Plant Molecular Biology Reporter (2019) 37:1–13
https://doi.org/10.1007/s11105-018-1133-8

ORIGINAL PAPER

External Supplement of Impulsive Micromanager *Trichoderma* Helps in Combating CO_2 Stress in Rice Grown Under FACE

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Published online: 4 January 2019

Abstract

The present study aims to explore the alternative way to improve the quality and productivity of rice grown under CO_2 stress through an external supplement of *Trichoderma* as a biofertilizer (BF). The impact of BF-treated rice under elevated CO_2 (eCO_2) was examined by different plant growth parameters, physiological observations, scanning electron microscopy, microbial community profiling and expression levels of stress-related genes. The effect of eCO_2 on percent change in yielding attributes of rice (Heena and Kiran) was found higher in control, whereas it was reduced in the presence of BF. Photosynthetic rate, stomatal conductance and transpiration rate were higher in BF-treated rice under eCO_2 condition. SEM analysis of BF-treated roots exhibits an increase in the number of metaxylem along with its diameter with thicker and rigid sclerenchymatous cells. Expression analysis of stress-related genes showed an increase in their mRNA transcripts under eCO_2 condition. A significant change in the microbial community was found in the rhizospheric region of Heena treated with BF under eCO_2. The current study demonstrates the potential of BF in ameliorating the stress generated as a result of CO_2 enrichment.

Keywords Elevated CO_2 · FACE · Microbial diversity · SEM · Stress genes · *T. reesei*

Introduction

With a rise in global population, the demand of increased productivity of different crops is expected to increase substantially. Since rice is being the most important cereal crop cultivated globally, world rice production in 2015 is set to contract by 4.0 million tons, or 0.5%, below the already disappointing 2014 season (FAO 2015). The decline is expected to be caused

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11105-018-1133-8) contains supplementary material, which is available to authorized users.

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by a combination of various biotic and abiotic factors which influence rice productivity severely. Among the abiotic factor, the rise of global atmospheric concentration of CO_2 resulted from the industrial revolution and various human activities are increasing rapidly and expected to reach optimum level of 550 ppm in the next 40–50 years even with efforts to decrease CO_2 emission (IPCC 2014). This is in parallel with the increase in the demand of rice which is expected to increase by 20 to 30%. To study the effect of elevated CO_2 on different crops, various experiments have been performed around the globe using Free-Air Carbon Dioxide Enrichment (FACE) (free air CO_2 enrichment) system which allows to study the effect of elevated CO_2 on plants grown under natural system (Lesaulnier et al. 2008; Yang et al. 2006). These FACE experiments expose vegetation to elevated (CO_2) at 475–600 ppm, enclosing a large number of species including rice. It has been observed that exposure to elevated (CO_2) resulted in a 31% increase in the light-saturated leaf photosynthetic rate and a 28% increase in the diurnal photosynthetic carbon assimilation (Ainsworth and Long 2005).

The effect of rising CO_2 on global production of rice has been studied by various researchers (Guo et al. 2015; Okubo et al. 2015; Usui et al. 2015). In rice, it has been observed that at elevated CO_2 levels, the productive tiller ratio was decreased (Wang et al. 2015) along with reduced percentage of fertile spikelets (Yang et al. 2006). Interestingly, since the number of tillers and panicles per area was significantly higher at elevated (CO_2), yield was increased despite the negative effects on productive tiller ratio and spikelet fertility (Usui et al. 2015).

Plant growth is highly affected by its rhizospheric inhabitants. It has been reported that with an increase in atmospheric CO_2, the release of root derived carbon compounds increases quantitatively and qualitatively (Zak et al. 2000). The mycorrhiza present in the rhizosphere helps in plant nutrient foraging, plant carbon allocation and architecture, changes in soil structure and soil carbon storage (Rillig et al. 2002; Staddon et al. 2002). With the presence of surplus carbon in the soil, the microbe has ample opportunity to utilize them; hence an increase in the growth rate is witnessed in elevated CO_2 condition (Lesaulnier et al. 2008). It has been reported that the increase in growth rate is basically because of mycorrhizal fungi which form symbiotic associations with plants and depend directly on its photosynthetic products. As a result, fungi will be affected by any CO_2-induced changes in the carbon allotment from their hosts (Hodge 1996; Zak et al. 2000).

It is a well-known fact that species of genus *Trichoderma* are known for their plant growth promotion and biocontrol abilities by one or more direct and indirect mechanisms. Their highly reproductive ability coupled with efficiency in utilizing nutrients along with strong aggressiveness against plant pathogenic fungi makes them a preferred fungus for plants in the rhizosphere (Contreras-Cornejo et al. 2015; Mahfooz et al. 2016; Mishra and Nautiyal 2009). A recent report indicated its effectiveness in enhancing the amino acid and mineral nutrients availability of the plant even in Arsenic (As)-contaminated field (Tripathi et al. 2015). It has been reported that some species of *Trichoderma* can accelerate its host growth under abiotic stresses through various mechanisms (Shukla et al. 2012). This includes regulation of stomatal aperture and leaf transpiration through an abscisic acid (Contreras-Cornejo et al. 2015) or through expression of several genes which delays stress-induced changes in stomatal conductance and net photosynthesis (Bae et al. 2011).

In view of the increasing CO_2 in the atmosphere and the plant growth promoting capabilities of *Trichoderma*, we attempted to evaluate whether external supplement of biofertilizer can ameliorate CO_2 stress in rice. Two contrasting rice varieties (Heena and Kiran) were selected and examined for different physiological parameters in the presence of BF under elevated CO_2 condition. Moreover, SEM analysis of root micrographs and differential expression of stress responsive genes were also analysed. Additionally, alteration of microbial communities in the rice rhizospheric region was also investigated under elevated CO_2 conditions.

Materials and Methods

FACE Site The field experiments were conducted at FACE site located at National Botanical Research Institute, Lucknow (26°51′28.55″ N, 80°56′52.22″ E). Six hexagonal rings, each with a diameter of 10 m, were established where three randomly allocated for elevated CO_2 treatments (eCO_2), while remaining three were used for ambient CO_2(aCO_2). The CO_2 concentration was maintained at ~550 ppm (in eCO_2) during the daylight hours by releasing compressed CO_2 gas from pipes at the perimeter of rings. Wind direction was continuously sensed so that gas was released only on the upwind side of the ring. The rings were covered with nylon net to protect the crop from birds. The CO_2 concentration in elevated and ambient CO_2 rings was monitored and controlled by a computer program (SCADA, Canada).

Crop Cultivation The seedlings of two contrasting drought-resistant and sensitive rice cultivars (*Oryza sativa* subspecies "indica") namely Heena and Kiran (Agrawal et al. 2016) were manually transplanted in rings on August 8, 2014, and July 17, 2015. Before transplantation, the seedlings of the cultivars were pre-treated with *Trichoderma reesei* MTCC5659 (Mishra and Nautiyal 2013) as biofertilizer (BF) at a concentration of ~6.0×10^6 cfu/ml in sterile distilled water for 60 min (Roberts et al. 2010).The seedlings of the two varieties were transplanted at the density of three seedlings per hill in four cone shaped plots (36 m^2) where two plots consist of BF treated and remaining two served as control in both eCO_2 and aCO_2 rings. All the plots were submerged in water with a brief aeration to create

aerobic condition for the BF through ploughing. The average temperature and relative humidity (RH) of two seasons were recorded 32.0°C and 44.6%, respectively. Different yielding attributes such as number of tillers, total panicle, number of spikelets per panicle, total spikelets and grain weight were recorded (from randomly selected 24 plants of each treatment) after vegetative stage of crop.

Photosynthesis and Stomatal Conductance Photosynthetic rate and stomatal conductance were measured using a portable photosynthetic gas exchange system (LI-6400, Licor Inc.). Fully expanded flag leaves were used for observation at vegetative stage. During the observations, photosynthetic photon flux density was maintained at 800–900 mmol m^{-2} s^{-1} with a leaf chamber temperature of 32°C. The average leaf temperature within the chamber was 32.2 ± 0.9°C and 33.4 ± 0.7°C for Heena and Kiran, respectively. The average relative humidity of leaf for Heena and Kiran was slightly higher (47.5% and 47.8%, respectively) with respect to the relative humidity of surroundings (45.4% and 46.1%). The average CO_2 in the surroundings was ~350.0–400.7 ppm, whereas in the elevated rings, it was maintained at ~550 ppm. All the observations were performed with three biological as well as five technical replicates.

Scanning Electron Microscope SEM analysis was performed to confirm the presence of BF and its effect on rice root under eCO_2 condition. The main root of control and treated rice cultivars (at vegetative phase) was sampled randomly and sectioned transversally. Sections of roots were placed in to a small cap tube vials, fixed for 1 h in 2.5% (*v*/*v*) glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma). This was followed by 40-min treatment in 1% (*w*/*v*) osmium tetroxide (OsO_4, Sigma). The root cells were dehydrated by sequential passage through increasing concentration of ethanol (30–100%) in 10% increments, dried in a critical point dryer, coated with gold-palladium for 60 s in a Pelco 3 sputter coater (SC 7620, mini sputter coater, Quorum Technology Ltd., UK), and visualized using a scanning electron microscope (Quanta 450FEG, FEI, The Neitherland) as described earlier (Pathan et al. 2010). All the experimental work was performed in triplicate.

Inductively Coupled Plasma Mass Spectrometry Micro-elemental depositions of BF-treated rice grains of both varieties were analysed by Inductively Coupled Plasma Mass Spectrometry (Agilent 7500CX, Agilent Technologies, Palo Alto, CA, USA). Unpolished seeds (0.3g) collected from different plants (as per the treatment) were crushed into powdered form with the help of mortar and pestle and were digested with 3.0 ml of HNO_3 (Sigma) and 1.0 ml of H_2O_2 (Sigma). Reference standards of chromium (Cr), manganese (Mn), iron (Fe), nickel (Ni), copper (Cu), zinc (Zn), selenium (Se) and molybdenum (Mo) were taken from E-Merck, Germany. All experiments were performed in triplicate.

Quantitative Reverse-Transcriptase PCR All the experiments were performed under RNase-free conditions. Total RNA was extracted from three independent samples of rice tissue (flag leaf) at panicle stage using TRI reagent (Sigma) according to the manufacturer's protocol. The concentration of RNA was measured by NanoDrop (Thermo Fisher Scientific, USA). Rice tissue cDNA was synthesized using 1 μg RNA and the SuperScript III First-strand synthesis system (Thermo Fisher Scientific, USA) as per the manufacturer's instructions. Quantitative RT-PCR was carried out on a Stratagene Mx3005P instrument (Agilent Technologies, USA). Rice cDNA was diluted in the ratio of 1:10 with nuclease-free water. One microlitre of cDNA was mixed with 8.0 μl of Brilliant III Ultra-Fast SYBR Green QPCR master Mix (Agilent Technologies, USA) and 1.0 μl of primer (10 pmol forward/reverse). The final volume was adjusted to 15 μl with nuclease-free water. The primers used for amplification are listed as supplementary information 1. Thermal cycling was carried out as follows: The first segment of the amplification cycle consisted of denaturation at 95°C for 10 min; the second segment consisted of denaturation (15s at 95°C), primer annealing (30s at 50-60°C) and extension (30s at 72°C) for 40 cycles. The third segment consisted of melting curve programme (95°C for 5s, 58°C for 15s). The final segment consisted of cooling to 40°C. The threshold cycle (C_T) was used to represent the relative mRNA amounts. All samples were run and analysed in triplicate using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Microbial Diversity Analysis Using Carbon Source Utilization Pattern Biolog Eco and MT plates (Biolog, Inc., Hayward, CA, USA) were used to determine the carbon source utilization pattern of rice rhizospheric soil samples in ambient as well as elevated conditions. Rhizospheric soil samples (1.0 g) of rice plant collected from FACE were shaken in 99 ml of sterile 0.85% saline water for 60 min, and a final dilution 10^{-3} was made. After incubation, 150 μl of diluted samples was inoculated in each well of Biolog Eco and MT plates and incubated at 28 ± 2°C. The rate of utilization is observed by the reduction of tetrazolium, a redox indicator dye, which changes from colourless to purple. Data were recorded from day 1 to day 7 at 590 nm. Microbial activity in each microplate, expressed as average well colour development (AWCD) was determined (Garland 1996). Diversity and evenness indexes were calculated as described earlier (Nautiyal 2009). Principal component analysis (PCA) was performed on data divided by the average well colour development (AWCD) (Garland and Mills 1991).

Statistical Analysis

The Student's *t* test was used for statistical analysis of the data in the experiments of different yielding attributes and gas-

exchange parameters. Analysis of variance and the means were analysed by Fisher's least significant difference test using statistical analysis software (SPSS 16.0). In all the experiments, two-sided *t* tests were performed, and quantitative differences between the two groups of data for comparison were deemed statistically significant at $P < 0.05$ as indicated for each comparison in the tables.

Results

Yielding Attributes of Rice Plant Grown Under eCO_2 Condition

The grain yielding potential of Heena was analysed under eCO_2 condition and was compared with Kiran. The major parameters taken were number of tillers, total panicle, number of spikelets per panicle, total spikelets, and grain weight. A percent change of 18.6, 6.5, −16.1, −16.3, and −10.8% was observed within the ambient and elevated CO_2 conditions in Heena without treatment, whereas in treatment with BF, we observed 2.1, −5.8, 13.6, 6.4, and 16.6% change in aforesaid parameters (Table 1). In non-treated Kiran, we observed 53.7, −21.3, −16.7, −35.9 and 4.8% change in yielding attributes under ambient and elevated CO_2 conditions, whereas with the treatment of BF, the percent change was 21.8, 31.6, 3.0, 26.9 and 3.8. While comparing the yielding attributes of both the varieties, we can conclude that Heena responded better in elevated condition with bio-fertilizer treatment.

Physiological Parameters of Rice Plant Grown under eCO_2

To evaluate the effect of CO_2 stress, we measured the photosynthetic responses of rice in ambient and eCO_2 conditions with BF treatment. In Heena, an increase in photosynthetic rate (25.7 μmol/m^2/s) along with internal CO_2 of leaf (209.54 μmol/m^2/s) and water use efficiency (23.3%) was observed as compared to the treated ambient control, whereas respiration rate (−1.6 μmol/m^2/s) was found reduced. Importantly, stomatal conductivity (0.06 mmol/m^2/s), and transpiration rate (2.3 mmol/m^2/s) which was generally reported to be increased initially in elevated condition, was also reduced in Heena by the interaction of BF. Similar results were obtained in Kiran as well where photosynthetic rate (19.6 μmol/m^2/s),

Table 1 Averages for grain yield and yielding components for two rice cultivars, Heena and Kiran, grown in situ at ambient and elevated CO_2 concentrations using FACE methodology

		Tillers	Total panicle	Spikelet per panicle	Total spikelet	Weight per grain (mg)	Yield (g/m^2)
Heena	Ambient control	11.8	18.4	11.2	219.8	10.66	590.74
	Elevated control	14	19.6	12.4	183.8	9.5	620.70
	% change	18.64	6.52	− 16.07	− 16.4	− 10.88	5.07
	Ambient treated	19	17	13.2	225	12.42	641.3
	Elevated treated	19.4	16	15	239.4	14.48	710.32
	% change	2.11	− 5.88	13.64	6.4	16.59	10.76
Kiran	Ambient control	10.8	17.8	10.8	194.6	11.9	490.2
	Elevated control	16.6	14	9	124.8	11.32	495.94
	% change	53.70	− 21.35	− 16.67	− 35.87	− 4.87	1.17
	Ambient treated	11	11.4	13.2	150	12.46	552.14
	Elevated treated	13.4	15	13.6	190.4	12.94	571.3
	% change	21.81	31.58	3.030	26.93	3.85	3.47
Cultivar		***	**	***	***	ns	***
CO_2		***	ns	ns	ns	ns	**
Treatment		***	**	***	*	***	***
Cultivar × CO_2		*	ns	**	ns	ns	*
Cultivar × treatment		***	ns	*	ns	***	*
CO_2 × treatment		*	*	**	**	***	*
Cultivar × CO_2 × treatment		ns	**	ns	*	*	ns

% Change was determined as (elevated − ambient)/ambient × 100%

NS non-significant

*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$

internal CO_2 (261.8 μmol/m^2/s) and water use efficiency (23.3%) was increased, whereas respiration rate (−1.4 μmol/m^2/s) was decreased. The BF again proves its potential in reducing stomatal conductance (0.09 mmol/m^2/s) and transpiration rate (3.6 mol/m^2/s) in Kiran under eCO_2 (Fig. 1).

Mineral Element Accumulation in Rice Grains

Micro-element investigation of rice seeds (both varieties) revealed that significant amount of elements was deposited in plants treated with BF. Among the nutritional elements, the level of Mn, Fe, Ni, Cu, Zn and Mo was found to be higher in treated Heena under eCO_2, whereas maximum amount of Cr and Se was recorded in treated Heena at ambient condition of CO_2 (Table 2). The second highest deposition of Fe, Cu, Zn and Mo was noted in ambient-treated Heena, along with Cr and Se in treated Heena at eCO_2. The least amount of elements (Cr, Mn, Fe, Ni, Cu, Zn, Se, Mo) were recorded in non-treated Kiran at eCO_2 with an exception of Fe which was minimum in non-treated Kiran at aCO_2.

Scanning Electron Microscopy of Root Treated with Biofertilizer

The result from LI-COR analysis prompted us to look into the roots treated with the BF. The localization of fungal spores was clearly evident in the SEM micrographs (Figs. 2 and 3). Detailed analysis of SEM images of Heena revealed irregular aerenchymatous cells in eCO_2 condition (Fig. 2a); however, this irregularity was reversed with the BF treatment which is evident as well arranged aerenchymatous cells under the same condition (Fig. 2b). Other important observation from the images exhibited an increase in the number of metaxylem along with its diameter in the BF-treated roots (Fig. 2f) as compared

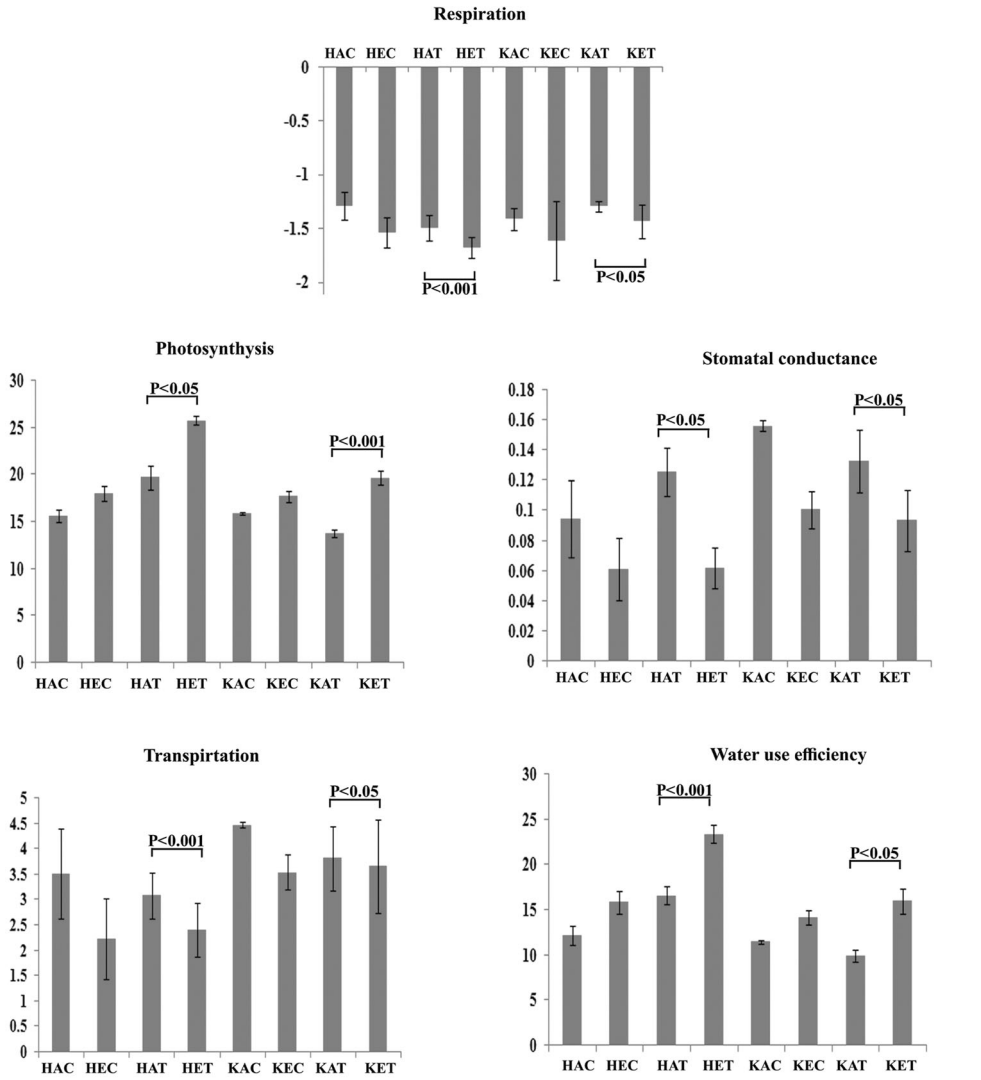


Fig. 1 Plant physiological response in ambient and elevated CO_2 with and without BF treatment. Error bars show standard error of the mean. Lines on the bars indicate that treatment is significantly ($P < 0.05$, $P < 0.001$ and NS-non-significant) different from ambient and elevated Heena and Kiran

HAC= Heena ambient control; HEC= Heena elevated control; HAT= Heena ambient treated; HET= Heena elevated treated
KAC= Kiran ambient control; KEC= Kiran elevated control; KAT= Kiran ambient treated; KET= Kiran elevated treated

Table 2 Micronutrient deposition in grain of for two rice cultivars, Heena and Kiran, grown in situ at ambient and elevated CO_2 concentrations using FACE methodology

	Cr	Mn	Fe	Ni	Cu	Zn	Se	Mo
ATK	2.44 ± 0.119^{b}	149.53 ± 4.5^{bc}	151.98 ± 1.13^{c}	$3.133 \pm .19^{cd}$	38.54 ± 2.6^{bc}	275.12 ± 1.8^{bc}	$5.2 \pm .53^{b}$	$12.37 \pm .4^{b}$
ATH	$3.61 \pm .48a$	155.51 ± 1.4^{bc}	337 ± 1.87^{b}	$3.523 \pm .22^{bc}$	52.05 ± 3.6^{a}	302.12 ± 2.7^{b}	$6.69 \pm .18^{a}$	14.72 ± 1.3^{a}
ACK	$1.52 \pm .23^{bcd}$	131.56 ± 1.3^{cd}	85.61 ± 3.2^{c}	$2.611 \pm .23^{d}$	38.3 ± 2.5^{bc}	$264.79 \pm .84^{c}$	$5.18 \pm .06^{b}$	$10.39 \pm .9^{cd}$
ACH	$1.66 \pm .072^{bcd}$	158.78 ± 2.5^{b}	291.44 ± 3.3^{b}	$3.2 \pm .2^{bcd}$	43.03 ± 2.7^{b}	$279.07 \pm .163^{bc}$	$6.28 \pm .58^{a}$	11.82 ± 1.9^{bc}
ETK	$1.13 \pm .34^{cde}$	124.13 ± 2.8^{d}	94.79 ± 2.3^{c}	$3.89 \pm .21^{b}$	37.31 ± 1.9^{bc}	$261.73 \pm 1.2_{c}$	$5.32 \pm .9^{b}$	$11.57 \pm .46^{bcd}$
ETH	$2.13 \pm 1.47bc$	187.17 ± 1.49^{a}	518.94 ± 2.2^{a}	$5.47 \pm .9^{a}$	52.29 ± 1.2^{a}	340.34 ± 1.3^{a}	$6.33 \pm .6^{a}$	$15.04 \pm .33^{a}$
ECK	$0.11 \pm .12^{e}$	120.31 ± 1.88^{d}	87.8 ± 3.8^{c}	$2.54 \pm .1^{d}$	$33.54 \pm .7^{c}$	222.01 ± 1.7^{d}	$5.14 \pm .07^{b}$	$10.03 \pm .47^{d}$
ECH	$0.6 \pm .38^{de}$	132.02 ± 1.87^{cd}	165.46 ± 2.5^{c}	$3.01 \pm .5^{cd}$	39.9 ± 1.8^{bc}	270.23 ± 1.7^{c}	$5.08 \pm .29^{b}$	$11.09 \pm .21^{bcd}$

Values are mean of three replicates with ± standard error are integrated. Means followed by the same letters within the column are not significantly different according to Duncan's multiple range test ($P < 0.05$)

ATK ambient-treated Kiran, *ATH* ambient-treated Heena, *ACK* ambient control Kiran, *ACH* ambient control Heena, *ETK* elevated treated Kiran, *ETH* elevated treated Heena, *ECK* elevated control Kiran, *ECH* elevated control Heena

to eCO_2 control (Fig. 2e). Similarly, thin sclerenchymatous cells were obtained in the elevated condition (Fig. 2c) which becomes thicker and rigid in the BF-treated roots under eCO_2 condition (Fig. 2d). The same pattern was also exhibited by Kiran, where distorted morphology of aerenchymatous cells was evident in the eCO_2 condition (Fig. 3a) which was later normalized to its shape with the help of BF (Fig. 3b). Number of metaxylem was also increased in the drought-sensitive Kiran treated with BF (Fig. 3f).

Real-Time Quantitative PCR of Stress-Related Genes

To examine the impact of external supplement of *Trichoderma* in CO_2 stress condition, the expression levels of stress responsive genes coding for dehydrin protein (*dha*), glutathione S-transferases enzyme (*gst*), late embryogenesis protein (*lea*), apical meristem protein (*nam*) and universal stress protein (*usp*) were analysed. We decided to select them due to their potential role during stress response of cells as shown in previous studies (Dixon et al. 2002; Hanin et al. 2011; Nishimura et al. 2002; Sharma et al. 2014; Zhu et al. 2015). The transcripts of all six genes were recorded in the normal condition. As shown in the heat map (Fig. 4), the mRNA levels of *dhn*, *nam*, *lea* and *gst* were significantly up-regulated (four fold) in HET (Heena elevated treated). Similar expression levels were documented for *gst* and *lea* genes in KET. Further analysis of heat map revealed a three fold up-regulation of *nam* (Heena ambient treated), *gst* (Kiran ambient treated) and *usp* (Kiran elevated treated).

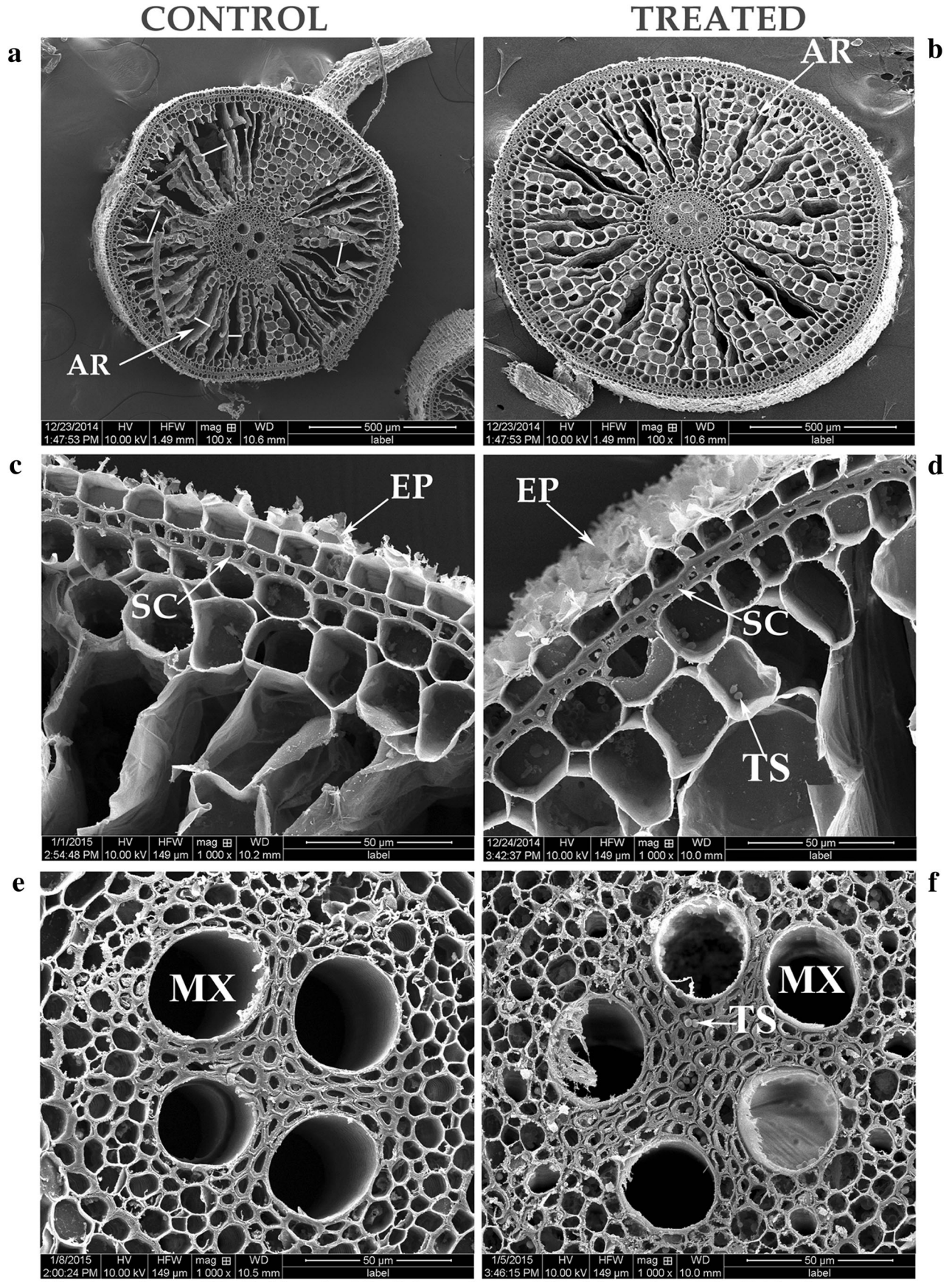
Microbial Community Substrate Utilization Profile

Microbial communities sampled from ambient and eCO_2 rings were able to utilize all the 31 carbon sources available in ECO plate. On the basis of Average Well Colour Development (AWCD), FACE soil of Heena rhizosphere treated with BF showed highest metabolic activity, whereas the lowest was obtained in Kiran cultivated under aCO_2 condition without BF (Fig. 5). Since the Biolog datasets are too large, we further subjected the data sets for principal component analysis, a mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in the data set. The percentage of variance explained by the first component is 44.8%, whereas it was 16.5% for the second. The result of PCA clearly showed that carbon substrate utilization profiling of microbial communities from BF-treated Heena under eCO_2 condition is distant from remaining treatments as it clustered separately (Fig. 6a). We further analysed the microbial community of FACE soil using complex natural substrates which are generally found in plant root exudates; this was achieved by using Biolog MT plates (Fig. 6b). Maximum substrate utilization was recorded for glucose, which was followed by proline, casein and chitin by microbial communities in FACE soil of BF-treated Heena. The second highest consumption of complex substrate was recorded for proline in FACE soil of Kiran, whereas minimum utilization was found in non-FACE soil.

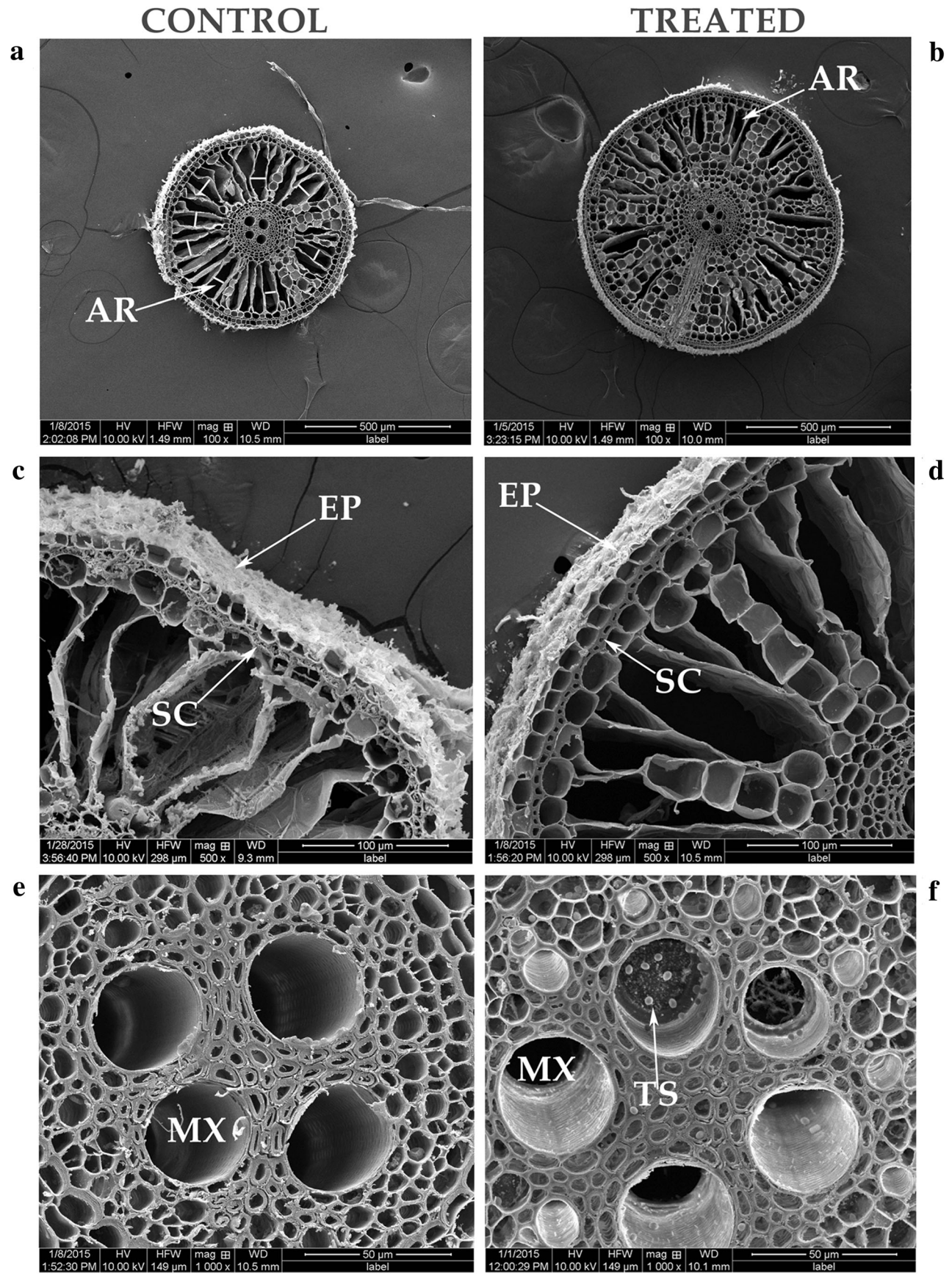
Discussion

The ability of rice to respond in eCO_2 condition has been studied by many researchers (Abdelrahman et al. 2016; Gao

Fig. 2 Scanning electron micrographs of main root transverse sections of Heena cultivar. Control represents non-treated root under elevated condition. Treated represents treatment with biofertilizer *Trichoderma*. Complete root section at 100X showing distorted and uniform morphology of aerenchymatous cells in control (**a**) and treated (**b**) plant tissues, respectively, rigid and smooth sclerenchymatous cells along with fine epidermis in control (**c**) and treated (**d**) plant sections, respectively, number of metaxylem were increased from four to five in treated root section (**f**) as compared to control (**e**)



AR= Arenchyma; EP= Epidermis; SC= Sclorenchyma; MX= Metaxylem; TS= *Trichoderma* spores



AR= Arenchyma; EP= Epidermis; SC= Sclorenchyma; MX= Metaxylem; TS= *Trichoderma* spores

◀ **Fig. 3** Scanning electron micrographs of main root transverse sections of Kiran cultivar. Control represents non-treated root under elevated condition. Treated represents treatment with biofertilizer *Trichoderma*. Complete root section at 100X showing distorted and uniform morphology of aerenchymatous cells in control (**a**) and treated (**b**) plant tissues, respectively, rigid and smooth sclerenchymatous cells along with fine epidermis in control (**c**) and treated (**d**) plant sections, respectively, number of metaxylem were increased from four to five in treated root section (**f**) as compare to control (**e**)

and Lan 2016). Simultaneously, there are numerous reports on the biocontrol and plant growth promotion abilities of *Trichoderma* (Abdelrahman et al. 2016; Alcantara et al. 2016; Contreras-Cornejo et al. 2016; Mishra et al. 2014). However, there are only few reports where *Trichoderma* has been utilized for reducing abiotic stress (Ahmad et al. 2015; Mastouri et al. 2010; Tripathi et al. 2015). Amelioration of CO_2 stress by any microbe is yet not reported; hence, in the present study, the efficacy of *Trichoderma reesei* MTCC5659 as a bio-fertilizer in diminishing the stress, induced as a result of higher atmospheric CO_2 concentration, was investigated.

The obtained data suggested a lesser percent change in different yielding parameters in BF-treated Heena under CO_2 stress as compared to its ambient counterpart. On the other hand, Kiran showed higher percentage change in elevated CO_2 condition under treatment. To find a reasonable explanation for this, different physiological parameters in rice were studied. Measurement of leaf gas exchange showed an enhanced photosynthetic rate under elevated condition along with a reduced stomatal conductance as compare to Heena. Many reports suggested a positive correlation between photosynthetic rate and stomatal conductance in rice (Hirasawa et al. 1988; Kusumi et al. 2012); however, this is not true in elevated condition. We observed a reduced stomatal conductance rate in elevated condition in Heena as well as in Kiran with BF. This might be due to the fact that high concentration of CO_2 might slow down its movement into stomata which already had excess internal CO_2 without effecting photosynthetic carbon gain. Furthermore, reduced transpiration rate was also evident in treatment under elevated CO_2 in both Heena and Kiran. The involvement of *Trichoderma* spp. in improving growth of *Arabidopsis* seedlings under salt stress through enhanced root development, osmolite production and Na^+ elimination through root exudates has been reported (Contreras-Cornejo et al. 2014). SEM analysis of root micrographs revealed intact sections in eCO_2 condition with treatment, whereas distorted morphology of sections was obtained in non-treated plants. This might have resulted in proper flow of water along with other essential nutrients required for combating stress more efficiently under CO_2 stress. Our result is well supported by an earlier finding where microscopic examinations revealed cell membrane cleavage and its increased permeability under drought stress condition (Blokhina et al. 2003). The authors suggested that the concentrations of appropriate solutes that could preserve membrane were not sufficient and the plant was not able to adjust osmotically. Additionally, the numbers of metaxylem were also increased from 4 to 5 due to various adaptation strategies induced by BF under elevated conditions. Our results are in accordance with earlier finding where a number of metaxylem were increased (108%) when the plants are treated with *T. asperellum*, *B. Pyrrocinia* + *P. fluorescens*, *B. pyrrocinia* and *P. fluorescene* (Rêgo et al. 2014). Additionally, it has also been reported that *Trichoderma* modulates stomatal aperture and leaf transpiration through an abscisic acid-dependent mechanism in *Arabidopsis* (Contreras-Cornejo et al. 2015). This might be true in the case of rice as both are monocots.

We further examined that the level of micro-nutritional elements in treated rice grains was higher in Heena even under elevated condition. It has been reported earlier that *Trichoderma* possesses a cysteine-rich cell wall protein that has a major role in lateral root growth along with hair

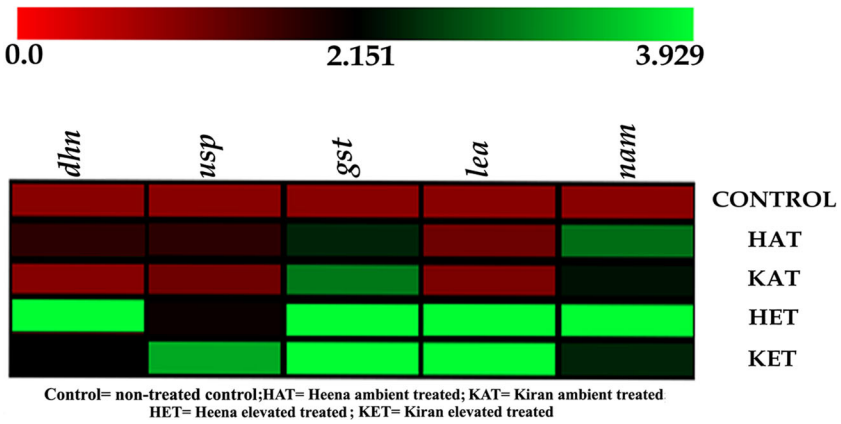


Fig. 4 cDNA real-time PCR analysis-related heat map showing magnitude of expression among the treatments. Fluorescence intensity of gene products determined by real-time SYBR green as a reporter. Selected stress inducible genes are listed above the heat map. Each horizontal row represent the individual treatment. The fluorescence range from high (green) to low (red) is indicated by the coloured bar and reflects the degree of fluorescence intensity upon gene expression

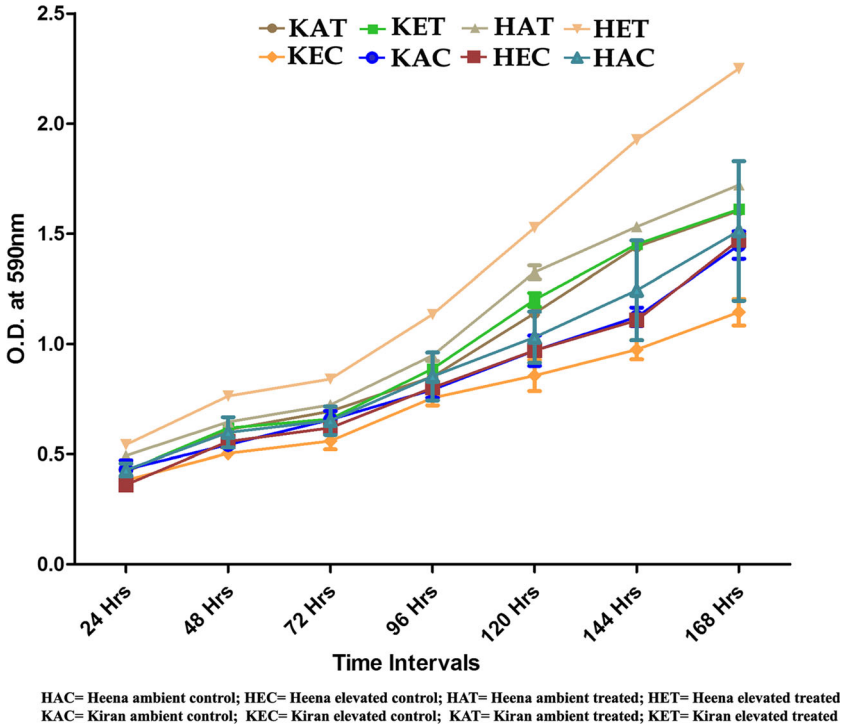
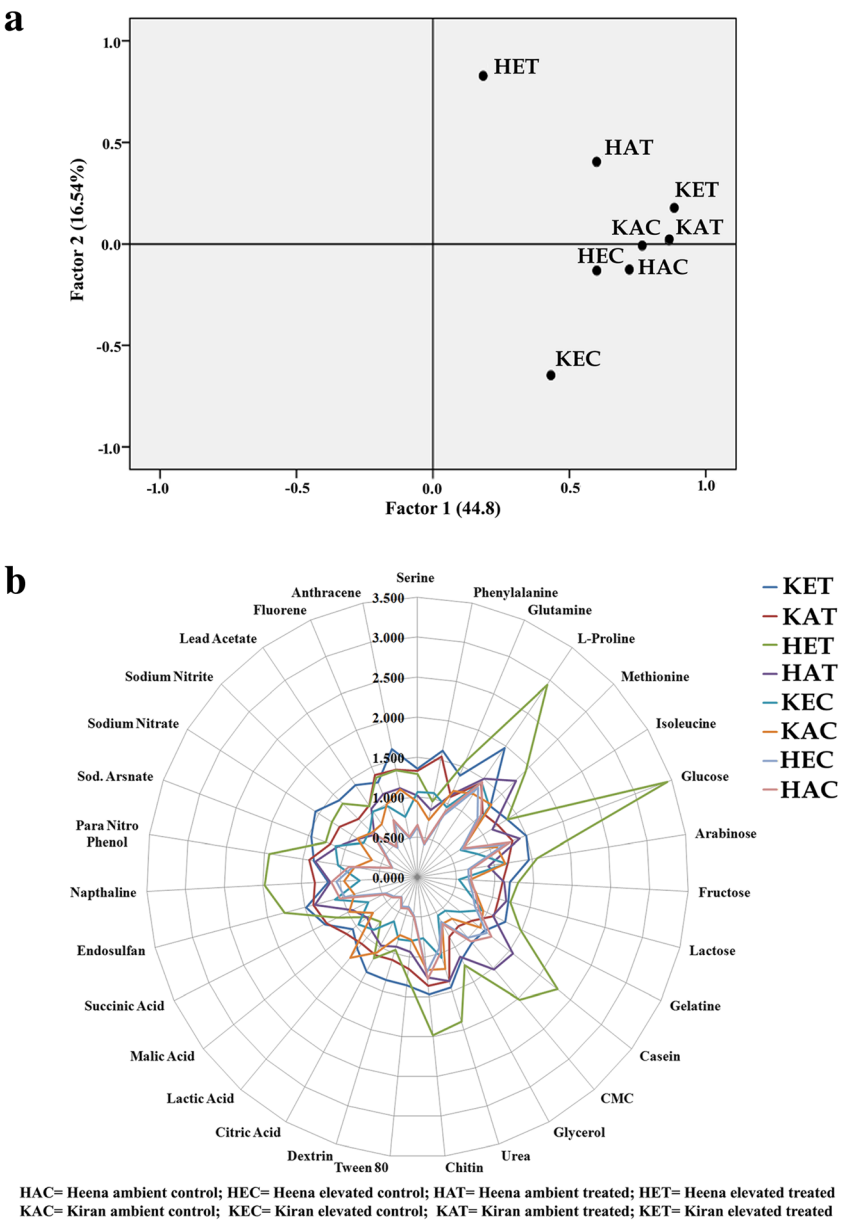


Fig. 5 Average well colour development (AWCD) based on substrate utilization pattern on Biolog Eco plates by rice rhizosphere microflora. The rate of utilization is indicated by the reduction of tetrazolium, a redox indicator dye, which changes from colourless to purple. Data were recorded for days 1–7 at 590 nm

formation and elongation which results in the enhancement of total absorptive surface. This enhancement resulted in higher uptake and translocation of nutrients in the host plant (Samolski et al. 2012). Similarly, microbial siderophore (Fe ion chelating agent) binding sites which is prominently present in *Trichoderma* did not compete only for Fe ions, but it was also able to bound other cations such as divalent i.e. Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}(Albrecht-Gary and Crumbliss 1998); trivalent i.e. Mn^{3+}, Co^{3+} and Al^{3+} and others i.e. Th^{4+}, U^{4+} and Pu^{4+}. This could probably be the reason why we found higher concentration of various micronutrients in BF-treated Heena grain under eCO_2 condition. Similar result was reported in tomato where an increase in the nutrient uptake by *T. harzianum* SQR-T047 was witnessed (Li et al. 2015).

We further analysed the expression of certain stress responsive genes through real-time PCR. *DHN* (group II of late embryogenesis abundant protein) is reported to accumulate typically in maturing seeds or are induced in vegetative tissues following salinity, dehydration, cold and freezing stress (Hanin et al. 2011). An enhanced expression level of *dhn* was observed in Heena under eCO_2 condition with BF treatment which confirms its role even in CO_2 stress. Our results are in accordance with earlier report where an enhanced expression level of *dhn* transcripts was reported with dose-dependent response to improve drought tolerance in rice genotypes with the help of *T. harzianum* (Pandey et al. 2016). *LEA* (group 5) is located at various organelles in plant such as embryo cells (Amara et al. 2013) and in mitochondria (Salleh et al. 2012) where it accumulates in response to various abiotic stresses. We observed higher expression of *lea* (group-V) in eCO_2 condition in both rice varieties in the presence of BF. The possible reason may be attributed to the fact that treatment of BF may enhance the resistant potential of plant against oxidative burst generated in response to high concentration of CO_2 (Liu et al. 2015). Glutathione S-transferases (GSTs) are ubiquitous enzymes encoded by a large family of genes, which plays an important role in cellular detoxification to a wide variety of endobiotic and xenobiotic substrates by conjugating the tri-peptide glutathione (Edwards et al. 2000). Higher expression level of *gst* mRNA in the eCO_2 condition under treatment of BF was obtained in both varieties. Again, the enhanced expression of *gst* mRNA reflects activation of antioxidant machinery to counter the CO_2 stress (Brotman et al. 2013). Our result collaborated well with a recent study where an increased level of *gst* mRNA in *Physcomitrella patens* under eCO_2 condition was observed (Shinde et al. 2015). Apical meristem gene is thought to play an important role in the formation of the apical meristem and in leaf adaxial cell specification. Moreover, it is also involved in the regulation of different phytohormones that plays a crucial role in plant development under abiotic stresses (Greve et al. 2003; Jensen et al. 2010). The expression of *nam* (no apical meristem) was higher in Heena treated with BF. The possible reason behind the higher expression of *nam* gene might be attributed to fact that these factors play a crucial role in complex singling processes during plant stress responses. Universal stress protein is likely known to play a significant role in response of various abiotic stresses (Loukehaich et al. 2012), but in the case of eCO_2, its expression is not yet reported. In this study, the expression of *usp* was recorded maximum in KET, which was followed by



HAC= Heena ambient control; HEC= Heena elevated control; HAT= Heena ambient treated; HET= Heena elevated treated
KAC= Kiran ambient control; KEC= Kiran elevated control; KAT= Kiran ambient treated; KET= Kiran elevated treated

Fig. 6 **a** Principal component analysis (PCA) of microbial carbon source utilization pattern on Biolog Eco plates (Biolog, Inc., Hayward, CA, USA). Control (C) represents non-treated root under ambient or elevated condition. Treated (T) represents treatment with biofertilizer *Trichoderma*. Biolog Eco plates were used to determine the effect of bio-fertilizer on the microbial diversity in rhizosphere of rice grown in eCO_2 condition. Data on Biolog Eco plates were recorded every 24 h at 590 nm with an automated microplate reader. At fourth day, PCA was performed on blank subtracted data divided by the average well colour development (AWCD). **b** Figure showing the microbial carbon substrate utilization pattern of complex substrates in Biolog MT. plates

HET. A reasonable explanation for lower expression of *usp* in treated Heena in comparison to Kiran under eCO_2 might be attributed to its drought-resistant potential.

Since plants are grown under sea of microbes, it has been reported that in the presence of plant growth promoting bacteria and fungi, an enhanced resistance to biotic and abiotic stresses can be achieved (Lugtenberg and Kamilova 2009). There are contradictory reports regarding the effect of microbial communities under eCO_2 condition. Some researchers reported only minor changes in the microbial community (Kanerva et al. 2008; Lesaulnier et al. 2008); however, others witnessed an increase in microbial growth rate under eCO_2 condition (Blagodatskaya et al. 2010). The organic substrates secreted from plants roots have been reported to alter the function and composition of the soil microbial communities (Zak et al. 2000). During exploration of microbial diversity, and carbon utilization pattern, the metabolic activity of microbial communities was higher in the BF-treated Heena rhizospheric soil from FACE. The extra carbon present in soil might have been utilized by the BF which resulted in maximizing its activity. Our results are in accordance with earlier report which strongly supported that as compared to bacteria, fungi responded more to increased root growth under elevated CO_2 (Lesaulnier et al. 2008).

Conclusion

With the ever increasing atmospheric CO_2 concentration, a reliable biological mitigation tool is the needed for proper rice

productivity along with nutritional value. Our study demonstrates the potential of *T. reesei* in minimizing the stress generated as a result of CO_2 enrichment. BF-treated rice minimized the change induced as a result of elevated CO_2 which is reflected in yielding attributes. Significant decrease in the stomatal conductance and water use efficiency also highlighted the efforts induced by the BF in modulating physiological changes in rice. We provide microscopic evidence of increase in the number of metaxylems which resulted in better uptake of water and other nutrients. Overall, our observations revealed that amendment of BF efficiently reduced the impact generated by higher concentration of CO_2 through diverse stress amelioration approaches.

Acknowledgments The authors are grateful to Dr. P. N. Saxena (CSIR-Indian Institute of Toxicology Research, Lucknow) for SEM micrograph imaging.

Funding Information This work is supported by funding from Science and Engineering Research Board, New Delhi (GAP3349), and Council of Scientific and Industrial Research (PSC0112).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interest

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