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Beneficial compatible microbes enhance antioxidants in chickpea edible parts through synergistic interactions

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ABSTRACT

Rhizosphere microbe-mediated induction of antioxidant mechanisms for disease resistance in plants is known but their impact on nutritional content of the edible parts is not clear. A study was conducted to evaluate potentiality of three compatible rhizosphere microbes, viz., fluorescent *Pseudomonas* (PHU 094), *Trichoderma harzianum* (THU 0816) and *Mesorhizobium* sp. (RL 091), singly and in combinations in modulating antioxidants in chickpea edible parts. Total phenolic and flavonoid content, ascorbic acid, free radical and hydroxyl radical scavenging activities as well as reducing power in seeds and pericarp in different microbial combinations were significantly high compared to their single application. However, the triple microbe treatment was most effective in enhancing the antioxidant status of chickpea along with enhanced accumulation of phenolics such as shikimic, gallic, tannic, p-coumaric, and ferulic acids as well as rutin and quercetin. Apart from the triple microbe treatment, dual combination of PHU 094 + THU 0816 also showed potentiality in enhancing the antioxidant and phenolic content majorly in pericarp. These findings suggest that synergistic interaction of microbes in the rhizosphere not only improved the antioxidant level in chickpea seeds but the same were also enhanced in the pericarp which is otherwise considered a waste material.

1. Introduction

Beneficial rhizosphere inhabiting microbes like *Trichoderma*, fluorescent *Pseudomonas*, *Rhizobium*, *Bacillus, Mycorrhizae*, etc. are being looked upon as a realistic alternative under the heavy fungicidal regime (Avis, Gravel, Antoun, & Tweddell, 2008; Jain, Singh, Singh, & Singh, 2013). The impact of the microbial world especially on plant health and growth, stress tolerance, disease resistance, and nutrient availability and uptake has widely been investigated, but what has largely been ignored is to assess their role in improving the nutritional value and crop quality (Gerhardson, 2002). Induced Systemic resistance (ISR) is one such aspect of mechanisms enhance by rhizosphere microbes where defence related secondary metabolites including antioxidant machineries of plants are mobilized systemically to distant parts on sensing of elicitor molecules from the beneficial microbes (Shoresh, Harman, & Mastouri, 2010). *Medicago truncatula* roots transcriptome analysis demonstrated that the genes involved in secondary metabolism are also affected when the mycorrhizal species *Glomus* was inoculated in artificial soil (Hohnjec, Vieweg, Puhler, Becker, & Kuster, 2005). Similarly, in another study changes in secondary metabolite levels were recorded in maize seedling roots on inoculation with an *Azospirillum*, *Pseudomonas* and *Glomus* consortium under field conditions. It was demonstrated that combining the strains proved useful in maintaining the effective root system even mineral under reduced fertilizer usage (Walker et al., 2012). Currently more attention are being paid to understand the role of diet in maintenance of human health and several epidemiological studies have also indicated that high intake of plant products rich in phenols and antioxidants is associated with a reduced risk of a number of chronic diseases, such as atherosclerosis, cancer, diabetes, Alzheimer's disease, Parkinson's disease, etc. (Prakash, Singh, & Upadhyay, 2007; Singh, Shankar, & Srivastava, 2011).

Microbes are increasingly being used in organic agriculture aiming safety and sustainability of the environment. However, the

Abbreviations: ISR, Induced Systemic resistance; ROS, reactive oxygen species; TPC, total phenolic content; TFC, total flavonoid content; AA, ascorbic acid; IC, iron chelation; FRSA, free radical scavenging activities; HRSA, hydroxyl radical scavenging activity; RP, reducing power; GAE, gallic acid equivalent; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid.

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answer to the question whether organically grown crops are nutritionally more rich than the conventionally grown food is still not clearly addressed. Some, preliminary reports carried out by Nautiyal, Govindrajan, Lavania, and Pushpangadan (2008) have pointed out that the single plant growth promoting microbe, *Bacillus lentimorbus* NRRL B-30488 played a vital role in inducing accumulation of total phenol and antioxidant enzymes in some vegetables and fruit crops. Few other reports have also highlighted the role of green manure, compost and mycorrhiza in improving food quality (Copetta, Bardi, Bertolone, & Berta, 2011; Shams, 2012). However, the impact of different bioactive molecules produced in the edible parts of plants following application of synergistic microbial combinations has not been investigated till date. We previously observed that microbes when applied singly or in combinations triggers activation of the phenylpropanoid pathway and activities of antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (PO) are differential under different pathogen stresses and microbial combinations (Jain, Singh, Sarma, & Singh, 2012; Jain, Singh, Singh, Singh, Upadhyay, et al., 2013; Singh, Sarma, Upadhyay, & Singh, 2013). It is therefore, expected that antioxidant level in edible parts should also be enhanced in chickpea by presence of the microbes. Therefore, the present study was undertaken for the first time to assess the phenolic profile, total phenolic content (TPC), total flavonoid content (TFC), ascorbic acid (AA) content, protein content, iron chelation (IC), free radical scavenging activities (FRSA), hydroxyl radical scavenging activity (HRSA) and reducing power (RP) using standard *in vitro* antioxidant assays in chickpea seeds and their pericarp to gain insights into the effects of such rhizosphere microbes when applied either singly or in combination.

2. Materials and methods

2.1. Materials

DPPH, ferrozine, Folin Ciocalteau's phenol reagent, aluminium chloride, TBA [2-Thiobarbituric acid], TCA [Trichloroacetic acid], DCPIP [2,6-dichlorophenol-indophenol], shikimic acid, gallic acid, tannic acid, rutin, ferulic acid, quercetin, kaempferol, catechin from Sigma–Aldrich, St. Louis, USA. Ascorbic Acid, sodium hypochlorite solution, gum arabic, sodium hydroxide, ferrous chloride, hydrogen peroxide solution, EDTA, sodium carbonate, sodium nitrite, sodium carbonate, sodium nitrite, potassium ferricyanide, bovine serum albumin were purchased from HIMEDIA, Mumbai, India. HPLC grade methanol, ethyl acetate, ethanol, acetonitrile, acetic acid and water were purchased from Merck, India.

2.2. Microbial cultures

Three microbial strains were used in the experiment, viz., *Trichoderma harzianum* THU 0816 (ARS Culture Collection Number NRRL 30598), which was obtained from National Botanical Research Institute (NBRI), Lucknow whereas *Pseudomonas aeruginosa* PHU 094 (GenBank Accession: JN038174) and *Mesorhizobium* sp. RL 091 (GenBank Accession: KC432645) were isolated from rhizospheric region and root nodules of chickpea, respectively. Compatibility between the strains of fluorescent *Pseudomonas*, *Trichoderma* and the rhizobial species was carried out on solid plate by placing the fungal block in the centre followed by streaking the bacterial partners on its both sides (Singh et al., 2013).

2.3. Inoculum preparation

Mesorhizobium and *Pseudomonas* strains were grown overnight in yeast extract mannitol and King's B broth, respectively. Bacterial cells were pelleted separately by centrifugation and the supernatant was discarded. Cell pellets were washed with 1 ml phosphate buffer saline (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and suspended in PBS to a cell density of 10^8 CFU ml^{-1} through optimization of optical density at 620 nm. Similarly, conidial suspension of *Trichoderma* harvested from 7 day old culture on potato dextrose agar medium and was adjusted to 2×10^6 CFU ml^{-1}. Cell suspensions of the three strains were mixed in ratios 1:1 for dual consortia and 1:1:1 for triple consortium representing 1/2 and 1/3rd of each initial microbial load, respectively. The suspensions were vortexed to obtain homogenous suspensions of microbial mixtures.

2.4. Experimental design

Chickpea (cv. Radhey) seeds were surface sterilized with 1% NaOCl solution for 5 min, followed by washing with sterilized distilled water (SDW) to remove traces of NaOCl. The moistened seeds were stored at room temperature (25 ± 2 °C) for 24 h under aseptic condition to promote germination. Plastic pots (15 cm × 10 cm) were used for Sterile Soil Assay (SSA). Soil mixture containing sandy soil and vermicompost (20%) was autoclaved for 30 min for three consecutive days and filled with 1.5 kg pot^{-1}. For seed treatment, germinated seeds of chickpea were first coated with 15% (w/v) gum arabic (HiMedia, RM682) and various combinations of the microbes were applied by dipping the seeds in the cell suspensions. Coated seeds were kept for air drying for 2 h under a stream of sterile air which were then planted in plastic pots at an average of six germinated seeds per pot at a depth of approximately 1.5 cm. Following seven suspensions prepared were used in the experiment: (A) *Mesorhizobium* (RL 091), (B) fluorescent *Pseudomonas* (PHU 094), (C) *Trichoderma* (THU 0816), (AB) RL 091 + PHU 094, (AC) RL 091 + THU 0816, (BC) PHU 094 + THU 0816, and (ABC) RL 091 + PHU 094 + THU 0816. Untreated seeds sown in pots served as control (D). Chickpea plants were grown in a glasshouse and watered regularly till the plants matured.

2.5. Sample preparation for biochemical and High Performance Liquid Chromatography (HPLC) analysis

Ripened seeds and pericarp of the pods were separated from matured chickpea pods. The extracts were prepared by crushing 2.0 g of fresh seed and pericarp in mortar and pestle with 10 ml of methanol (MeOH):water (1:1). The extracts thus obtained were filtered through sterilized Whatman No. 1 filter paper. An equal volume of ethyl acetate was mixed with the extracts and after vigorous shaking in a separatory funnel, the ethyl acetate fractions were collected separately. The MeOH fractions were extracted for a second time and the ethyl acetate fractions were pooled with the previous extract (Singh et al., 2009). The ethyl acetate fractions were vacuum evaporated under reduced pressure at 1,034, 214 Pa and 40 °C in a Rota evaporator (Eyela N–N series, Tokyo, Japan). 1 mg of dried ethyl acetate fractions was suspended in 1.0 ml of HPLC grade MeOH by vortexing and used for further chemical analysis.

2.5.1. Total phenolic content (TPC)

Total phenolic content (TPC) was determined according to Zheng and Shetty (2000). The absorbance was recorded at 725 nm and the calibration curve was prepared for each assay using different concentrations of gallic acid (GA) prepared in 95% ethanol. Absorbance values were converted to mM Gallic acid equivalent (GAE) g^{-1} fresh weight (FW).

2.5.2. Total flavonoid content (TFC)

Total flavonoid content (TFC) was determined following the procedure of Dewanto, Adom, and Liu (2002), with catechin as