acid	heptadecanoic acid	glycolic acid	glutamic acid	ferulic acid	conduritol-beta epoxide	cerotinic acid	capric acid	arachidic acid	alpha-ketoglutarate	adenosine	aconitic acid	4-aminobutyric acid	3-aminoisobutyric acid	3,4-dihydroxycinnamic acid	1-monopalmitin
Shoot FW	0.70	0.72	0.21	0.45	0.20	0.79	0.84	0.33	0.70	0.82	0.53	0.70	0.82	0.37	0.61	0.25	0.35	0.36	0.28	0.46	0.48	0.60	0.58	0.36	0.13	0.39	0.72	0.55	0.78	0.11	0.66	0.34	0.58	0.55	0.79	0.82	0.69	0.78
Shoot DW	0.33	0.35	0.05	0.67	0.06	0.57	0.73	0.10	0.33	0.66	0.13	0.33	0.75	0.00	0.32	0.21	-0.02	0.04	0.12	0.11	0.06	0.71	0.20	-0.08	0.16	-0.01	0.47	0.24	0.82	0.10	0.82	-0.08	0.18	0.68	0.52	0.55	0.84	0.43
Root FW	0.33	0.36	-0.04	0.70	-0.02	0.58	0.76	0.02	0.33	0.68	0.10	0.35	0.89	-0.06	0.30	0.14	-0.08	-0.03	0.03	0.04	0.06	0.75	0.18	-0.10	0.08	-0.07	0.49	0.20	0.88	0.01	0.94	-0.13	0.15	0.71	0.60	0.65	0.95	0.46
Root DW	0.47	0.50	0.07	0.63	0.08	0.68	0.81	0.17	0.48	0.79	0.26	0.48	0.86	0.14	0.44	0.21	0.11	0.12	0.15	0.20	0.23	0.73	0.34	0.07	0.12	0.09	0.61	0.35	0.86	0.07	0.85	0.05	0.32	0.68	0.67	0.67	0.86	0.60
P-R	0.65	0.67	0.97	0.51	0.97	0.83	0.77	0.94	0.68	0.79	0.51	0.70	-0.05	0.72	0.91	0.96	0.71	0.86	0.98	0.81	0.33	0.66	0.66	0.33	0.89	0.66	0.87	0.89	0.59	0.91	-0.02	0.53	0.63	0.67	0.07	0.07	-0.01	0.57
K-R	0.74	0.75	0.98	0.34	0.97	0.82	0.71	0.98	0.75	0.75	0.65	0.78	-0.09	0.83	0.94	0.90	0.82	0.94	0.98	0.89	0.48	0.51	0.77	0.49	0.82	0.79	0.88	0.95	0.46	0.86	-0.15	0.68	0.74	0.52	0.13	0.12	-0.14	0.64
Ca-R	0.33	0.37	0.55	0.94	0.58	0.72	0.88	0.50	0.35	0.82	0.05	0.39	0.39	0.17	0.59	0.74	0.14	0.33	0.59	0.29	-0.09	0.99	0.24	-0.19	0.73	0.09	0.71	0.49	0.94	0.67	0.64	-0.05	0.19	0.98	0.13	0.16	0.63	0.36
Mg-R	0.96	0.95	0.74	-0.03	0.70	0.83	0.63	0.87	0.96	0.70	0.95	0.96	0.16	0.97	0.93	0.55	0.97	0.95	0.76	0.98	0.87	0.21	0.99	0.86	0.39	0.97	0.86	0.96	0.29	0.46	-0.19	0.94	0.98	0.19	0.54	0.51	-0.15	0.90
S-R	0.56	0.60	0.84	0.76	0.85	0.86	0.90	0.80	0.59	0.88	0.35	0.62	0.18	0.52	0.84	0.93	0.50	0.68	0.87	0.63	0.17	0.88	0.52	0.12	0.88	0.45	0.87	0.78	0.82	0.86	0.31	0.30	0.48	0.88	0.13	0.14	0.31	0.53
Zn-R	0.76	0.79	0.72	0.66	0.72	0.97	0.98	0.76	0.77	0.97	0.55	0.80	0.45	0.60	0.89	0.76	0.58	0.70	0.76	0.71	0.41	0.82	0.69	0.32	0.67	0.56	0.95	0.84	0.87	0.66	0.43	0.45	0.66	0.80	0.46	0.47	0.45	0.76
Mn-R	0.96	0.95	0.48	-0.25	0.44	0.71	0.48	0.66	0.95	0.58	0.99	0.94	0.29	0.91	0.79	0.25	0.92	0.82	0.50	0.89	0.98	0.00	0.98	0.97	0.08	0.95	0.73	0.83	0.15	0.15	-0.18	0.96	0.98	-0.04	0.71	0.67	-0.14	0.93
Fe-R	0.99	0.99	0.61	-0.05	0.57	0.84	0.65	0.77	0.99	0.73	0.97	0.98	0.33	0.93	0.89	0.43	0.93	0.88	0.64	0.94	0.92	0.20	0.99	0.89	0.25	0.95	0.85	0.92	0.33	0.32	-0.07	0.93	1.00	0.16	0.69	0.66	-0.03	0.96
Cu-R	0.51	0.55	0.72	0.85	0.74	0.85	0.93	0.69	0.53	0.90	0.26	0.57	0.32	0.40	0.77	0.85	0.37	0.55	0.76	0.52	0.10	0.95	0.44	0.02	0.81	0.32	0.85	0.69	0.91	0.77	0.49	0.18	0.40	0.94	0.19	0.21	0.48	0.51
Al-R	0.99	0.99	0.66	-0.02	0.62	0.86	0.67	0.80	0.99	0.74	0.96	0.99	0.30	0.94	0.92	0.48	0.94	0.90	0.68	0.95	0.90	0.23	1.00	0.87	0.31	0.95	0.87	0.94	0.35	0.38	-0.08	0.93	0.99	0.20	0.65	0.63	-0.04	0.95
P-Sh	0.79	0.80	0.58	0.36	0.56	0.85	0.80	0.68	0.78	0.78	0.69	0.80	0.43	0.62	0.80	0.54	0.64	0.68	0.63	0.71	0.57	0.54	0.75	0.50	0.42	0.65	0.82	0.78	0.62	0.44	0.27	0.58	0.73	0.51	0.55	0.59	0.31	0.78
K-Sh	0.64	0.66	0.45	0.48	0.44	0.77	0.79	0.55	0.62	0.74	0.51	0.66	0.51	0.44	0.67	0.47	0.46	0.50	0.51	0.54	0.40	0.62	0.58	0.31	0.38	0.45	0.74	0.63	0.69	0.37	0.44	0.37	0.55	0.59	0.51	0.56	0.46	0.67
Ca-Sh	0.59	0.62	0.73	0.66	0.74	0.83	0.86	0.75	0.60	0.85	0.40	0.65	0.27	0.53	0.81	0.80	0.51	0.63	0.76	0.59	0.27	0.79	0.55	0.19	0.73	0.45	0.86	0.75	0.76	0.71	0.33	0.34	0.51	0.78	0.23	0.23	0.33	0.58
Mg-Sh	0.58	0.62	0.30	0.67	0.30	0.82	0.90	0.38	0.57	0.84	0.36	0.63	0.77	0.26	0.61	0.40	0.25	0.32	0.36	0.37	0.28	0.78	0.47	0.15	0.32	0.26	0.76	0.52	0.90	0.27	0.74	0.18	0.44	0.75	0.62	0.67	0.76	0.66
S-Sh	0.90	0.92	0.51	0.33	0.49	0.93	0.87	0.67	0.89	0.89	0.78	0.91	0.61	0.70	0.86	0.46	0.70	0.69	0.57	0.75	0.71	0.54	0.84	0.61	0.31	0.69	0.91	0.82	0.67	0.32	0.37	0.65	0.82	0.50	0.74	0.74	0.40	0.93
Zn-Sh	0.92	0.94	0.74	0.31	0.72	0.95	0.85	0.84	0.93	0.90	0.80	0.95	0.37	0.84	0.97	0.66	0.82	0.86	0.77	0.89	0.70	0.54	0.90	0.65	0.52	0.81	0.97	0.95	0.61	0.55	0.14	0.74	0.88	0.51	0.56	0.55	0.17	0.90
Mn-Sh	0.90	0.91	0.51	0.13	0.48	0.84	0.72	0.66	0.90	0.78	0.82	0.92	0.49	0.77	0.84	0.40	0.76	0.73	0.54	0.78	0.79	0.35	0.87	0.72	0.24	0.77	0.86	0.82	0.49	0.27	0.16	0.75	0.86	0.31	0.70	0.69	0.19	0.92
Fe-Sh	0.73	0.75	0.88	0.57	0.88	0.92	0.88	0.90	0.75	0.90	0.57	0.77	0.16	0.71	0.94	0.89	0.70	0.83	0.92	0.80	0.41	0.73	0.72	0.36	0.81	0.65	0.93	0.91	0.70	0.82	0.17	0.53	0.68	0.73	0.26	0.24	0.18	0.68
Cu-Sh	0.66	0.68	0.92	0.51	0.92	0.83	0.78	0.92	0.67	0.79	0.52	0.71	0.02	0.70	0.90	0.91	0.70	0.83	0.93	0.77	0.35	0.66	0.66	0.33	0.84	0.63	0.88	0.87	0.60	0.86	0.04	0.52	0.62	0.67	0.12	0.11	0.04	0.59
Al-Sh	0.81	0.83	0.66	0.53	0.65	0.95	0.94	0.76	0.81	0.94	0.64	0.84	0.49	0.66	0.89	0.67	0.65	0.71	0.71	0.73	0.53	0.72	0.75	0.43	0.55	0.61	0.95	0.84	0.78	0.55	0.39	0.53	0.72	0.69	0.55	0.54	0.41	0.82

In the first column, the element symbol followed by R and Sh represents element uptake by root and shoot, respectively. Pink colored cells represent significant positive correlation.

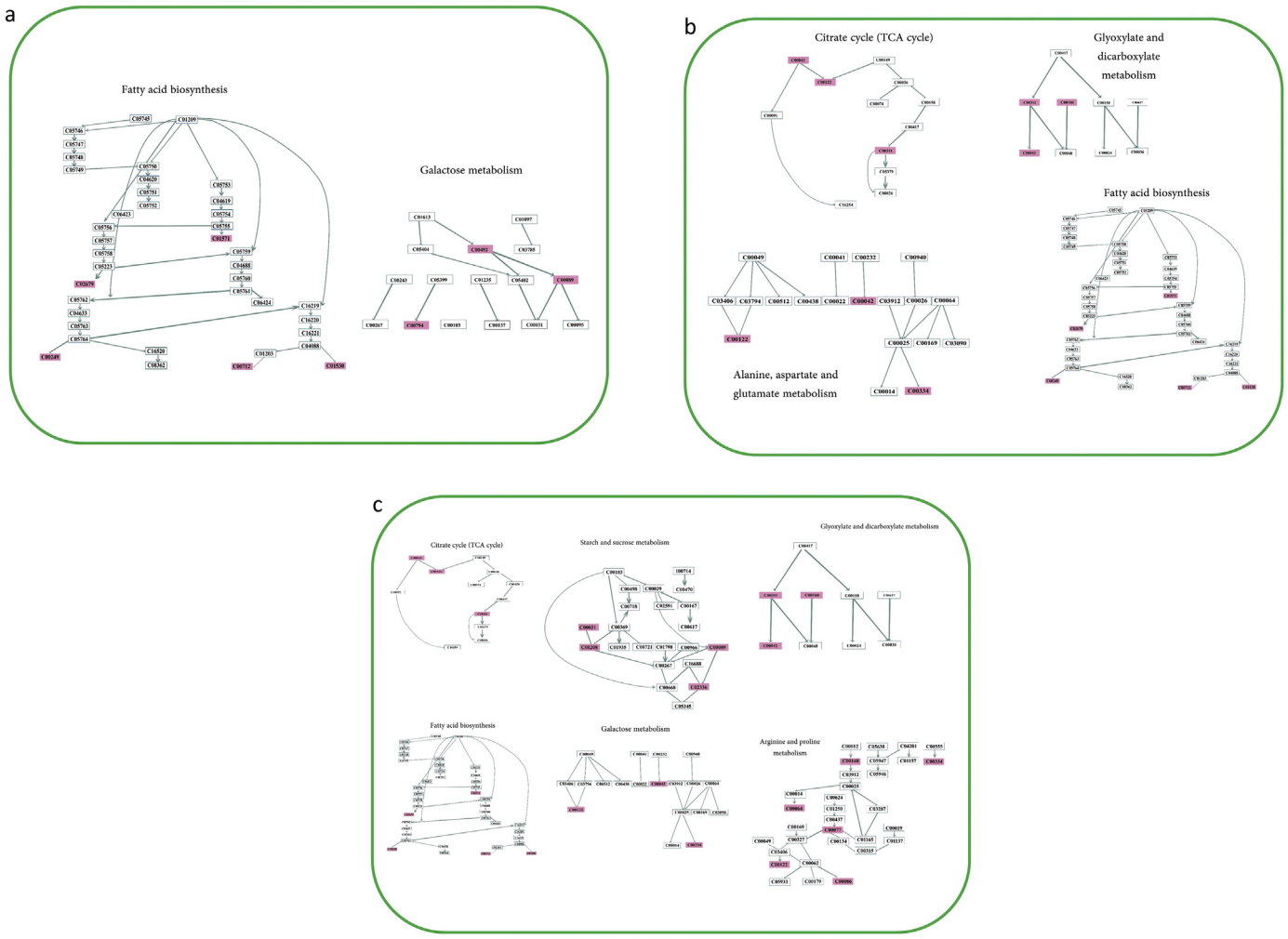

Fig. 5. Metabolic pathways affected by different treatments (a) B group, (b) My, and (c) My + B. The red boxes indicate induced metabolites that significantly contribute to each pathway. The pathways were modified from Metaboanalyst website (http://mirror.metaboanalyst.ca/MetaboAnalyst/faces/Secure/upload/PathUploadView.xhtml) to highlight the significant compounds in each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlation with root uptake for Mg, Mn, Fe and Al. Root uptake for P, Ca, S and Cu positively correlated only with beta-sitosterol. The PGPB downregulated metabolites were 17 with no correlation with sorghum biomass except 2-hydroxyglutaric acid and ethanolamine, which were negatively correlated with root fresh weight. In addition, 2-hydroxyglutaric acid correlated negatively with shoot dry weight. The downregulation of maltose and succinic acid correlated positively with root P, K, Mg, Mn, Fe, Al and shoot S, Zn, Mn uptake. In addition, shoot Fe, Cu and Al uptake showed positive correlation with maltose only. Root Al, Fe, Mn and Mg showed positive correlation with downregulation of 11 metabolites (succinic acid, quinic acid, palmitic acid, maltose, malic acid, levoglucosan, hydroxycarbamate, galactinol, fructose, ethanolamine and erythritol) whereas five metabolites (urea, quinic acid, maltose, malic acid and hydroxycarbamate) showed positive correlation with shoot S, Zn and Mn uptake.

3.9. Downregulation of mycorrhiza group metabolites show negative correlation with biomass and uptake of elements

Asparagine and allantoic acid downregulation correlated negatively with sorghum biomass and uptake of elements except root Mn (Table S3). In addition, down regulation of octadecanol, glutamine and benzoic acid correlated positively with root Mg, Mn, Fe and Al uptake while octadecanol and benzoic acid correlated positively with shoot S, Zn and Mn uptake. On the other hand, upregulation of glycerol-3-galactoside and 4-hydroxybenzoate correlated negatively with root biomass whereas upregulation of erythrose and beta-sitosterol correlated positively with root P, K and S.

4. Discussion

Sorghum is a versatile cereal used as food, feed, biofuel feedstock and for phytoremediation. The investigation of the response of sorghum grown in marginal soil and treated with PGPB and mycorrhiza provided insights into the effect of plant-microbe interactions on enhancing the uptake of both nutrients and heavy metals as well as the metabolite dynamics in roots. The highest and lowest levels of uptake of heavy metals such as copper and zinc were observed in roots treated with mycorrhiza alone and PGPB alone, respectively. However, biomass was highest for both shoot and root in B treated plants. This probably resulted in lower biomass observed in My group compared to B group. It is likely that an increase in the levels of elements, particularly the heavy metals, increase abiotic stress.

During stress, plants accumulate metabolites to enhance osmotic and ionic adjustment. The accumulation of metabolites regulate optimal water balance and reduce toxicity (Augé et al., 2014). Colonization with arbuscular mycorrhizal (AM) enhanced tolerance of soybean to salt stress, which increased symbiotic nitrogen fixation thereby promoting plant growth (Younesi et al., 2013). Further, soil inoculation with bacteria and mycorrhiza (*Azotobacter chroococcum* and *G. intraradices*) enhanced oil content in safflower (Mirzakhani et al., 2014). Similarly, glycolipids and unsaturated fatty acids increased in olive trees inoculated with mycorrhiza (Mechri et al., 2014a). In addition to mycorrhizal colonization, the presence of heavy metals like Cd increased unsaturated fatty acids in flax (Kaplan et al., 2015). The heavy metals probably helped in converting fatty acids from saturated to unsaturated state by improving the activity of enzymes involved in this process. In our study, treatment with microorganisms increased sorghum root saturated fatty acids, stearic acid, capric acid and pentadecanoic acid. The fatty acid biosynthesis pathway was affected significantly in My + B group, similar to a previous study that reported increase of lipid metabolism during mycorrhiza symbiosis (Mechri et al., 2014b). The increase of transcriptional regulation and lipid regulation in colonized roots suggested cell reprogramming by mycorrhiza during symbiosis (Gaude et al., 2012). In addition, sorbitol, a sugar alcohol, found abundantly in plants, increased significantly in all of the three treatment groups, which correlated positively with sorghum biomass and element uptake. Sorbitol and trehalose have been reported to act as defenders against oxidative stress (Leyman et al., 2001). In the current study, trehalose increase correlated with the uptake of most of the elements in shoot and root, and shoot fresh weight, which suggests a role in water preservation and osmoprotection. Trehalose accumulation increased shoot starch content in wheat, which indicates a role in carbon metabolism regulation and stress defence (Ahmed et al., 2013; O'Hara et al., 2013). This was evident in the current study where regulation of My + B metabolites are likely to contribute significantly to starch and sucrose metabolism.

Positive correlation with a phenolic compound, 3,4-dihydroxycinnamic acid with strong antioxidative properties (Sánchez-Alonso et al., 2011), with biomass, was observed in the current study. Although it is known to increase in a copper-tolerant plant (*Elsholtzia splendens*) as part of phytoremediation (Xing et al., 2012), its accumulation correlated positively with root Ca and shoot Mg in sorghum. The carboxylic acid, hexanoic acid, which correlated positively with shoot fresh weight and uptake of most of the elements in shoots, acts as a priming agent for defence response against plant pathogens by reducing oxidative stress (Scalschi et al., 2013; Finiti et al., 2014). Organic compounds, hydroxylamine, ferulic acid and sinapinic acid showed upregulation and positive correlation with uptake of most elements in sorghum. Hydroxylamine was shown to improve germination and growth of *Sesamum indicum* (Aliero, 2006). Although ferulic acid enhances plant cell wall rigidity and strength and has antioxidative and other beneficial biological properties (Mathew and Abraham, 2004; Kumar and Pruthi, 2014), sinapinic and ferulic acids have been reported to inhibit root growth in *Arabidopsis thaliana* (Reigosa and Pazos-Malvido, 2007) which was evident in our study in low (ferulic acid) or no correlation (sinapinic acid) with sorghum biomass. Another upregulated organic compound is maleic acid which is converted to malic acid by hydration. PGPB are likely to increase the production of malic acid as shown in case of Arabidopsis where the roots secrete malic acid which enhances PGPB growth (Rhodes and Hanson, 1993). In My group, allantoic acid and asparagine were downregulated in contrary to previous studies which reported upregulation of allantoic acid and asparagine with mycorrhizal association (Sprent, 2001; Salvioli et al., 2012). The downregulation of allantoic acid and asparagine in mycorrhiza group correlated negatively with biomass and element uptake except for Mn in the current study. Allantoic acid is known to play an essential role in plant nitrogen assimilation and metabolism (Schubert and Boland, 1990). Amidohydrolase is a manganese (Mn) dependent enzyme which degrades allantoic acid (Lukaszewski et al., 1992). In the current study, the downregulation of allantoic and asparagine might be due to a shift into pathways to utilize it as a nitrogen source due to poor soil condition. On the other hand, B group specifically upregulated metabolites that showed no correlation with biomass and negative correlation with the uptake of some elements. The positive effect of downregulation of metabolites in B group suggested that PGPB regulates specific pathways to maximize its benefit to the plant.

My + B group upregulated arginine and proline metabolism in the current study, which was evident by upregulation of glutamic acid in sorghum root. Glutamic acid showed significant positive correlation with uptake of most elements. The increase of glutamic acid can be explained by the enhanced uptake of elements in the presence of mycorrhiza and PGPB. Glutamic acid is an essential precursor in proline pathway, which accumulates in response to a wide range of stress factors (Munns, 2005; Sharma and Dietz, 2006). Glutamate can activate ion channels, function in signalling pathways in root to enhance root growth and play a central role in nitrogen metabolism (Forde and Lea, 2007). We hypothesize that the plant is homeostatically regulated by the upregulation of the metabolites in sorghum.

5. Conclusion

Mycorrhiza and PGPB increased efficient nutrient use for sorghum grown in marginal soil. The mycorrhiza inoculation increased uptake of elements by sorghum root with a complementary effect of PGPB in enhancing biomass. The use of PGPB decreased the competition between plant and mycorrhiza on organic matters that was evident in the increase of biomass in B group and My + B group in comparison with My group. Most of the root metabolites were upregulated when sorghum was subjected to both mycorrhiza and PGPB treatment affecting seven metabolic pathways. Galactose metabolism and fatty acid biosynthesis were upregulated in all three groups emphasizing the role of microbial inoculation in utilizing the carbon sources. On the other hand, three pathways involved in metabolism of amino acids and energy generation were also common between My and My + B groups. This indicated a role for mycorrhiza in inducing essential amino acid precursors and biosynthesis of plant carbohydrates from fatty acids through glyoxylate and dicarboxylate metabolism. Our study and future metabolite analysis will lead to the development of metabolite based markers for association of plants with PGPB and mycorrhiza.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.04.112.

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