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Modulation of nutritional and antioxidant potential of seeds and pericarp of pea pods treated with microbial consortium

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ABSTRACT

Microbial populations have diverse roles within rhizosphere where interactions among distinct microorganisms along with the host may lead to mutualistic associations. The present study aimed to investigate the nutritional and antioxidant qualities of seeds and pericarp of pea raised from seeds treated with beneficial microbes namely *Bacillus subtilis* BHHU100, *Trichoderma harzianum* TNHU27 and *Pseudomonas aeruginosa* PJHU15 either singly and/or in consortia. A significant increase in total phenolic, flavonoid, ascorbic acid and protein contents, free radical scavenging activity, hydroxyl radical scavenging activity, iron chelation and reducing power were observed in the seeds and pericarp of pods treated with a consortium of microbes in comparison to control pods. Also, the differential accumulation of phenolic compounds, namely, shikimic acid, gallic acid, tannic acid, syringic acid, p-coumaric acid, quercetin and kaempferol, was observed from the HPLC chromatogram of the seed extracts of different treatments. We especially emphasized on dietary importance of the pod pericarp, other than seeds, along with their modulation by microbial consortium. The study also highlights the role of beneficial microbes in improving nutritional value by providing protection against oxidative stress.

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1. Introduction

Plants respond to both biotic and abiotic stresses by generating reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical and superoxide anion (O^{2-}) (Apel & Hirt, 2004; Torres, 2010). These ROS when produced in excess can oxidize nucleic acids, proteins, lipids and sugars, causing damage to the cell membrane integrity and inactivation of various cellular functions (Halliwell & Gutteridge, 1999). Antioxidants can protect cellular systems from the potentially harmful effects of ROS-causing excessive oxidations (Krinsky, 1992) and maintain cellular redox homeostasis. They may also inhibit pathogenesis in many diseases involving oxidation reactions (Diplock et al., 1998). Thus, antioxidants protect the cells from injurious effects of ROS and reactive nitrogen species by scavenging O and N free radical species, metal chelation and iron sequesteration.

Plants with improved antioxidant machinery when consumed protect the human cells against oxidative damage caused in various chronic diseases, such as cancer, cardiovascular diseases and diabetes (Podsedek, 2007; Temple, 2000). Therefore, better ROS scavenging activity in plants will not only make them efficient in protecting themselves from adverse environmental factors but is also desirable in terms of nutrition and health point of view. Plants have well-equipped defense and ROS scavenging systems to defend them from pathogenic ingress. Host defense mechanisms can be strengthened by treating them with beneficial microorganisms. Beneficial rhizospheric microorganisms not only protect plants by inducing systemic resistance in the host but also improve food quality by increasing the antioxidant levels. Rhizospheric inhabitant plant growth promoting microorganisms (PGPMs) have shown to enhance ROS scavenging activity during pathogenic ingress via increased antioxidant levels (Jetiyanon, 2007; Singh, Sarma, Upadhyay, & Singh, 2013). The inoculation of antagonistic yeasts on peach fruits decreased fruit decay, and it was shown that antioxidant defense response was also involved in combating oxidative stress caused by the fungal pathogen *Monilinia fructicola* (Xu, Qin, & Tian, 2008). Similarly, the plant growth-promoting bacterium *Bacillus lentimorbus* NRRL B-30488 was found to induce the accumulation of total phenol and antioxidant enzymes. NRRL B-30488 also enhanced 1,1-diphenyl-2- picrylhydrazyl (DPPH) and superoxide scavenging activities along with inhibition of lipid peroxidation in some vegetables (*Trigonella foenum-graecum*, *Lactuca sativa*, *Spinacia oleracea* and *Daucus carota*) and a fruit (*Citrus sinensis*) (Nautiyal, Govindarajan, Lavania, & Pushpangadan, 2008). An extension of this approach could be using a consortium of PGPMs, which could enhance reliability and efficacy of protection against pathogenic ingress (Jain, Singh, Singh, Singh, Upadhyay, et al., 2013). Some earlier reports have proven the effectiveness of using microbial consortium for plant growth promotion,

Abbreviations: BCAs, Biocontrol agents; FRSA, Free radical scavenging activity; HRSA, Hydroxyl radical scavenging activity; ROS, Reactive oxygen species; RP, Reducing power.

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induction of systemic responses, increased yield and protection from diseases (Sangeetha, Thangavelu, Usha Rani, Muthukumar, & UdayKumar, 2010; Srinivasan & Mathiavanan, 2009; Srivastava, Khalid, Singh, & Sharma, 2010). However, the impact of PGPMs in consortia in improving nutritional and dietary values has not been studied.

We developed a beneficial microbial consortium consisting of *Trichoderma harzianum* (NRRL 30596), *Bacillus subtilis* (JN099686) and *Pseudomonas aeruginosa* (JN099685), which reduced plant mortality, increased phenolic and proline content and enhanced activities of defense related and antioxidant enzymes in leaves of pea challenged with *Sclerotinia sclerotiorum* (Jain, Singh, Sarma, & Singh, 2012; Jain, Singh, Singh, & Singh, 2013). However, it was not clear whether these beneficial microbes, when applied either singly and/or in consortium, have any effect on nutritional and antioxidant components in the seeds and pericarp of pea pods. The present study was therefore conducted to analyze the role of microbial consortium consisting of *T. harzianum*, *B. subtilis* and *P. aeruginosa* in modulating the antioxidant components in seeds and pericarp of the pea pod using standard *in vitro* antioxidant assays. A large amount of raw materials are wasted during pea processing, and utilization enhancement in the dietary importance of pod pericarp can make it a good source of antioxidants and functional compounds, for food other than seeds.

2. Materials and methods

2.1. Inoculum preparation of beneficial microbes

P. aeruginosa PJHU15 (GenBank accession no. JN099685) and *B. subtilis* BHHU100 (GenBank accession no. JN099686) were isolated from rhizosphere of *Pisum sativum* from Jaipur and Hyderabad, respectively, as described previously (Jain, Singh, Sarma, & Singh, 2012). *Trichoderma* isolate TNHU27, used in the present experiment and previously identified as *T. harzianum* (ATCC No. PTA-3701), was isolated from an agricultural farm (Pantnagar). These isolates were selected on the basis of their antagonistic potential against *S. sclerotiorum* and can be used in consortium mode as they are compatible *in vitro* and *in vivo* as studied previously by giving seed treatment (Jain, Singh, Sarma, & Singh, 2012).

A single colony of bacterial isolates (*B. subtilis* and *P. aeruginosa*) was transferred to 250 ml flasks containing 100 ml of nutrient broth, and the flasks were kept on a rotating shaker (150 rev min^{-1}) for 48 h at 27 ± 2 °C. The bacterial cells were harvested by centrifugation at 6000×g for 10 min at 4 °C and washed twice with sterile distilled water. The final pellet was resuspended in a small quantity of sterile distilled water, and the final cell density was adjusted to 4×10^8 CFU ml^{-1} using the Thermo Scientific UV 1 spectrophotometer. Similarly, *T. harzianum* was grown on potato dextrose agar (PDA) for 6 days at 27 ± 2 °C, and the spores were harvested and brought to a final cell density of 2×10^7 CFU ml^{-1}.

2.2. Greenhouse Experiment

Soil mixture containing sandy soil, vermicompost and farmyard manure (2: 1: 1) was sterilized for three consecutive days at 15-lb pressure for 30 min in an autoclave, and 1.5 kg of the soil mixture was filled in each plastic pot. Seeds of pea (*P. sativum* L. cv. Arkel) were surface sterilized with 1% sodium hypochlorite for 30 s, rinsed twice with sterile distilled water and dried under a sterile air stream. The seeds were coated with *B. subtilis*, *P. aeruginosa* and *T. harzianum* either singly or in dual or triple combinations with suspensions of the organisms prepared in 1% carboxymethyl cellulose. For coating, seeds were soaked in the respective suspensions of organisms for 10 h [in case of consortia, equal amounts of suspensions (v/v) were mixed], after which the microbial suspension was drained off and the seeds were dried overnight in sterile Petri dishes. The following treatments were examined: (i) *B. subtilis* (BHHU100), (ii) *T. harzianum* (TNHU27), (iii) *P. aeruginosa* (PJHU15), (iv) BHHU100 + TNHU27, (v) BHHU100 + PJHU15, (vi) TNHU27 + PJHU15 and (vii) BHHU100 + TNHU27 + PJHU15. One set of untreated control plants was also maintained. For each treatment, five pots were used, and six seeds were sown in each pot. The pots were placed in a greenhouse, and irrigation was provided as and when required or at 2 days interval till partial saturation. A cycle of 10 h dark/14 h light and a temperature of 18 ± 2 °C were maintained in the greenhouse. After 10 weeks, the pods were harvested from each treatment and used for further analysis.

2.3. Ascorbic acid content (AA)

AA content was quantified using the method of Keller and Schwager (1977). Fresh seed (0.1 g) and pericarp samples were homogenized in 2 ml of oxalic acid and Na EDTA extraction solution. To 1 ml of supernatant, 5 ml of 20 μg/ml of 2,6-dichlorophenol-indophenol (DCPIP) was added, and the absorbance of the color developed was recorded at 520 nm against a reagent blank using the spectrophotometer. AA content was expressed in terms of μmol g^{-1} fresh weight (FW).

2.4. Protein content

Protein was estimated following the method of Lowry, Nira, Rosenburgh, Lewis, and Rose (1951) using bovine serum albumin as the standard and expressed in terms of mg protein g^{-1} FW.

2.5. Sample preparation

Seeds and pericarps from the pods were separated, the extracts were prepared by crushing 2.0 g of fresh seed and pericarp in mortar and pestle with 10 ml of methanol: H_2O (1:1) and incubated overnight at room temperature. The extracts thus obtained were filtered through sterilized Whatman No. 1 filter paper. The extracts were mixed with equal volume of ethyl acetate in a separating funnel and were shaken vigorously. The ethyl acetate fractions were collected separately, and the residue was extracted again using the ethyl acetate. The pooled fractions were then evaporated under vacuum. Dried samples were suspended in 1.0 ml of HPLC grade methanol, vortexed and used for analysis.

2.5.1. Total phenolic content (TPC)

TPC was determined following the method of Zheng and Shetty (2000) using gallic acid (GA) as standard. To 1 ml of extract, 1 ml of 95% ethanol, 5 ml of sterile distilled water and 0.5 ml of 50% Folin-Ciocalteau reagent were added, and the content was mixed thoroughly. After 5 min, 1 ml of 5% sodium carbonate was added to the above reaction mixture, and the content was allowed to stand for 1 h at room temperature. The absorbance of the color developed was recorded at 725 nm against a reagent blank. TPC was expressed in terms of mg GA equivalents (GAE) g^{-1} FW.

2.5.2. Total flavonoid content (TFC)

TFC was determined following the procedure of Dewanto, Adom, and Liu (2002), expressed in catechin equivalents based on standard curve. To 1 ml extract placed in a 10 ml volumetric flask, 4 ml of distilled water and 0.3 ml of 50% $NaNO_2$ solution were added. After 5 min, 0.3 ml of 10% $AlCl_3$ solution was added to the above mixture. After another 5 min, 2 ml of 1 M NaOH was added, and the volume was made up to 10 ml with 95% ethanol. The solution was mixed thoroughly, and absorbance was recorded at 510 nm against a reagent blank. TFC was expressed as mg catechin equivalents g^{-1} FW.

2.5.3. Free radical scavenging activity (FRSA) and reducing capacity

The hydrogen donating or free radical scavenging activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method using the stable radical DPPH, which is reduced in presence of antioxidant active substances. DPPH as stable free radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule. For analysis, 100 μl of the sample was added to 2.9 ml freshly prepared

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