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

Pratibha Tripathi, Poonam C. Singh, Aradhana Mishra, Rudra D. Tripathi *, Chandra S. Nautiyal *

CSIR-National Botanical Research Institute, Lucknow 226001, India

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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

* Corresponding authors. Fax: +91 522 2205839.
E-mail addresses: tripathi_rd@rediffmail.com (R.D. Tripathi), nautiyalnbri@lycos.com (C.S. Nautiyal).

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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.