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Trichoderma inoculation augments grain amino acids and mineral nutrients by modulating arsenic speciation and accumulation in chickpea (*Cicer arietinum* L.)

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ABSTRACT

Trichoderma reesei is an industrially important fungi which also imparts stress tolerance and plant growth promotion in various crops. Arsenic (As) contamination of field soils is one of the challenging problems in agriculture, posing potential threats for both human health and the environment. Plants in association with microbes are a liable method to improve metal tolerance and enhance crop productivity. Chickpea (*Cicer arietinum* L.), is an important grain legume providing cheap source of protein in semi-arid regions including As affected areas. In this study we report the role of *T. reesei* NBRI 0716 (NBRI 0716) in supporting chickpea growth and improving soil quality in As simulated conditions. NBRI 0716 modulated the As speciation and its availability to improve grain yield and quality (amino acids and mineral content) in chickpea (*C. arietinum* L.) plants grown in As spiked soil (100 mg As kg^{-1} soil). Arsenic accumulation and speciation results indicate that arsenate [As(V)] was the dominant species in chickpea seeds and rhizosphere soil. The *Trichoderma* reduced total grain inorganic As (As_i) by 66% and enhanced dimethylarsonic acid (DMA) and monomethylarsinic acid (MMA) content of seed and rhizosphere soil. The results indicate a probable role of NBRI 0716 in As methylation as the possible mechanism for maneuvering As stress in chickpea. Analysis of functional diversity using carbon source utilization (Biolog) showed significant difference in diversity and evenness indices among the soil microbial rhizosphere communities. Microbial diversity loss caused by As were prevented in the presence of *Trichoderma* NBRI 0716.

1. Introduction

Arsenic (As) a carcinogenic metalloid is non-essential for plant growth that interferes with various metabolic processes and causes physiological and morphological disorders leading to reduced plant growth and death (Tripathi et al., 2007, 2012; Zhao et al., 2010). Arsenic mainly has two inorganic [arsenate As(V) and arsenite As(III)] and two organic species [monomethyl arsinic acid (MMA) and dimethyl arsonic acid (DMA)] all of which are associated with As metabolism/toxicity in soil and crops. Toxic effect of As is highly dependent on its species, inorganic As [As(III) and As (V)] being more toxic and carcinogenic than the organic species (DMA, MMA) (Meharg and Hartley-Whitaker, 2002; Qin et al., 2006; Zhao et al., 2010). Among crop plants, studies have mainly focused on arsenic metabolism in rice (Abedin et al., 2002; Chakrabarty et al., 2009; Shri et al., 2009) with special emphasis on its effect on grain mineral composition (Williams et al., 2009; Dwivedi et al., 2010a; Norton et al., 2010a) amino acid status (Dwivedi et al., 2010b) and identification of quantitative trait loci (QTLs) (Norton et al., 2010b). Crops, vegetables and pulses grown on As contaminated soils, exceeding the food safety limits of 50 $\mu g\ l^{-1}$ (WHO, 2001) can be a source of As in food chain (Das et al., 2004; Bhattacharya et al., 2010).

Chickpea (*Cicer arietinum* L.), an annual plant, is the third most important grain legume in the world on the basis of total yield (Zhang et al., 2007). This crop is cultivated during winter season in Northern India (Singh and Ocampo, 1997) including Uttar Pradesh and other As affected states. The ground water As contamination in some districts of Uttar Pradesh is up to 468 $\mu g\ l^{-1}$ (Chauhan et al., 2009) while soil As level ranges 9–390 mg kg^{-1} dw (dry

Abbreviations: As, arsenic; A_{si}, inorganic arsenic; As(III), arsenite; As(V), arsenate; CFU, colony forming units; dw, dry weight; EAAs, essential amino acids; NBRI 0716, *Trichoderma reesei* isolate NBRI 0716; NEAAs, non-essential amino acids; NPTs, non-protein thiols; DMA, dimethyl arsonic acid; MMA, monomethyl arsinic acid

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weight) in other As affected states of India (Patel et al., 2005). Gupta et al. (2008) have reported As accumulation in root (1.17 μg g^{-1} dw) and shoot (47.34 μg g^{-1} dw) of chickpea under hydroponic conditions in presence of As(V). Chickpea proteins are considered to be a suitable source of dietary protein due to excellent balance of essential amino acids (EAAs) composition (Wang et al., 2010). High soil As exposure differentially affect the essential and non-essential amino acids (NEAAs) in rice (Dwivedi et al., 2010b, 2012). Similarly Davies et al. (1987) reported significant induction of histidine, proline, cysteine and glycine upon other heavy metal exposure.

Application of fungi to decontaminate soil and water of heavy metals has received increasing attention because of their ubiquity and high surface area to volume ratio. Besides, their cell wall components contain a large quantity of polysaccharides and proteins that offer many functional groups for binding metal ions (Congeevarama et al., 2007). *Trichoderma* has been extensively exploited in agriculture for plant growth promotion, biological control, a modifier of plant metabolism (Harman et al., 2004a), environmental bioremediation (Harman et al., 2004b; Mishra and Nautiyal, 2009) and as a heavy metal tolerant organism (Arriagada et al., 2009; Cao et al., 2008). Effect of As *in vitro* bioaccumulation (Urik et al., 2007), biovolatilisation (Su et al., 2011; Srivastava et al., 2011) and gene expression (Tripathi et al., 2012) in presence of *Trichoderma* isolates have been documented. Considering the tripartite interactions among soil As, plant and fungi in the rhizosphere and their influence on plant nutrient uptake, we hypothesized possible role of *Trichoderma* in As contaminated soil. It is hypothesized that *Trichoderma* alters As uptake and speciation to improve qualitative and quantitative chickpea yield and also maintain functional diversity of microbial communities in the rhizosphere. The study focuses on effect of As and *Trichoderma* in different treatments by determining the amino acids and mineral nutrient composition and presence of As in chickpea seeds and other plant parts. Besides, the study also manifests the role of *Trichoderma* in amending the quality of As contaminated soil.

2. Materials and methods

2.1. Microorganism and culture conditions

The fungal isolate used for this study was *Trichoderma reesei* (NBRI 0716) isolated from diesel contaminated soil near railway tracks, Hussainganj, Lucknow, U.P. India (Tripathi et al., 2012). NBRI 0716 could grow on potato dextrose agar (PDA) medium containing 100 mg l^{-1} As. *Trichoderma* isolate was maintained and propagated on PDA slants and plates. The *Trichoderma* spores were harvested from 7 day old culture plates and filtered with four layers of cheese cloth for inoculation of the chickpea seeds. Soil and rhizosphere microbial population was determined as described earlier (Mishra and Nautiyal, 2009).

2.2. Plant growth conditions and treatments under green house

A green house experiment was setup at National Botanical Research Institute, Lucknow, India (26°55′N, 80°59′E) and methods of seed selection, surface sterilization, sowing and plant growth conditions of chickpea (*C. arietinum* L.) remained same as described earlier (Nautiyal et al., 2010). The experiment consisted of following treatments: T_0 (Garden soil; GS), T_1 (GS+NBRI 0716), T_2 (GS+As), T_3 (GS+NBRI 0716+As). 5 kg of soil on dry weight basis was used to fill 23 cm diameter earthen pot maintaining six replicates of each treatment, with six plants in each pot. For soil amendment, $Na_2HAsO_4 \cdot 7H_2O$ (MW=312.02; purity 99% HiMedia Labs, Mumbai, India) used as As(V) source and mixed with soil to get final concentration of 100 mg kg^{-1} soil before maintaining 20% moisture (Tripathi et al., 2012). The sterilized seeds were dipped in saline (0.85% NaCl w/v) containing fungus spore suspension to obtain a CFU of 4.0 $\log_{10}$ units per seed, a recommended inoculum dose for seed treatment. Plant growth parameters (plant height, number of nodules, dry weight *etc.*) were recorded after 60 days of plant growth, while yield, mineral element with As content and amino acid profiling was carried out in rhizosphere soil and plant parts after final harvesting at day 90. In order to collect rhizosphere soil, plants were carefully removed at the specified time and all root segments 5 mm severed below seed remnants served as rhizosphere. This was done to ensure that only the *Trichoderma* that colonized the roots were assayed; rest of the soil served as bulk soil (Nautiyal, 1997). Roots were washed thoroughly to remove all soil particles and then macerated in 0.85% (w/v) saline MQW with a mortar and pestle. The harvested plants chosen at random were rinsed with Milli-Q water and oven-dried at 70 °C for 72 h for dry weight and metal content analysis.

2.2.1. Determination of total As in different parts of chickpea and soil

For determination of total As in different parts of chickpea plant (root, leaves, empty pods and seeds) and soil, samples were oven dried at 70 °C and wet digested in HNO_3 (69%, ACS quality Germany) at 120 °C. The digested samples were diluted to 20 ml and total As was determined using inductively coupled plasma mass spectrometer (ICP-MS; Agilent 7500 ce) according to Dwivedi et al. (2010a). Rhodium (MECS-4, part no. 8500-6942) was used as an internal standard in each sample.

2.2.2. Speciation of As in chickpea grain and soil

For As speciation, powdered samples were weighed (0.2 g) into a 50 ml polyethylene centrifuge tube, and extracted with 1 ml of 1.52 mM NaH_2PO_4 buffer containing 0.198 mM Na_2EDTA, 3 mM $NaNO_3$, 10 mM CH_3COONa and 1% C_2H_5OH (pH 6.0) modified from Zheng et al. (2011). The extraction solutions were centrifuged and passed through a 0.45 μm nylon syringe filter and immediately kept on auto sampler at 4 °C and analyzed. The speciation was performed using High performance liquid chromatography (HPLC Agilent Technologies 1200 series) coupled to ICP-MS (HPLC-ICP-MS). Chromatographic columns consisted of Column 150 × 4.6 mm^2, (Anion exchange resin hydrophilic polyacrylate as basic resin, PEEK1, Agilent Technologies, Tokyo, Japan). The mobile phase, consisted of 1.52 mM NaH_2PO_4 buffer containing 0.198 mM Na_2EDTA, 3 mM $NaNO_3$, 10 mM CH_3COONa and 1% C_2H_5OH (pH 6.0), was run isocratically at a flow rate of 1 ml min^{-1}. Arsenic species in samples were identified by using the standards of As(V), As(III), DMA and MMA and quantified using external calibration curves with peak area of each standard. Matrix-matched DMA standards were used to calibrate the instrument. The representative chromatogram of reference material has been given in the supplementary data (Fig. S3).

2.2.3. Determination of mineral elements in chickpea plant parts and soil

For analysis of mineral elements *viz.* Fe, Se, Zn, Ni, Mn, Cu and Co, samples were prepared as mentioned above and estimated using ICP-MS. The multi-element calibration standard (MECS-2A, part no. 8500-6940) was used for standardization of Fe, Se, Zn, Ni, Mn, Cu and Co. Rhodium (MECS-4, part no. 8500-6942) was used as an internal standard in each sample. Total phosphorous (P) extracted from HNO_3 and $HClO_4$ (3:1 ratio) digest and available P from soil filtrate was determined following phospho-molybdate blue color method (Jackson, 1958). The standard curve was constructed using absorbance values from standards of known P concentration.

2.2.4. Quality control and quality assurance

The standard reference materials for different metals Fe, Se, Zn, Ni, Mn, Cu and Co (E-Merck, Germany) were used for the calibration and quality assurance (QA) for each analytical batch. Analytical data quality of metals was ensured with repeated analysis ($n=3$) of quality control samples, and the results were found within (± 2.82) the certified values. The recoveries of the standard reference material were P $98 \pm 7\%$, Fe $92 \pm 6\%$, Se $86 \pm 6\%$, Zn $88 \pm 5\%$, Ni $84 \pm 6\%$, Mn $94 \pm 5\%$, Cu $92 \pm 6\%$ and Co $80 \pm 8\%$ as determined by spiking of samples with a known amount of each metal.

For total As, rice flour NIST 1568a was used as a reference material with known spiked samples, and recovery of total As were 94% (± 2.8; $n=5$) and 91% (± 3.1; $n=5$), respectively. The detection limit for each metal was 1 $\mu g\, l^{-1}$.

2.3. Amino acids analysis

Amino acid analysis (essential amino acids, EAAs) and non-essential amino acids (NEAAs) was done using HPLC by the pico tag method as described in Bidlingmeyer et al. (1984).

2.4. Microbial diversity using carbon source utilization pattern

Microbial diversity by carbon source utilization pattern using Biolog Eco, GN and MT plates (Biolog, Inc., Hayward, CA, USA) were used to determine the carbon source utilization pattern of chickpea rhizosphere samples. The MT plates were prepared using the manufacturer's instructions (Biolog Inc., Hayward, CA 94545, U.S.A.) as described earlier (Mishra and Nautiyal, 2009). The additional carbon sources used in MT plates were chosen to represent compounds reported in literature as main components of chickpea root exudates *viz.* phytic acid, oxalic acid, glycolic acid, tartaric acid, tryptophan (Dakora and Phillips, 2002); heavy metal chelators *viz.* phytic acid and tartaric acid (Tu et al., 2004); As contaminants *viz.* sodium arsenate and sodium meta arsenite and P containing compounds *viz.* sodium hexameta phosphate and tri calcium phosphate (TCP) since P is analog of As (Tripathi et al., 2007; Zhao et al., 2010). Data were recorded for 15 days at 590 nm and data recorded after day 5 was used for further analysis. Microbial activity in each micro-plate, expressed as average well color development (AWCD) was determined as described by Garland (1996). Statistical analyses were performed using SPSS 16.0 and Statistica 7.0.

2.5. Statistical analysis

All the experiments were conducted following a randomized block design. Two-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments. Correlation analysis was performed by following Gomez and Gomez (1984). All the data of each treatment with respect to change in As accumulation and between selected parameters, has been given within text at relevant places ($p < 0.001$***; $p < 0.01$**; $p < 0.1$*; ^{NS} non-significant).

3. Results

3.1. Mineral status and As speciation in soil

The pH of garden soil (GS) used in the experiment was around neutral (7.3 ± 0.1), which decreased slightly (7.2 ± 0.1) after addition of arsenic (100 mg kg^{-1}) (Table S1). The concentration (mg kg^{-1} dw) of different elements in GS were Fe 1107, Se 3, Zn 217, Ni 14, Mn 394, Cu 11, Co 2. The natural As (mg kg^{-1} dw) concentration in GS (T_0 and T_1) was around 5.27, which increased up to 24 after supplementation of As in T_2 and T_3. After harvesting soil As (mg kg^{-1} dw) was 18 in T_2 and 11 in NBRI 0716 inoculated As soil T_3. Arsenic speciation results showed As(V) to be the dominant species in GS while traces of As(III) were also detectable in As treated soils after harvesting. DMA and MMA were significantly enhanced in the presence of NBRI 0716 in comparison to uninoculated As treated soil. Inoculation of NBRI 0716 lead to an overall reduction of soil inorganic arsenic (As_i) by *ca.* 1.7 folds (Table 1).

Table 1
Total arsenic and speciation in garden soil (GS) and chickpea seeds amended with *T. reesei* during As^V exposure.

Arsenic species	Arsenic (mg kg^{-1} dw)			
	Rhizosphere soil			
	T_0	T_1	T_2	T_3
Total As	$5.27^a \pm 0.40$	$3.05^a \pm 0.16$	$17.88^c \pm 1.61$	$10.76^b \pm 1.69$
As^V	$2.48^a \pm 0.54$	$1.53^a \pm 0.23$	$9.63^c \pm 0.86$	$5.21^b \pm 0.73$
As^{III}	BDL	BDL	$0.34^a \pm 0.02$	$0.37^a \pm 0.02$
DMA	BDL	$0.04^a \pm 0.00$	$0.87^b \pm 0.03$	$2.19^c \pm 0.01$
MMA	BDL	BDL	$0.22^a \pm 0.01$	$0.89^b \pm 0.01$
Total As_i	2.48	1.53	9.97	5.58
Total As_o	–	0.04	1.09	3.08
	Seed			
Total As	$0.10^a \pm 0.02$	$0.08^a \pm 0.00$	$1.74^c \pm 0.03$	$0.58^b \pm 0.02$
As^V	$0.07^a \pm 0.00$	$0.064^a \pm 0.01$	$0.74^c \pm 0.09$	$0.20^b \pm 0.06$
As^{III}	BDL	BDL	$0.26^b \pm 0.04$	$0.08^a \pm 0.03$
DMA	BDL	$0.002^a \pm 0.00$	$0.08^{ab} \pm 0.00$	$0.11^b \pm 0.07$
MMA	BDL	BDL	$0.03^a \pm 0.00$	$0.06^b \pm 0.04$
Total As_i	0.07	0.06	1.00	0.28
Total As_o	–	0.002	0.11	0.17

T_0 (GS), T_1 (GS+*T. reesei*), T_2 (GS+As), T_3 (GS+*T. reesei*+As), BDL=Below Detection Limit, All values are mean of six replicates ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values among treatments (DMRT, $p \leq 0.05$).

3.2. Total As accumulation and speciation in chickpea seeds

Total As was maximum in As exposed plants (T_2) being 12, 5, 3, 2 mg kg^{-1} dw in roots, leaves, empty pods (Fig. 1A) and seeds (Table 1) respectively. However, As accumulation decreased by 64%, 44%, 24% and 67% in roots, leaves, empty pod and seeds respectively with NBRI 0716 inoculation. Out of the four As species [DMA, MMA, As(III) and As(V)], As(V) was found to be the dominant As species in both the As treatments (T_2 and T_3). However, the percentage of organic As species increased during the inoculation of NBRI 0716 which changed the order of various As species dominance *viz.*, As(V) > As(III) > DMA > MMA in solely As exposed plants (T_2) as compared to As(V) > DMA > As(III) > MMA order in NBRI 0716+As treatment (T_3) (Table 1). The results show an overall reduction of grain inorganic As (As_i) by about 3.5 folds due to NBRI 0716 inoculation as also observed in soil.

3.3. Mineral-element accumulation in chickpea plant parts

Mineral nutrients content in chickpea seeds was found to reduce under As stress showing decreased accumulation of Fe (6–80%), Se (27-33%), Zn (23–61%), Ni (11–68%), Mn (5–41%), Cu (33–48%) and Co (25–65%) in different plant parts (Fig. 1B–H and Fig. S1H) while presence of NBRI 0716 (T_3) enhanced these nutrients in

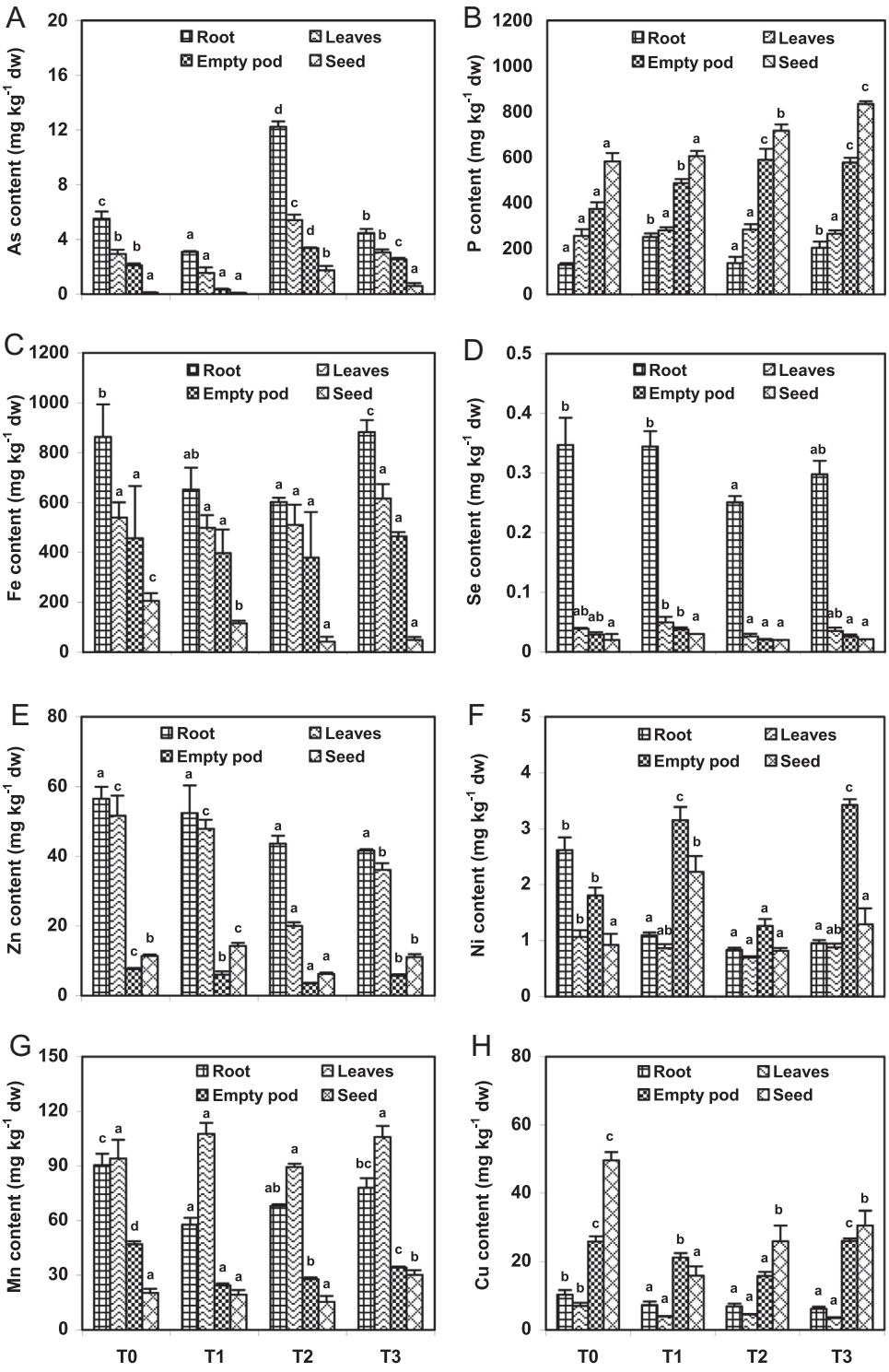

Fig. 1. Arsenic and micro-nutrient accumulation in different parts of chickpea during As exposure amended with NBRI 0716. Total As (A); P (B); Fe (C); Se (D); Zn (E); Ni (F); Mn (G); Cu (H). All values are mean of six replicates ± S.D. after 90 days of plant growth. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values among As treatments and NBRI 0716 amendments (DMRT, $p \leq 0.05$).

all plant parts. An increase in seed mineral content, (Mn 98%), (Zn 78%) (Ni 57%) (Co 33%) (Se 28%) (Cu 18%) (P 16%) (Fe 16%) in T_3 as compared to T_2 is indicative of the role of NBRI 0716 in enhancing grain quality in As exposed soil. On the contrary P accumulation in seeds correlated differentially with As in presence and absence of NBRI 0716. Seed P and As showed positive correlation in T_2 and negative correlation in T_3.

3.4. Effect of As on amino acid profile of chickpea seeds

Arsenate exposure significantly affected the EAAs in comparison to NEAAs (Fig. 2A–D). The EAAs Lys (151%), Ile (186%), Phe, Leu, Met (108% each), Thr (88%) and Val (46%) significantly declined as compared to control (T_0). However, the inoculation of NBRI 0716 in As exposed soil enhanced various amino acids such as Lys (68%), Ile (67%), Phe (81%), Leu (44%), Met (92%), Thr (23%)

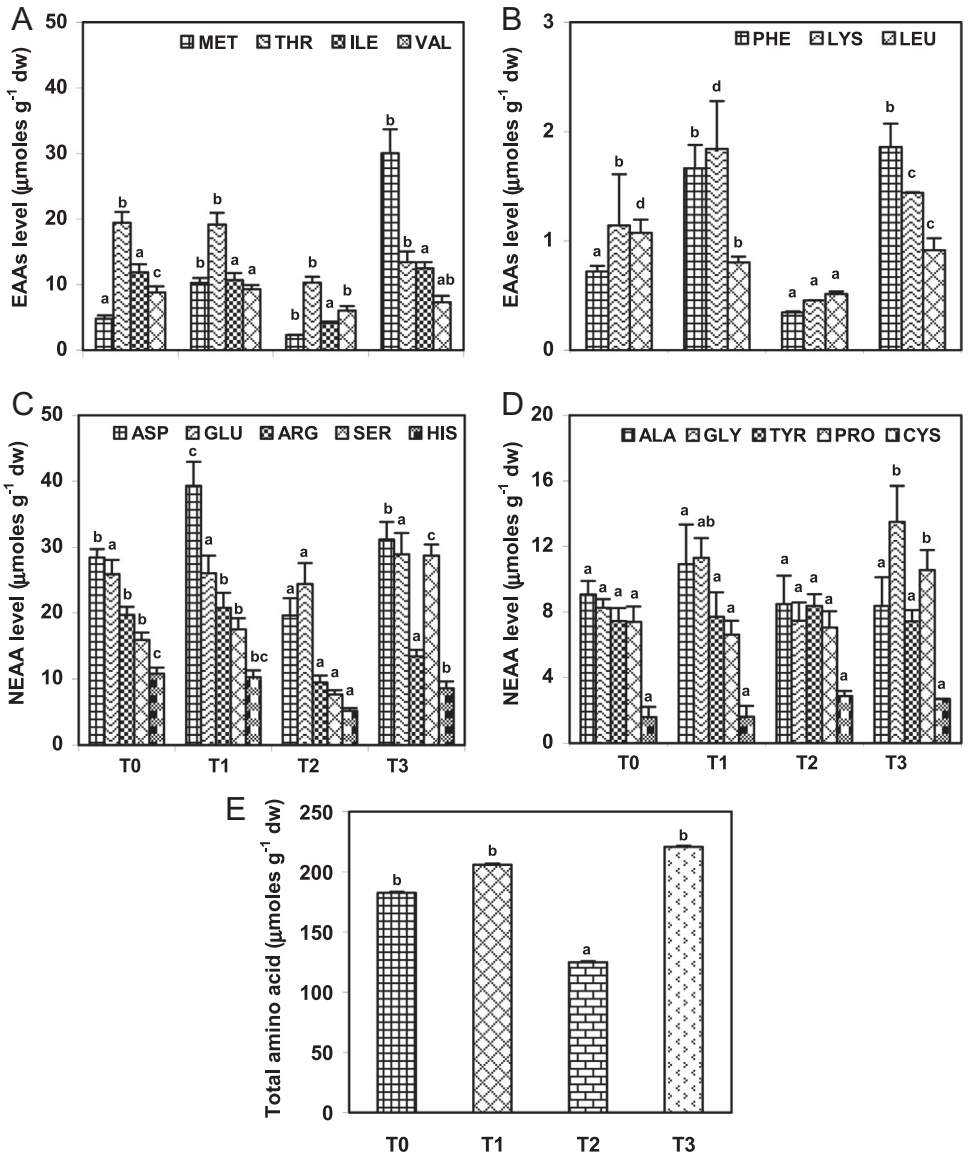

Fig. 2. Effect of As on amino acids of chickpea during NBRI 0716 inoculation. EAAs (A,B); NEAAs (C,D); total amino acid (E). All values are mean of six replicates ± S.D. ANOVA significant at $p \leq 0.01$. Different letters indicate significantly different values among As treatments and NBRI 0716 amendments (DMRT, $p \leq 0.05$).

and Val (18%) significantly as compared to As treatment (T_2). The NEAAs like His, Ser and Asp acid also significantly decreased as compared to control (T_0) (Fig. 2C), while NEAAs like Cys (44%) and Tyr (11%) were increased in As exposed chickpea seed (Fig. 2D). The inoculation of NBRI 0716 significantly enhanced Gly and other NEAAs as well. The total amino acids decreased by 46% in As exposed plants (T_2) while it significantly increased by upto 43% in NBRI 0716 +As treatment (Fig. 2E).

3.5. Plant growth parameters and rhizosphere microbial population

Soil application of As reduced seed germination (44%), shoot length (21%), shoot diameter (11.5%) and decreased pod formation with poor and wrinkled seed setting as compared to control. The adverse effect of As was more pronounced in root region as evident by highly reduced nodulation (86%), decrease in lateral root formation (82%), root dry weight (29%) and root length (29%). Inoculation of NBRI 0716 significantly enhanced plant growth both in presence and absence of As. NBRI 0716 ameliorated the toxic effect of As and increased the chickpea germination (43%), root length (28%), number of nodules (92%), root dry weight (40%), yield (63%), and other plant growth parameters (Figs. S2A–J and S1A–F). Total chlorophyll reduced by 44% due to As exposure (T_2), was significantly enhanced by NBRI 0716, T_3 (204% and 70% with respect to T_2 and T_0 respectively) (Fig. S1I). Colonization of chickpea rhizosphere by isolate NBRI 0716 remained unaffected by As, besides it promoted the population of heterogeneous microbes (Table S2).

3.6. Microbial diversity using carbon source utilization pattern

Microbial diversity of chickpea rhizosphere was analyzed by carbon source utilization pattern using *E*co, GN and MT micro-titer plates in different treatments with As and NBRI 0716. Average well color development (AWCD) data from day 5 was used for analysis based on patterns of color development on the biolog microtiter plates. Results from AWCD analysis indicated that rhizosphere samples from control soils (T_0) metabolized most carbon sources at higher rates than those treated with As (T_2), NBRI 0716 (T_1) and As+NBRI 0716 (T_3) (Fig. 3). Results of MT plate carbon source utilization showed increased metabolization of phytic acid both in presence and absence of As when treated by NBRI 0716. Maximum

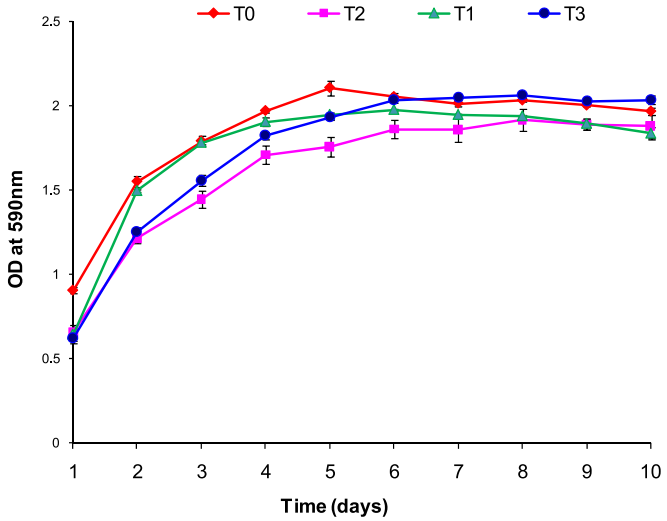

Fig. 3. Average well color development showing carbon source utilization pattern by rhizospheric microbial community of Un-inoculated control-T_0 (♦); NBRI 0716-T_1 (●); As control-T_2 (■); As+NBRI 0716-T_3 (▲) treatments over different time intervals. The plotted data are averages of three independent experiments ± SE.

utilization of sodium meta arsenite and sodium arsenates were observed in treatments inoculated with NBRI 0716 (T_1 and T_3) (Fig. 4). McIntosh, Shannon, and Simpson indices showed significant differences among treatments based on Tukey's test (at $P=0.05$) (Table 2).

Table 2
Shannon diversity index showing change in microbial community structure among different treatments.

	T_0	T_1	T_2	T_3
Shannon diversity	4.588 ± 0.023	4.563 ± 0.029	4.575 ± 0.025	4.562 ± 0.029
Shannon evenness	0.992 ± 0.005	0.987 ± 0.006	0.989 ± 0.005	0.986 ± 0.006
Mcintosh diversity	1.003 ± 0.011	1.001 ± 0.011	1.002 ± 0.011	1.001 ± 0.011
McIntosh evenness	0.996 ± 0.003	0.994 ± 0.004	0.995 ± 0.003	0.994 ± 0.003
Simpson diversity	1.001 ± 0.002	1.000 ± 0.002	1.000 ± 0.002	1.000 ± 0.002

Different letters show significant difference at $P=0.05$ using Tukey's test.

4. Discussion

4.1. Effect of As and Trichoderma on plant growth promotion

Trichoderma is a widely used fungus in agriculture to improve plant growth promotion, solubilize phosphate and mineral nutrients (Altomare et al., 1999; Harman et al., 2004a) and bio-remediate environmental contaminants (Tripathi et al., 2013). The present study was conducted to examine the effect of NBRI 0716 on amino acid profile and mineral nutrient status of chickpea plants *vis-à-vis* As accumulation during simulated pot experiment. The results show that growth inhibition due to As was more pronounced in roots than in shoots showing the direct effect of As interaction with the plant roots, the immediate point of contact resulting in retarded root length and diameter, reduced number of rootlets, nodules and dry weight. Similarly direct effect of As on rice roots has been reported by Shri et al. (2009). Decrease in germination rate and root length under As exposure in comparison to control has been reported earlier (Liu et al., 2005; Shri et al., 2009). Furthermore, decrease of chlorophyll contents due to As application was probably due to decreased uptake of essential nutrients such as Fe, Mn, Cu, Ni and Zn which reduced overall growth, vigor and productivity of the plants as reported earlier in oat and rice (Stoeva and Bineva, 2003; Dwivedi et al., 2010b). The decline in level of nutritional elements caused by As in chickpea, a dicot plant, appears well in accordance with earlier report on monocot plants such as rice (Norton et al., 2010a; Dwivedi et al., 2010a). Arsenic and P being chemical analogs resulted in enhanced P uptake in As contaminated soil unlike other minerals. However it is interesting that NBRI 0716 restricted the co-mobilization of As with P in chickpea seeds. Improved As tolerance of legume plants by enhancing P accumulation and restricting As uptake has been earlier reported (Xu et al., 2008; Gunes et al., 2009). Interestingly, in the present study a positive correlation between As and Fe was observed. Iron oxyhydroxides are reported as strong adsorbent of As on plant root and soil surface (Zhao et al., 2010) and almost all microbes *viz.* rhizospheric bacteria and fungi including *Trichoderma* spp. are reported to excrete low molecular weight ferric-iron-specific chelators termed as siderophores, which mobilize and sequester iron from soil and make available to the plant tissue (Altomare et al., 1999; Hoyos-Carvajal et al., 2009). Enhanced content of P, Zn, Ni, Mn and Co in chickpea seeds in NBRI 0716 inoculated treatments indicate its tolerance and characteristics such as mineral solubilization and mobilization during As stress.

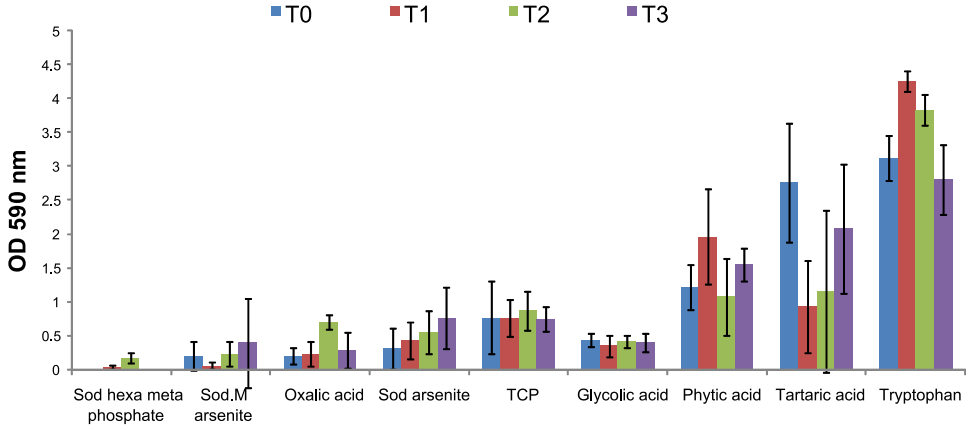

Fig. 4. Utilization of 9 different carbon sources by chickpea rhizosphere microbial population of Un-inoculated control (T_0); NBRI 0716 (T_1); As control (T_2); As+NBRI 0716 (T_3) using Biolog MT plates [values are means ± standard error ($n=3$)]. Plants were harvested after 45 days. Different letters represent significant difference at $P=0.05$ using Waller–Duncan test.

4.2. Trichoderma and As detoxification

The present experiment shows detoxification of As at two levels one by dilution of As concentration due to enhanced biomass in the *Trichoderma* treatments both in presence and absence of As and second by transformation of As_i forms to organic and/or volatile forms. Many fungi like *Penicillium, Fusarium* and *Trichoderma* have been found to methylate As(V) or As(III) to organic derivatives such as MMA, DMA or TMAO or TMA (Bentley and Chasteen, 2002; Fitz and Wenzel, 2002; Maheshwari and Murugesan, 2009; Srivastava et al., 2011; Su et al., 2011; Feng et al., 2015). Thus higher partitioning of total As into organic As such as DMA and MMA in NBRI 0716+As (T_3) seeds than in As alone (T_2) seeds shows that rhizospheric fungi NBRI 0716 facilitated the methylation of As. This conversion is probably carried out by NBRI 0716 in the rhizosphere region where it is colonized and mediates the conversion of As_i to organic As through methylation pathway which is subsequently taken up by the plant or lost to the environment as volatile. This observation is in accordance with earlier reports of methylated As species being taken up by rice plant (Abedin et al., 2002) with protonated forms involving silicic acid transport pathway (Li et al., 2009). These observations regarding As transformation are of relevance since the major pentavalent product DMA(V) and trimethyl arsine oxide (TMAO) are approximately 100 and 1000 fold less toxic respectively than As(III) (Qin et al., 2006). Not passing the role of bacteria in As methylation (Qin et al., 2006) the enhanced microbial diversity observed in presence of NBRI 0716 showing As_i metabolism in Biolog MT plates might be well recruited to perform the function. It may further be emphasized that As leaching and volatilization pathways occurring in rhizosphere must have lead to reduced availability of As to chickpea resulting into reduced As content in chickpea.

4.3. Effect of As and Trichoderma on chickpea nutrients

Recently, negative impact of As application on amino acid profile of rice grains in a genotypic specific manner has been observed emphasizing on importance of breeding As tolerant crop varieties (Dwivedi et al., 2010b, 2012). Our results however, emphasizes on the importance of application of an efficient *Trichoderma* isolate to ameliorate As stress and improve grain quality and yield. An augmented EAAs content in chickpea seeds grown in As contaminated soil inoculated with NBRI 0716 is a direct consequence of the enhanced uptake of mineral nutrients and As modulation. Non-essential amino acids such as Cys and Tyr on the other hand, increased in solely As exposed plants which is in accordance with earlier reports (Srivastava et al., 2007, 2010). Cysteine, the main component of metalloid detoxifying ligands such as glutathione (GSH) and phytochelatins (PCs), was induced significantly upon higher As accumulation (Srivastava et al., 2007) and has been found to induce cysteine synthase activity leading to higher cysteine levels in many plants (Srivastava et al., 2010). Plants are also reported to detoxify As by complexation with thiol reactive peptides such as γ-glutamylcysteine (γ-EC), GSH and PCs (Paulose et al., 2010). Non-protein thiols (NPTs) are considered as an index of GSH and PCs in plants (Mishra et al., 2009; Scheller et al., 1987). Therefore, an increase in NPTs content in As exposed chickpea plants may suggest an induction of GSH and PCs for protection of plant cells through direct chelation with As as reported in *Helianthus annus* (Raab et al., 2005).

4.4. Effect of As and Trichoderma on microbial diversity

Changes in heterogenous microbial population due to As and NBRI 0716 was clearly seen in the rhizospheric count of heterogenous bacterial and fungal populations (Table S2). An increase in 1 log units in bacterial count and 2 log units in fungal count in NBRI 0716 treated soil (T_1) suggests that microbial community of NBRI 0716 treated soil could be a mixture of related organisms associated through specific functional behavior. However decrease in 2 log units of bacterial count was observed in As treatment (T_2) in contrast to 2 log units increase in fungal population as compared to uninoculated control showing deleterious effect of As causing imbalance of microbial community (Table S1). The effect of As amelioration by NBRI 0716 on microbial population was further confirmed by the study of microbial diversity using carbon source utilization pattern of rhizospheric bacteria. There was distinct resolution of soil microbial communities in the presence of either As or, NBRI 0716 and results indicate that in general, addition of either As or, NBRI 0716 impacted native microbial community structure as earlier reported in presence of diesel and *T. reesei* by Mishra and Nautiyal (2009).

Interesting observations were made by analyzing utilization of different substrates in Biolog microtiter plates by the rhizosphere bacteria. It is well documented that both phytic acid and tartaric acid are main components of chickpea root exudates (Dakora and Phillips, 2002). Tartaric acid is a heavy metal chelator and used as adsorbent for As removal in aqueous solution (Schulz, 1977; Xin et al., 2006), its decreased utilization in presence of As (T_2) and increased utilization in presence of both As and NBRI 0716 (T_3), indicates a role of NBRI 0716 in modulation of tartaric acid utilizing microbial diversity. Phytic acid is principal form of phosphate storage in many plant tissues and being a chemical analog of As mechanism of arsenate mobilization may be similar to that of phosphate. In addition plant produces more organic acids to mobilize As as observed in fern (Tu et al., 2004) which might be a possible reason in increasing the phytic acid utilization in presence of As in chickpea rhizosphere. Reason for the differential microbial utilization of the two chelating organic acids is obscure and warrant further investigation. Utilization of As salts such as sodium arsenate and sodium meta arsenite was highest in T_3 treated chickpea rhizosphere which indicates that As metabolizing microbes were maximum in this treatment.

5. Conclusions

From the present study, it may be concluded that As accumulation in chickpea altered the amino acid content and mineral nutrient uptake. However, inoculation of *Trichoderma reesei* isolate NBRI 0716 modulated the chemical and biological features of the chickpea rhizosphere in As contaminated soil such that As speciation, mineral mobilization and their uptake resulted in reduced As accumulation in seeds and augmented amino acids and mineral nutrient content. Thus, the isolate, *T. reesei* NBRI 0716 may be useful bioinoculant for As affected areas. However further field trials with NBRI 0716 are required with chickpea and other crops grown in As contaminated regions before it can find wide field application.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2014.10.027.

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