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RESEARCH ARTICLE

Metabolomics, biomass and lignocellulosic total sugars analysis in foxtail millet (*Setaria italica*) inoculated with different combinations of plant growth promoting bacteria and mycorrhiza

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Foxtail millet (*Setaria italica*) is the second most widely produced millet with potential as a biofuel source. Employment of plant growth promoting bacteria (PGPB) and mycorrhiza could serve as environment-friendly alternatives for the use of excessive NPK fertilizers and producing biofuel. The highest increase of biomass was associated with endomycorrhiza combined with PGPB in comparison to control. Nuclear magnetic resonance (NMR) analysis detected 28 metabolites in foxtail shoot with most of them upregulated in ecto/endomycorrhiza group and combined with PGPB. The upregulation of metabolites associated with synthesis of amino acids correlated positively with biomass. The inoculation with both PGPB and endomycorrhiza gave the best results with reference to total sugar yield. Our study indicates that PGPB and endomycorrhiza combination is well suited for enhancing biomass and boosting sugar yield, which are useful attributes for utilizing foxtail millet as a biofuel source.

Highlighted Conclusions
1. Effect of PGPB and mycorrhiza on foxtail millet biomass, metabolites and total sugars was studied.
2. Biomass and total sugars increased by combined PGPB and endomycorrhiza treatment.
3. Higher biomass correlated positively with metabolites associated with amino acid biosynthesis.

Foxtail millet (*Setaria italica*) is a diploid C4 grass with a relatively small genome (~515 Mb) (Li and Brutnell 2011). Foxtail millet is an annual grass that is widely used for both food and livestock feed and grown in arid and semi-arid regions of the world (Pandey et al. 2017). Being a close relative of an important biofuel crop switchgrass, it is also a potential biofuel source. The early maturity of foxtail in 60-70 days makes it a good model for experimental studies. Several recent studies have explored the molecular responses of foxtail millet under abiotic stress. Colonization of arbuscular mycorrhizal (AM) fungus *Glomus mosseae* and its effect on phosphate transport was reported previously, where a significant increase in the expression of plant phosphate transporters belonging to PHT1 family in low phosphate environment was reported in the presence of AM fungi (Ceasar et al. 2014). PHT1 transporter gene family of foxtail millet consists of 12 members with varying levels of expression depending on the availability of phosphate and AM colonization, which helps in enhancing phosphate availability.

The plant growth promoting bacteria used in this study was isolated from Torch Lake sediments rich in copper deposits and was shown to enhance maize growth and metal uptake (Li and Ramakrishna 2011). Previously, we reported higher biomass and element uptake in sorghum and maize and upregulation of stress tolerance associated proteins in sorghum as a result of root colonization of PGPB and AM fungi (Dhawi et al. 2015, 2016,

2017). However, the effect of root colonization in foxtail millet by PGPB and arbuscular mycorrhiza nutrient uptake has not been reported. The objective of this study was to explore the effect of rhizospheric colonization of PGPB and AM fungi on growth parameters, key metabolites and potential use of the biomass as biofuel feedstock. The results showed a significant increase in chlorophyll, shoot height, biomass and levels of 28 metabolites in colonized plants. Highest biomass was observed in foxtail millet treated with arbuscular mycorrhiza in combination with PGPB. Lignocellulosic saccharification of AM fungi and PGPB treated foxtail millet shoot pretreated with 0.5% H_2SO_4 gave the best total sugar yield suggesting its suitability for biofuel generation.

MATERIAL AND METHODS

Plant material and treatments. Seeds of foxtail millet (*Setaria italica*) Hylander were surface sterilized by treatment with 2% sodium hypochlorite and then rinsed with distilled water. Seeds were soaked in distilled water for 48 hours for germination. Seedlings were subjected to different treatments for an hour. The treatments were (1) control group (C) subjected to autoclaved ectomycorrhiza mix and 100 mL NaCl 0.85% solution, (2) PGPB *Pseudomonas* TLC6-6-5.4 (B) (Li and Ramakrishna 2011) suspended in 100 mL of 0.85% NaCl solution (7.5×10^8 cfu mL^{-1}) as described by Dhawi et al. (2015), (3) endomycorrhiza (EN) (9 grams endomycorrhiza powder suspended in 100 mL distilled water) consisting of a blend of spores of *Glomus intraradices, G. mosseae, G. aggregatum* and *G. etunicatum* (100,000 propagules lb^{-1}), (4) ecto/endomycorrhizal mix (EC) consisting of a blend of spores of four endomycorrhizal fungi described above and seven ectomycorrhizal fungi, *Rhizopogon villosullus, R. luteolus, R. amylogpogon, R. fulvigleba, Pisolithus tinctorius, Scleroderma Cepa,* and *S. citrinum* (110 million propagules lb^{-1}), (5) groups 2 + 3 (BEN), (6) groups 2 + 4 (BEC). Mycorrhizae (endo or ectomycorrhiza) were obtained from MycoApply® Endo (Valentine Country Inc.). Seedlings were sown in potting mix soil (Miracle Gro Premium Potting Mix, Scotts Company LLC, USA) with each pot containing 6 kg of soil. Each treatment had ten replicates. Treatments were repeated ten days later for each group with the addition of 100 mL inoculum to each pot. Plants were maintained in greenhouse for 60 days (28^{0}C and 65% humidity with a photoperiod of 16h-light/8h-dark). Plants were watered every alternate day. Shoot length was recorded after 60 days for six replicates for each treatment. Chlorophyll was recorded after 60 days using atLEAF chlorophyll meter. Shoot and root fresh weights were recorded after 60 days. Dry weights were recorded after the samples were oven dried (70 °C for 48 h). A portion of the fresh shoot samples were frozen in liquid nitrogen for metabolite analysis.

Analysis of metabolites. Metabolites were extracted according to Wu et al. (2014). One hundred grams of fresh shoots were ground in liquid nitrogen followed by addition of 1.2 mL 50% methanol. Metabolite analysis was performed as four replicates for each treatment. The extracts were lyophilized and samples were reconstituted in phosphate buffer containing 10% deuterium oxide and 0.15 mM sodium 3-trimethlysilyl [2,2,3,3-D4] propionate (TSP). for analysis of metabolites by Nuclear Magnetic Resonance (NMR) center at University of Minnesota. The NMR spectra were acquired using a gradient-enhanced 1D NOESY-presaturation plus sequence for water suppression on a Bruker Avance III 700-MHz spectrometer with a TCI 1.7-mm cryoprobe. Baseline correction and chemical shift referencing to the TSP peak were performed using the processor module in Chenomx NMR Suite 8. The identified metabolites were subjected to correlational analysis to look at linear relationships between physiological measurements and identified metabolites in all the groups.

Determination of total sugars and optimum conditions for lignocellulosic saccharification. Shoot samples were oven dried at 70°C for 24h and then milled with processor. The pretreatment used 40mg of dried sample. The pre-treatments were performed with 0.5%, 1% and 2% H_2SO_4, and 0.2%, 0.5% and 1% NaOH at 121°C for 30min as described by Gomez et al. (2011). Hydrolysis and estimation of total sugars was performed using spectrophotometer plate reader as per Gomez et al. (2011) with some modifications. Each analysis was performed in triplicate for biomass, chlorophyll, metabolites, and lignin quantification. SPSS17 was used to perform analysis of variance followed by Tukey test for factors with significant values ($p \leq 0.05$).

RESULTS

Endomycorrhiza alone or combined with PGPB showed best impact on biomass, plant height and chlorophyll content. AM fungi are known to colonize a wide range of plants and benefit their host. The addition of PGPB to mycorrhiza increased the metabolic activities related to element uptake and root biomass in maize (Dhawi et al. 2015). In the current study, the mycorrhiza showed a significant increase in plant height and chlorophyll in

groups BEN, EC, EN and BEC in comparison with control (Table 1). Maximum increase of ~60% in plant height and ~85% in chlorophyll was observed in BEN group. Foxtail millet shoot fresh and dry weight increased significantly in BEN, EC, and EN groups where BEN group showed 2.7- and 4-fold increase in shoot fresh and dry weight, respectively, in comparison with control. Root fresh weight increased significantly in BEN and EC groups only with highest value recorded in BEN group. Root dry weight increased significantly in BEN, EC and EN groups with highest biomass in BEN group. Endomycorrhiza alone or combined with PGPB showed best effect on biomass, plant height and chlorophyll content.

Table 1. Effect of different microbial treatments on plant height, chlorophyll and biomass of foxtail millet.

Treatments	Control	B	BEC	EN	EC	BEN
Height (inch)	13.5±1.14c	14.8±1.14b,c	18.0±0.74a,b	20.2±1.74a	21.0±1.0a	21.5±1.7a
Chlorophyll ($\mu g\ cm^{-2}$)	18.4±1.17c	18.5±1.99c	26.3±1.4b	33.4±1.23a	34.3±1.3a	34.6±2.7a
Shoot FW (g)	3.100±0.4c	3.430±0.47c	4.800±0.26c	8.030±0.72b	10.800±0.75a	11.500±1.55a
Root FW (g)	0.030±0.004c	0.045±0.008b,c	0.110±0.06b,c	0.170±0.016b,c	0.250±0.17b	0.570±0.02a
Shoot DW (g)	0.280±0.04c	0.330±0.051c	0.460±0.023c	0.800±0.057b	1.100±0.05a	1.130±0.15a
Root DW (g)	0.026±0.003c	0.036±0.006b,c	0.110±0.06a,b,c	0.130±0.026a,b	0.160±0.03a	0.170±0.038a

All the values are mean of six replicates ±SD.
Values in rows in each column indexed by different letters ($p<0.05$) are significantly different according to Tukey's test.
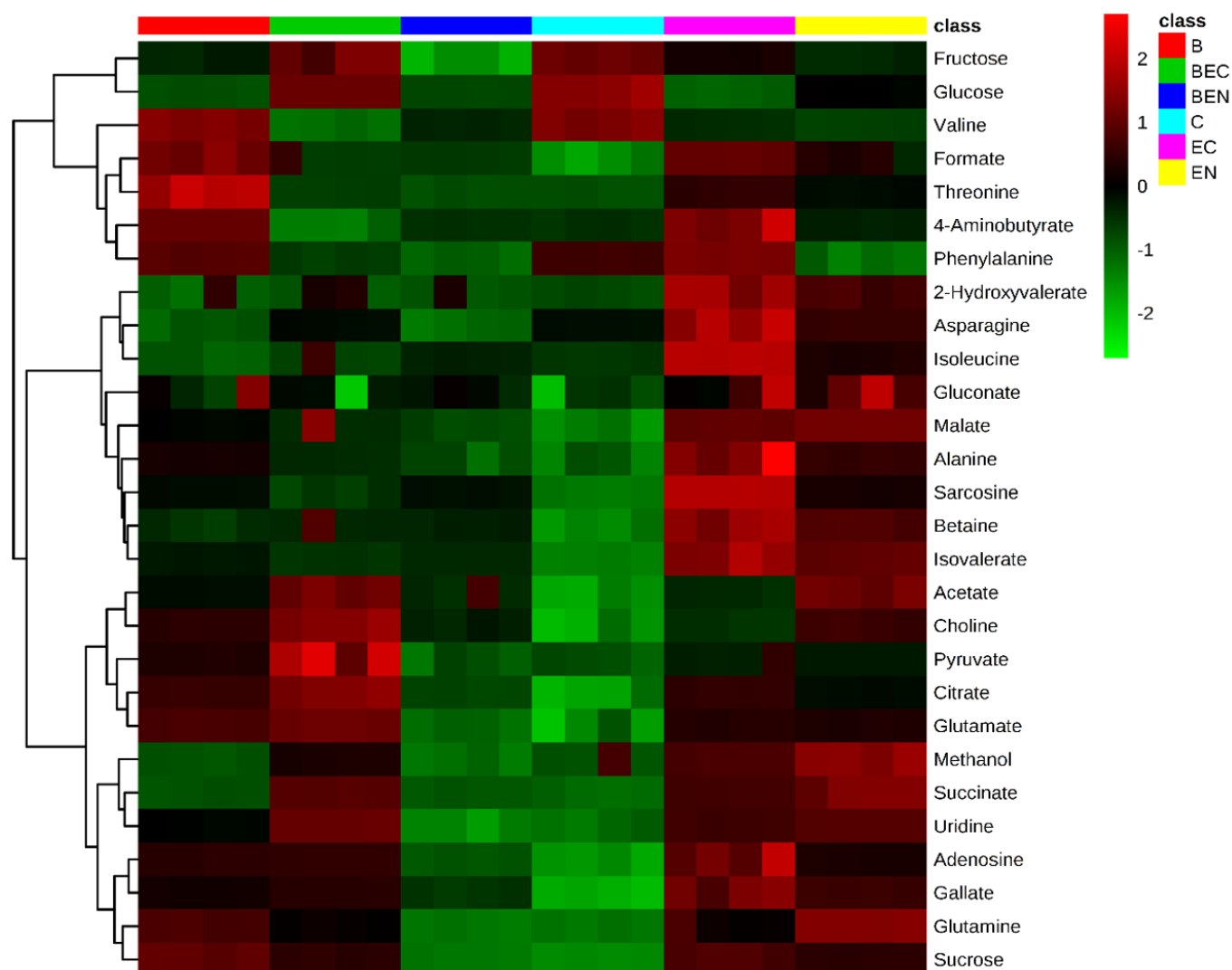

Figure 1. Heat map and hierarchical clustering analysis using Euclidean and ward algorithm of the relative intracellular metabolite concentrations in foxtail millet shoots subjected to different microbial treatments. Clustering was performed using Pearson correlation as the distance metric. Relative metabolite levels in the heat map are shown by red (upregulation) and green (downregulation). The color bar on top and left represents different treatments (B, BEC, BEN, EC and EN) and control (C).

Amino acid levels correlated positively with biomass. NMR analysis identified twenty eight metabolites in foxtail millet shoots, where majority of them were upregulated in treated groups in comparison with control group (Figure 1). The monosaccharides such as fructose and glucose were downregulated in most of the treated groups (except BEC) compared to the control group. The separation among the groups was evident in principal component analysis (PCA) plot where treated group metabolites clustered separately and showed upregulation compared to control group (Figure 2). EC and EN groups showed mostly upregulated metabolites in component 1 (47%) while BEC showed most upregulated metabolites in component 2 (20%). The correlation analysis showed that eleven metabolites correlated with various attributes including biomass and plant height. Five metabolites, 2-hydroxyvalerate, betaine, isoleucine, isovalerate and sarcosine positively correlated with plant height, shoot fresh and dry weight and root dry weight (Table 2). However, the amino acid valine negatively correlated for the same measurements. Gallate, gluconate and malate positively correlated with plant height and chlorophyll. The sugars, fructose and glucose negatively correlated with all plant attributes with significant negative correlation with whole plant biomass.
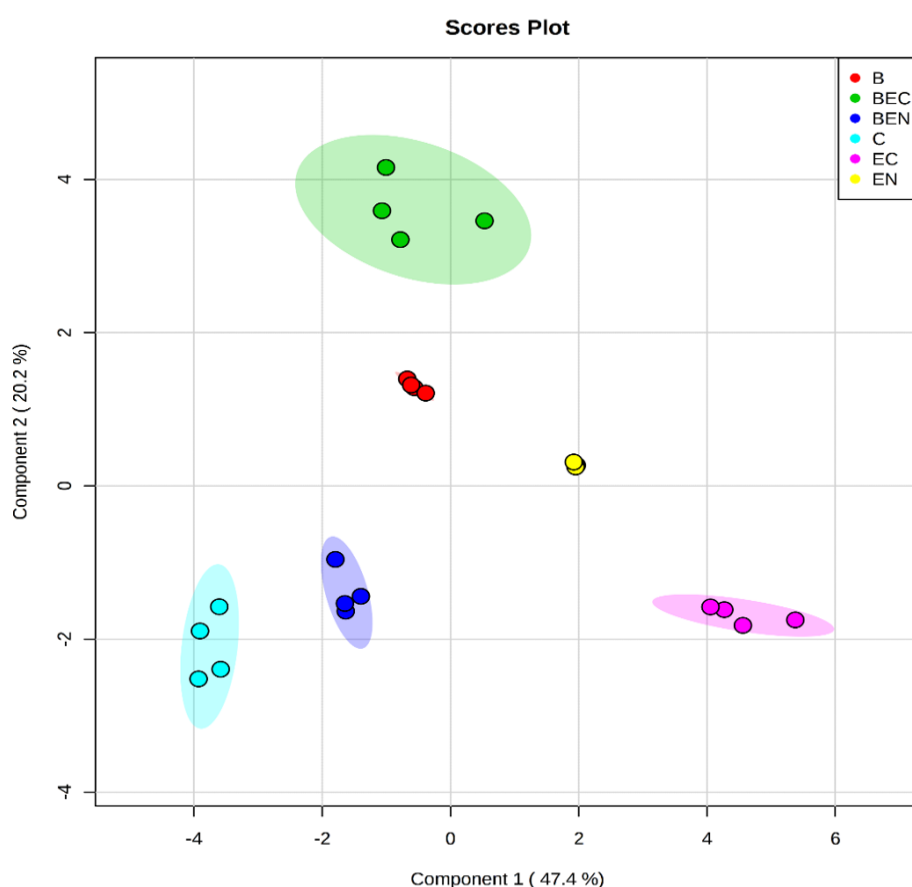

Figure 2. Two-dimensional scores plot displaying the relation between selected components (PCs) of shoot metabolites using principal component analysis (PCA). Metabolites were extracted from five groups of treatments (B, BEC, BEN, EN, EC) in comparison with control (C) in foxtail millet. The interaction between different groups of treatments are indicated by different colors.

Lignocellulosic saccharification and total sugar quantification. Foxtail millet dried leaves from control samples were processed to determine the optimal sugar yield using different saccharification methods. Lignocellulosic saccharification with sulfuric acid and sodium hydroxide showed most total sugar yield when treated with sulfuric acid with low concentration (0.5%), where it reached 1.3 nmol kg^{-1} (Figure 3). The pretreatment with 0.2% sodium hydroxide showed the next best yield with 800 nmol g^{-1} of total sugar. Based on these results, pre-treatment with 0.5% sulfuric acid was used for sugar quantification of treated samples. The total sugars increased significantly to reach the highest level in BEN (0.337 mg kg^{-1}) followed by EN (0.270 mg kg^{-1}), BEC (0.246 mg kg^{-1}), and EC (0.236 mg kg^{-1}) groups which represent 190% 152% 139% and 133%, respectively, in comparison to the control (0.177 mg kg^{-1}). Only B group showed total sugars (0.091 mg kg^{-1}) less than the control group.

Table 2. Correlation of eleven metabolites with height, biomass and chlorophyll of foxtail millet subjected to different microbial treatments.

Plant Variables	Metabolites										
	2-Hydroxyvalerate	Betaine	Fructose	Gallate	Gluconate	Glucose	Isoleucine	Isovalerate	Malate	Sarcosine	Valine
Plant height	0.62	0.76	-0.56	0.59	0.61	-0.54	0.63	0.68	0.57	0.66	-0.80
Chlorophyll	0.66	0.76	-0.52	0.54	0.62	-0.48	0.67	0.69	0.57	0.65	-0.79
Shoot FW	0.58	0.66	-0.64	0.41	0.55	-0.63	0.66	0.62	0.39	0.68	-0.58
Plant FW	0.56	0.64	-0.66	0.40	0.53	-0.63	0.64	0.60	0.37	0.66	-0.58
Root FW	0.10	0.25	-0.78	0.07	0.19	-0.51	0.23	0.19	-0.02	0.29	-0.44
Plant DW	0.59	0.69	-0.63	0.46	0.56	-0.63	0.66	0.64	0.43	0.69	-0.63
Root DW	0.59	0.72	-0.50	0.55	0.50	-0.49	0.65	0.61	0.48	0.64	-0.81
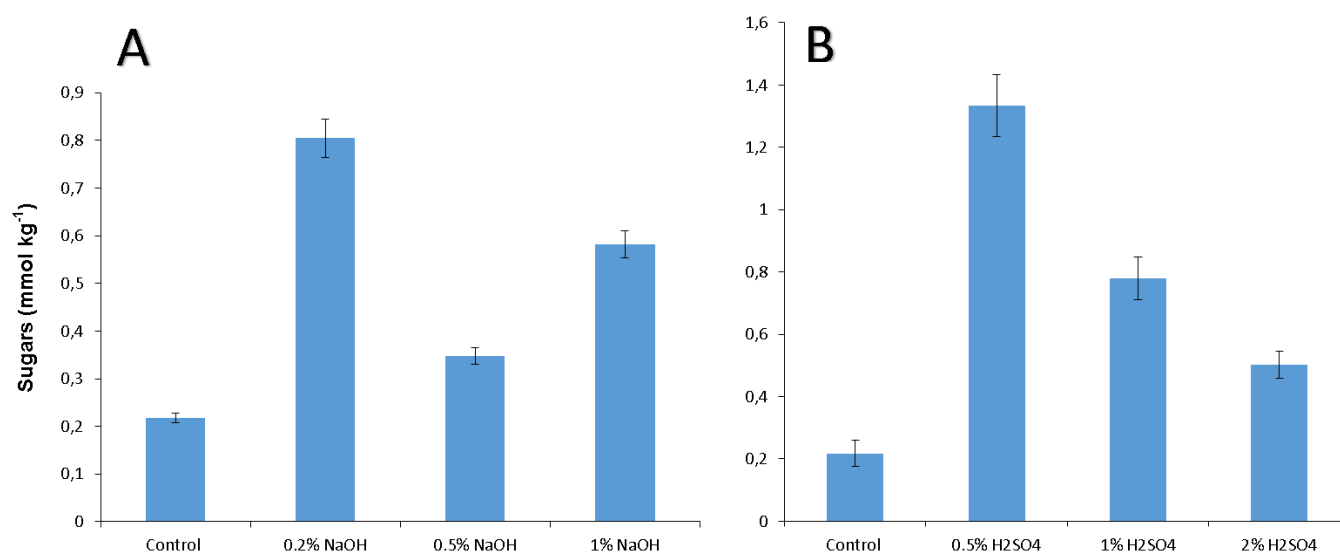
Figure 3. Total sugar yield from foxtail millet shoots subjected to different pre-treatments followed by lignocellulosic saccharification. A. Pre-treatment with sodium hydroxide (0.2%-1%). B. Pre-treatment with sulfuric acid (0.5%-2%). Total sugars (nmol/kg) are shown on Y-axis.

DISCUSSION

The ability of foxtail millet to serve as biofuel source is based mainly on the biomass production followed by inexpensive saccharification treatment. Significant increase in biomass was observed in mycorrhizas only groups (EC and EN) and in combination with PGPB (BEN). The increase in sugar yield was supported at the metabolic level with increase in malate and other metabolites associated with tricarboxylic acid (TCA) cycle. In addition, EC group biomass was associated with increase in 4-aminobutyrate, succinate and asparagine, which are involved in alanine, aspartate and glutamate metabolism (Xia et al. 2015). The alanine and aspartate were also upregulated in maize root inoculated with mycorrhiza (Dhawi et al. 2015) and sorghum roots inoculated with PGPB and mycorrhiza (Dhawi et al. 2016). In the current study, downregulation of monosaccharides such as fructose and glucose in most of the treated groups might be an indication of metabolites shifting towards more complex carbohydrates involved in biomass enhancement. This was supported by the negative or no correlation of fructose and glucose with foxtail millet biomass (Table 2). Another metabolite that positively correlated with biomass was hydroxyvalerate (HV) found in foxtail shoot. The HV source might be poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) known to be available in soil film and degraded by microbial activity especially by some fungal strains (Sang et al. 2002). The production of PHBV in soil is associated with some bacterial strains such as *Methylobacterium organophilum* under nitrogen limitation (Yezza et al. 2006). In our study, the increase of hydroxyvalerate was reflected on foxtail shoots uptake in the groups treated with mycorrhiza only (EC and EN) and showed a positive correlation with foxtail height and fresh and dry biomass. Sarcosine is an inducible metabolite in soil bacteria and found in *Arabidopsis thaliana* where it is known to enhance growth by working as a nitrogen source (Goyer et al. 2004) with other amino acids that showed positive correlation with increase in foxtail millet biomass. Glycine betaine which is known to improve plant abiotic stress tolerance (Ashraf and Foolad 2007)

showed significant increase in EC and EN groups although there was no source for abiotic stress during the experiment. Glycine betaine supports ascorbate peroxidase and catalase activity to preserve lipids from oxidation (Cruz et al. 2013) which might be pertaining to mycorrhiza lipids more than foxtail shoots since GB increased in EC and EN groups more than the other groups.

The total reducing sugars reported in foxtail millet in a previous study was 0.195 mg kg^{-1} (Rao et al. 2011) which is comparable to that reported in our study in the control sample. In the current study, foxtail millet subjected to different microbial inoculations showed significant increase in total sugars as part of metabolites compared to control in most of the treatments, which may be due to metabolic shift towards biomass enhancement. Although the lignocellulosic saccharification methods cannot convert 100% biomass into fermentable sugars (Maurya et al. 2015) alkali pretreatment for switchgrass and big bluestem obtained 88% and 90% total sugars, respectively (Karunanithy and Muthukumarappan 2009, 2011). The lignocellulosic saccharification of biomass for bioethanol production has more environmental and economic benefits in comparison to ethanol production from sugar or starch (Zheng and Rehmann 2014). The fermentation process needs further quantification for sugar content to check the level of fermentable sugars (glucose and xylose), end-fermentation products (ethanol, lactate and acetate), and fermentation inhibitors furfural and hydroxymethyl furfural which might interfere with yeast growth and ethanol production. Foxtail millet showed varied response to microbial inoculation with the combination of mycorrhiza and PGPB showing synergistic effect which is likely due to their positive interactions resulting in not only highest levels of total sugars but also biomass and plant height. In our earlier studies, we showed that maize and sorghum responded much better to the PGPB compared to mycorrhizae and enhanced biomass in maize and sorghum in different soil conditions such as heavy metal contaminated or nutrient poor soil (Dhawi et al. 2015, 2016). In the current study, the lack of heavy metals might be the reason for the less influence of PGPB on the growth of foxtail millet in normal soil.

In conclusion, plant growth promoting bacteria (PGPB) and mycorrhiza enhanced biomass of foxtail millet, a widely produced millet, in soil deficient in nutrients. The best effect on biomass was observed with endomycorrhiza and PGPB combined. Majority of the metabolites were upregulated in ecto/endomycorrhiza group and combined with PGPB. These upregulated metabolites showed positive correlation with plant height and biomass. The total sugar yield was found to be the highest in PGPB and endomycorrhiza treated plants pretreated with 0.5% sulfuric acid which indicates their potential for generating biofuel. Overall, our study demonstrated the utility of PGPB and mycorrhiza to promote plant growth of foxtail millet thereby enhancing its value as a biofuel crop.

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