




Multifarious functional traits of free-living rhizospheric fungi, with special reference to *Aspergillus* spp. isolated from North Indian soil, and their inoculation effect on plant growth

Mohd Imran^{1,2}, Hussein H. Abulreesh^{3,4} , Mohammad K. Monjed^{3,4}, Khaled Elbanna^{3,4,5}, Samreen¹ and Iqbal Ahmad^{1,3,4*}

Abstract

Purpose: Rhizospheric soil fungi are critical for plant and soil health. However, their multiple functional traits and impact on plant growth have not been systematically explored.

Methods: During this study, biochemical traits of 73 indigenous soil fungal isolates and 15 unidentified isolates related to plant growth promotion and production of extracellular enzymes were studied.

Results: Forty four (65.67%) of the total isolates produced indole acetic acid (IAA) followed by siderophore (52.23%), phosphate solubilization (37.31%), and antibiotic (11.93%). 91.04% of the studied isolates produced ammonia whereas 28.35% produced organic acid. Extracellular enzyme activities of lipase, amylase, chitinase, and cellulase were detected among 95.52%, 61.11%, 35.82%, and 41.79% isolates, respectively. Based on these activities, 73 fungal isolates were categorized into different biotypes. Quantitative analysis of IAA production and phosphate solubilization was carried out for *Aspergillus*, *Penicillium*, and *Rhizopus* isolates. *Aspergillus* isolates exhibited varying activities of IAA production and phosphate solubilization. Most of the *Aspergillus* isolates and some other fungi demonstrated multiple activities. Based on the multiple traits of selected fungal isolates, *Aspergillus* sp-07, *Penicillium* sp-03, and *Rhizopus* sp-02 were further evaluated in different combinations for their inoculation effect on the growth and yield of wheat under field conditions.

Conclusions: The results indicated that these isolates could be developed into bio-inoculants to enhance plant growth. The consortium of these three isolates was also found to be compatible and beneficial for plant growth.

Keywords: Soil fungi, Plant growth promotion, Indole acetic acid, Phosphate solubilization, Extracellular enzymes

* Correspondence: ahmadiqbal8@yahoo.co.in

¹Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 202002, India

³Department of Biology, Faculty of Applied Science, Umm Al-Qura University, Makkah, Saudi Arabia

Full list of author information is available at the end of the article



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Background

Fungi are an integral part of the terrestrial ecosystems and are known for their wide applications in the food, pharmaceutical, agricultural, and health care industries. Soil fungi play an important role in the biogeochemical cycling of the elements, maintaining soil fertility, and humus formation by decomposing organic materials (Frąc et al. 2018; Imran et al. 2020). Phosphate solubilization, nutrient mobilization, metal solubilization, and production of organic acid and extracellular enzymes by fungi have also been reported from different soil niches (Newbound et al. 2010; Frąc et al. 2018). Free-living filamentous soil fungi are diverse, adaptable, and beneficial for plants. They possess the extraordinary ability to degrade complex natural and synthetic organic compounds by producing a wide range of extracellular enzymes (Khalil et al. 2013; Zhao et al. 2014; El-Morsy et al. 2017). Indigenous soil fungi produce different enzymes including amylases, proteases, lipases, pectinases, and cellulases, which are important for industrial utilization (Park et al. 2017; McKelvey and Murphy 2018). These enzymes play vital roles in the soil ecosystem via nutrient turnover and maintaining soil fertility; however, their direct role in promoting plant growth is less explored.

The rhizosphere is a nutrient-rich zone for microorganisms and contains more microbial population density and diversity as compared with bulk soil. Extensive research data have been documented about the relation of mycorrhizal fungi in enhancing plant growth, effect on crop yield, and their significance to the environment (Bona et al. 2017; Carrara et al. 2018; Mohamed et al. 2019). However, such data about free-living rhizospheric soil fungi are limited. Soil bacteria have gained much attention as biofertilizers and biocontrol agents during the last few decades (Hafeez et al. 2006; Bhardwaj et al. 2014; Osman et al. 2015; Singh et al. 2019). The free-living fungi have yet not been systematically screened for their potential in promoting plant growth. Different species of filamentous fungi have been reported with only one major character such as phosphate solubilization by *Aspergillus* and *Penicillium* (Elias et al. 2016). *Trichoderma* spp. are also known for phosphate-solubilizing properties and have been developed as phosphate solubilizer bioinoculant and biocontrol agent (Li et al. 2015; Shaw et al. 2016).

During this study, we hypothesized that multiple beneficial activities of fungal isolates are distributed among common free-living rhizospheric fungi, and the selection of fungi might enhance plant growth under field conditions. Considering the huge diversity of free-living rhizospheric filamentous fungi and the lack of concerted efforts to screen and utilize the rich diversity of indigenous free-living rhizospheric fungi in promoting plant

growth, we used previously isolated rhizospheric fungi from the agricultural fields of Aligarh, Northern India (Imran et al. 2020). During this study, these fungi were further screened for plant growth-promoting traits and extracellular enzyme activities. The isolates with desired traits were also tested in different combinations to assess their potential in promoting plant growth under field conditions.

Materials and methods

Rhizospheric free-living fungi used in this study

Seventy-three (73) rhizospheric fungi previously isolated from the agricultural fields adjacent to Aligarh city (Northern India) and tentatively identified based on the cultural, morphological, and molecular methods were used in this study. The details of these fungal isolates are available in a recent publication by Imran et al. (2020).

Cultivation of fungi

The fungal isolates were maintained at 4 °C in the refrigerator by subculturing on Sabouroud Dextrose Agar (SDA) (Hi-Media Lab. Pvt., Mumbai, India). Fresh fungal cultures for the experiments were prepared by transferring a loop/fragment of mycelium/spore. The purity of freshly grown cultures (28 °C for 3 to 7 days) was assessed by subsequent streak plate method followed by microscopic examination (Gilman 2001; Zafar and Ahmad 2005; Imran et al. 2020).

Screening of isolates for extracellular enzymes

All fungal isolates (73) were screened for the production of extracellular enzymes using conventional agar plate assays as follows:

Amylase activity

Starch-degrading activity of the cultures was estimated by the hydrolysis of starch on a medium ($\text{g}\cdot\text{l}^{-1}$) containing 5.0 g peptone, 3.0 g beef extract, 2.0 g starch (soluble), and 20 g agar. Freshly grown fungal spores of 3-day-old culture were spot inoculated in the center of the plate and incubated at 28 °C for 7 days. Seven-day-old fungal cultures were flooded with an iodine solution (Yarrow 1998; Singh et al. 2014). The development of a pale yellow zone around a colony in the blue medium indicated the starch-degrading activity, and isolates were considered positive for amylase production.

Lipase activity

Fungal isolates were inoculated on tributyrin agar medium ($\text{g}\cdot\text{l}^{-1}$) containing 5.0 g peptone, 3.0 g beef extract, 10 tributyrin, and 15 g agar at pH 6.5. The development of a clear halo zone around the colony indicated the lipase activity after incubation at 28 °C for 7 days (Cappuccino and Welsh 2018).

Cellulase activity

Cellulase activity was examined using a modified agarose plate technique. The plates contained the medium [14 ml of 10% $(\text{NH}_4)_2\text{SO}_4$, 15 ml of 1 M KH_2PO_4 , 6.0 ml of 35% urea, 3.0 ml of 10% CaCl_2 , 3.0 ml of 10% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 ml of trace elements solution (10 ml of concentrated HCl, 0.51% FeSO_4 , 0.186% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.166% ZnCl_2 , and 0.2% CoCl_2), 2.0 ml of Tween 80, 0.2% cellulose, and 1.5% agarose] (Mandels et al. 1962; Jahangeer et al. 2005). A single isolate of fungi was inoculated in the middle of the cellulose/agarose plate and incubated for 7 days at 28 °C. The plates were flooded with 0.1% Congo red and allowed to react for 30 min followed by destaining with 1 M NaCl for 60 min according to Teather and Wood (1982). The width of fungal growth and the zone of clearing indicated the cellulase activity in the cellulose medium.

Chitinase activity

The production of chitinases was determined on the plates pre-poured with a layer of 15 $\text{g} \cdot \text{l}^{-1}$ agar and successively poured with a 3.0-ml overlayer of the test medium containing 25 $\text{g} \cdot \text{l}^{-1}$ chitin powder (Hi-Media) (Campbell and Williams 1951; Krithika and Chellaram 2016). After incubation at 28 °C for 7 days, the formation of a clear zone around the colony in an opaque medium indicated the presence of extracellular chitinase.

In vitro detection of plant growth-promoting (PGP) traits of soil fungi

Plant growth-promoting activities of isolated soil fungi were detected and quantified *in vitro*. PGP activities such as the production of indole acetic acid, siderophore, antibiotics, ammonia, organic acid, and phosphate solubilization were determined according to the standard methods as follows:

Production and quantification of indole acetic acid (IAA)

Fungal cultures were grown in Sabouroud Dextrose broth amended with 500 $\mu\text{g} \cdot \text{ml}^{-1}$ tryptophan at 28 °C for 7 days. The fungal growth was separated from 7-day-old culture by filtration followed by centrifugation at 10,000 rpm for 20 min. Two milliliters of supernatant was taken from each tube and mixed with 4.0 ml of Salkowski's reagent (50 ml of 35% perchloric acid, and 1.0 ml of 0.5M FeCl_3), and 2–3 drops of orthophosphoric acid were added to each aliquot. The samples were incubated at room temperature for 25 min. The development of the rose pink color indicated the production of IAA (Ahmad et al. 2006). IAA was quantified by reading the Optical Density (OD) at 530 nm using a Spectronic 20D⁺ spectrophotometer, whereas its concentration was measured through a standard IAA graph ranging from 10 to 100 mg/ml.

Phosphate solubilization and quantification

Phosphate solubilization was detected on Pikovskaya agar plates containing ($\text{g} \cdot \text{l}^{-1}$) 10 g glucose, 5.0 g tricalcium phosphate, 0.5 g yeast extract, 0.5 ammonium sulfate, 0.2 g MgSO_4 , 0.1g NaCl, and a trace amount of manganese sulfate and ferrous sulfate according to Ahmad et al. (2006). The fungal cultures were grown on the Pikovskaya agar plates at 28 °C for 7 days. After the incubation period, a clear halo zone of tricalcium phosphate solubilization around the fungal colony indicated a positive result. The solubilization index was evaluated as the ratio of the total diameter (colony + halo zone) and colony diameter of the test fungi (Premono et al. 1996).

The release of soluble phosphate in the culture medium was measured to quantify the phosphate solubilization according to Gaur (1990). Briefly, the fungal cultures were separately inoculated (2.5×10^6 spore- ml^{-1}) in 25 ml of Pikovskaya broth in a 100-ml flask and incubated at 28 °C and 125 rpm for 7 days. The uninoculated medium with tricalcium phosphate served as the control. The culture was filtered and centrifuged at 10,000 rpm for 30 min, and the pH of the supernatant was determined. An aliquot of 10 ml supernatant and 10 ml of chloromolybdic acid were mixed in a separate tube, and 5–6 drops of chlorostannous acid were added. The volume was adjusted to 50 ml with double distilled water and the developed blue color was read at 600 nm. The amount of solubilized phosphate was calculated using the calibration curve of KH_2PO_4 . Biomass was separately processed to determine its dry weight.

Production of siderophores

Siderophore production was detected by the FeCl_3 test (Jalal and van der Heim 1990). Briefly, 7-day-old culture grown in Sabouroud dextrose broth was filtered through Whatman filter paper (No. 42). Culture filtrate was centrifuged at 10,000 rpm for 20 min. Then, an aliquot of 1.0 ml was taken and 1.0 ml of 2% aqueous ferric chloride solution was added. The formation of reddish brown or red color indicated the siderophore production. Uninoculated SD broth was used as a negative control.

Production of organic acid

The Sabouroud dextrose agar media amended with 2% alizarin red S dye was used to examine the production of organic acid. Both media and Alizarin red S were separately autoclaved and mixed in molten media (50 °C) before plates were poured. Spore suspension of different fungal isolates was spot inoculated in the center and incubated at 28 ± 2 °C for 7 days. Medium acidification was visualized by the presence of pH indicator Alizarin red S, whereas the diameter of the yellow (acid) zone or change in color from red to yellow around the fungal

colony indicated the production of organic acid (Prusky et al. 2001).

Production of ammonia

Ammonia production was detected in peptone water (Hi-Media). Fungal cultures were inoculated and incubated at 28 °C for 7 days. After incubation, 1.0 ml of Nessler's reagent was added to each tube, and the development of brown to the yellow color indicated the positive test (Cappuccino and Welsh 2018).

Antibiotic activity of fungal culture filtrates

A modified agar well diffusion method was adopted to determine the antibacterial activity of fungal culture filtrates (Ahmad and Beg 2001). An aliquot of 0.1 ml was grown overnight at 37 °C and appropriately diluted cultures (10^5 CFU·ml⁻¹) of *Escherichia coli* and *Staphylococcus aureus* were spread on Muller-Hinton Agar (Hi-Media) plates. The wells of 8.0 mm diameter were punched into agar medium and filled with 100 µl of fungal culture filtrates obtained from 7-day-old culture. The plates were incubated at 37 °C for 18–24 hrs. Antibiotic (chloramphenicol) concentration of 100 µg·ml⁻¹ was used as the positive control. The zones of inhibition for bacterial growth were measured in millimeters around each well.

The role of soil fungi in promoting Wheat (*Triticum aestivum*) plant growth under field conditions

The field experiments were carried out on wheat (*Triticum aestivum*) var. PBW 343, at the Agricultural fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India. Wheat seeds were sown in 12 plots by following a completely randomized design. Crops were irrigated with tube-well water by drip irrigation.

Inoculum and plant culture

The plant growth-promoting fungal isolates (*Aspergillus niger*, Asp-07), *Penicillium* sp-03, and *Rhizopus oryzae*, Rsp-02) were cultured on Sabouraud dextrose agar plates at 30 °C. The spores were scraped from the plates with a pasture pipette by using 2.0 ml sterile normal saline solution and transferred to a sterile test tube. The concentration of the spores was adjusted to 1×10^6 spore·ml⁻¹. The consortium of *Aspergillus niger*, *Penicillium* sp-03, and *Rhizopus oryzae* (Table 1) were prepared by adding equal volumes of spore suspensions of all the strains. Then, the surface-sterilized seeds were either soaked in the individual suspensions or consortium suspension for 2 h at 28 °C on a shaker. The seeds were soaked in 0.85% normal saline solution for minimum dose treatment of inorganic fertilizers (NPK and DAP) and controls (Table 1). The seeds of each treatment were kept in 10 ml sterile NSS to assess the CFU/seed of the inoculated fungi on agar media. Control seeds (uninoculated) were surface sterilized by treating with mercuric chloride (0.1%). Then, control seeds were soaked in 0.85% saline followed by three-time washing with sterile distilled water before sowing. The experimental scheme is summarized in Table 1.

Inoculum density on the seeds was determined by agitating ten seeds from each treatment in 10 ml sterile NSS that was plated on agar plates after serial dilution. Mean fungal spore density per seed was determined after 4 days of incubation at 28 ± 2 °C.

Harvesting and data collection

The wheat sampling was carried out at 50 DAS (vegetative), 70 DAS (flowering), and harvesting. The plants were randomly removed from each treatment during the sampling. Plants were extended and pressed for

Table 1 Different sowing treatments of *Triticum aestivum* var PBW 343

Treatment no.	Description of treatments	Types of treatments
T _C	Control	Uninoculated and unfertilized
T _F	NPK	Uninoculated (Inorganic fertilizer dose)
T ₁	NPK + <i>Aspergillus niger</i> (Asp-07)	Single fungal inoculation with NPK
T ₂	NPK + <i>Penicillium</i> sp-03	Single fungal inoculation with NPK
T ₃	NPK + <i>Rhizopus oryzae</i> (Rsp02)	Single fungal inoculation with NPK
T ₄	<i>Aspergillus niger</i>	Single fungal inoculation
T ₅	<i>Penicillium</i> sp-03	Single fungal inoculation
T ₆	<i>Rhizopus oryzae</i>	Single fungal inoculation
T ₇	<i>Aspergillus niger</i> + <i>Penicillium</i> sp-03	Dual fungal inoculation
T ₈	<i>Aspergillus niger</i> + <i>Rhizopus oryzae</i>	Dual fungal inoculation
T ₉	<i>Penicillium</i> sp-3 + <i>Rhizopus oryzae</i>	Dual fungal inoculation
T ₁₀	<i>Aspergillus niger</i> + <i>Penicillium</i> sp-03 + <i>Rhizopus oryzae</i>	Triple fungal inoculation

N nitrogen, P phosphate, K potassium

biometric analysis. The following growth and yield parameters were determined using standard methods.

Vegetative growth parameters

The wheat plant growth parameters such as shoot weight, shoot length, leaf numbers, Spikelet numbers, and other parameters were taken into account at three stages (50, 70, and 130 DAS).

Estimation of nitrogen content

Nitrogen content was estimated according to Lindner and Harley (1942). Dry plant material (1.0 g) was digested in concentrated H_2SO_4 and 30% H_2O_2 . A 10-ml aliquot of the digested material was added to a 50-ml flask. Two milliliters of 2.5 N sodium hydroxide was added into the flask to neutralize the excess acid, and 2.0 ml of sodium salicylate was added to prevent the turbidity. The volume of the solution was made up to the mark. An aliquot of 5.0 ml of this solution was taken, and 0.5 ml Nessler's reagent was added to it. The volume was made up to 10 ml with distilled water and allowed to stand for 5 min. Absorbance was read at 525 nm, whereas the standard curve was prepared by using ammonium sulfate solution.

Estimation of protein content

The protein content of wheat seeds was estimated according to Lowry et al. (1951). Briefly, 500 mg of the seeds soaked in PBS were finely ground in 5–10 ml PBS. The resultant sample extract was centrifuged, and the supernatant was used for protein estimation. Aliquots of 0.1 and 0.2 ml were taken from the sample extract, and the volume was made up to 1.0 ml. A tube containing 1.0 ml of water served as a blank. Five milliliters of copper solution was mixed into each tube including the blank. The contents were mixed well and allowed to stand for 10 min. A 0.5-ml aliquot of Folin's reagent was added to the mixture and incubated at room temperature for 30 min. The OD was read at 660 nm after the development of blue color. The standard curve of bovine serum albumin (BSA) solution was used to calculate the protein concentration as milligram per gram ($mg \cdot g^{-1}$) of seed.

Yield parameters

The yield parameters were studied at the time of harvesting including the weight of seeds per plant and weight of thousand seeds.

Statistical analysis

The experimental data were subjected to statistical analysis using Duncan's new multiple range test (MRT). The differences among means were calculated at $P < 0.05$.

Results

During this study, previously isolated fungal isolates (73) belonging to various genera including *Aspergillus*, *Curvularia*, *Penicillium*, *Rhizopus*, and *Mucor* (Imran et al. 2020) were screened for their potential in producing extracellular enzymes and plant growth-promoting activities. The study included 20 distinct isolates of *Aspergillus* followed by *Curvularia* (09), *Mycelia Sterillia* (05), and *Penicillium* (03), whereas the remaining 14 genera were represented by one or two isolates. Fifteen unidentified isolates were also included in the primary screening.

Screening of fungal isolates for extracellular enzyme and plant growth-promoting (PGP) activities

The production of extracellular enzymes by 73 fungal isolates is shown in Table 2. Lipase was the most frequently produced enzyme by 70 fungal isolates (95.89%) followed by amylase which was produced by 45 isolates (61.64%). The production of chitinase and cellulose was observed in 27 (36.98%), and 30 (41.09%) fungal isolates, respectively (Table 2). The majority of the isolates produced more than one enzyme, while *Mucor* spp. and *Trichothecium* could not produce any enzyme (Table 2). The production of lipase and amylase was observed in the majority of the *Aspergillus* isolates [20 (100%, $n = 20$), and 16 (80%, $n = 20$) isolates, respectively]. However, only a few isolates were able to produce both chitinase and cellulose (40%, $n = 20$) enzymes (Table 2).

Free-living rhizospheric soil fungal isolates exhibited varying plant growth-promoting activities (Table 3). Sixty-one (83.56%) isolates out of the total 73 were found positive for ammonia production, the most frequently detected trait followed by the production of indole acetic acid (IAA) in 44 (60.27%) isolates. Siderophore production was observed in 35 (47.94%) isolates followed by the detection of phosphate solubilization in 25 (34.25%) isolates. 26.02% (19) isolates produced organic acid, whereas only 10.95% (8) isolates could produce antibiotics. The production of ammonia (60%), IAA (50%), and siderophore (40%) were the most frequently detected plant growth-promoting traits in *Aspergillus* isolates ($n = 20$) (Table 3). The overall enzyme and PGP activities of *Aspergillus* isolates are presented in Table 4.

Assessment of plant growth-promoting activities of soil fungi on solid media

Phosphate solubilization and production of organic acid

The phosphate-solubilizing activity of fungal isolates was determined on Pikovskaya agar plates, and efficient isolates were identified. The maximum phosphate solubilization activity among *Aspergillus* isolates was observed in *Aspergillus* sp-01 (SI = 2.40) followed by

Table 2 Extracellular enzyme activities of 73 free-living rhizospheric fungi

Fungal isolates (n)	Number of positive isolates (%) for Enzyme activities			
	Lipase	Amylase	Chitinase	Cellulase
<i>Alternaria</i> (2 isolates)	2 (100)	2 (100)	2 (100)	ND
<i>Aspergillus</i> (20 isolates)	20 (100)	16 (80)	8 (40)	8 (40)
<i>Chlamydospora</i> (1 isolate)	1 (100)	ND	ND	ND
<i>Curvularia</i> (9 isolates)	9 (100)	5 (55.5)	2 (22.2)	5 (55.5)
<i>Fusarium</i> (1 isolates)	1 (100)	ND	1 (100)	1 (100)
<i>Hormodendrum</i> (1 isolate)	1 (100)	1 (100)	ND	ND
<i>Microsporium</i> (2 isolates)	2 (100)	1 (50)	1 (50)	ND
<i>Monilia</i> (2 isolates)	2 (100)	1 (50)	1 (50)	2 (100)
<i>Monotospora</i> (2 isolates)	2 (100)	2 (100)	2 (100)	1 (50)
<i>Mucor</i> (1 isolate)	ND	ND	ND	ND
<i>Nigrospora</i> (1 isolates)	1 (100)	1 (100)	ND	ND
<i>Penicillium</i> (3 isolates)	3 (100)	2 (66.6)	ND	ND
<i>Rhizopus</i> (2 isolates)	1 (50)	ND	1 (50)	ND
<i>Trichoderma</i> (2 isolates)	2 (100)	1 (50)	ND	1 (50)
<i>Trichophyton</i> (2 isolates)	2 (100)	1 (50)	ND	1 (50)
<i>Trichothecium</i> (1 isolate)	ND	ND	ND	ND
<i>Verticillium</i> (1 isolate)	1 (100)	ND	ND	ND
<i>Mycelia sterillia</i> (5 isolates)	5 (100)	3 (6)	5 (100)	3 (60)
Unidentified (15 isolates)	15 (100)	9 (60)	4 (26.6)	8 (53.3)
Total number of isolates (73)				
Enzyme-producing isolates, total no. (%)	70 (95.89%)	45 (61.64%)	27 (36.98%)	30 (41.09%)

n number of isolates of each identified genera

P positive isolates for enzyme production, (%) = percentage of positive isolates

ND not detected

Aspergillus sp-03 (SI = 2.29). A few isolates belonging to other fungal genera also exhibited efficient phosphate solubilization on solid agar medium including *Mucor* sp-01 (SI = 2.57), *Penicillium* sp-03 (SI = 2.53), *Alternaria* sp-01 (SI = 2.33), *Curvularia* sp-02 (SI = 2.36), and other species (Table 5).

The production of organic acid was tested on SDA agar plates amended with Alizarin dye. The halo yellow zones around the fungal colonies were measured for the production of organic acid. Fungal isolates such as *Aspergillus* sp-07 (SI = 2.30), *Alternaria* sp-02 (SI = 2.35), *Mycelia sterillia* sp-01 (SI = 2.66), and Unidentified sp-07 (SI = 2.33) efficiently produced organic acid. However, the Unidentified sp-03 (SI = 2.69) isolate remained the best producer of organic acid among all tested isolates (Table 5).

The fungal isolates were grouped into 10 PGP groups/types based on the presence or absence of one or more PGP traits such as phosphate solubilization and production of IAA, ammonia, and siderophore. Group I consisting of 12 isolates (*Aspergillus* sp-01, *Aspergillus* sp-02, *Alternaria*, *Curvularia*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, *Trichoderma*, and three unidentified spp (sp-

02, sp-10, and sp-14)) possessed all four PGP traits as shown in Table 6.

Quantitative assessment of plant growth-promoting activities and biomass production in liquid medium

Based on the multiple PGP traits, certain isolates belonging to different fungal genera were analyzed for the quantification of phosphate solubilization and IAA production. These isolates were *Aspergillus* (sp-01), *Aspergillus* (sp-02), *Aspergillus* (sp-03), *Aspergillus* (sp-04), *Aspergillus* (sp-05), *Aspergillus* (sp-06), *Aspergillus* (sp-07), *Aspergillus* (sp-16), *Aspergillus* (sp-17), *Alternaria* (sp-01), *Curvularia* (sp-02), *Fusarium* (sp-01), *Penicillium* (sp-03), and *Rhizopus* (sp-02).

Phosphate solubilization and biomass production

Fungal isolates were grown in Pikorsvkaya broth for 7 days to estimate the solubilized phosphate. The maximum tricalcium phosphate (TCP) solubilization (435 $\mu\text{g}\cdot\text{ml}^{-1}$) activity among tested *Aspergillus* isolates was exhibited by *Aspergillus* sp-07 followed by *Aspergillus* sp-06 (322 $\mu\text{g}\cdot\text{ml}^{-1}$). The maximum TCP solubilization among the fungal isolates of other genera was observed

Table 3 Plant growth-promoting activities of free-living rhizospheric fungi

Fungal isolates (n)	Plant growth-promoting activities					
	IAA production	PO ₄ solubilization	Organic acids	Siderophore	NH ₃	Antibiotic production
<i>Alternaria</i> (2 isolates)	1 (50)	2 (100)	2 (100)	2 (100)	2 (100)	ND
<i>Aspergillus</i> (20 isolates)	10 (50)	6 (30)	5 (25)	8 (40)	12 (60)	1 (5)
<i>Chlamydospora</i> (1)	1 (100)	ND	ND	1 (100)	1 (100)	1 (100)
<i>Curvularia</i> (9)	2 (22.2)	3 (33.3)	ND	5 (55.5)	8 (88.8)	1 (11.1)
<i>Fusarium</i> (1)	1 (100)	ND	1 (100)	ND	1 (100)	ND
<i>Hormodendrum</i> (1)	1 (100)	1 (100)	ND	1 (100)	1 (100)	ND
<i>Mycellia sterillia</i> (5)	4 (80)	ND	1 (20)	2 (40)	5 (100)	ND
<i>Microsporium</i> (2)	2 (100)	1 (50)	ND	ND	2 (100)	ND
<i>Monilia</i> (2)	ND	ND	ND	ND	2 (100)	ND
<i>Monotospora</i> (2)	1 (50)	1 (50)	1 (50)	2 (100)	2 (100)	ND
<i>Mucor</i> (1)	1 (100)	1 (100)	ND	1 (100)	1 (100)	ND
<i>Nigrospora</i> (1)	1 (100)	ND	ND	ND	1 (100)	1 (100)
<i>Penicillium</i> (3)	1 (33.3)	1 (33.3)	ND	2 (66.6)	3 (100)	ND
<i>Rhizopus</i> (2)	1 (50)	ND	1 (50)	ND	1 (50)	ND
<i>Trichoderma</i> (2)	1 (50)	1 (50)	1 (50)	2 (100)	2 (100)	ND
<i>Trichophyton</i> (2)	2 (100)	2 (100)	ND	ND	2 (100)	ND
<i>Trichothecium</i> (1)	1 (100)	ND	1 (100)	1 (100)	1 (100)	1 (100)
<i>Verticillium</i> (1)	1 (100)	1 (100)	1 (100)	ND	1 (100)	ND
Unidentified fungi (15)	12 (80)	5 (33.3)	6 (40)	8 (53.3)	13 (86.6)	3 (20)
Total number of fungal isolates (73)						
Total number (%) of positive isolates	44 (60.27)	25 (34.25)	19 (26.02)	35 (47.94)	61 (83.56)	8 (10.95)

ND not detected

In parenthesis, percentage of the number of isolates among the total positive fungal isolates is given.

Table 4 Multiple plant growth-promoting activities of *Aspergillus* isolates

<i>Aspergillus</i> isolates	Phosphate solubilization	Organic acid	Siderophore	NH ₃	Antibiotic production	Lipase	Amylase	Chitinase	Cellulase	IAA
<i>Asp-01</i>	+	-	+	+	-	+	+	-	-	+
<i>Asp-02</i>	+	+	-	+	-	+	+	+	-	+
<i>Asp-03</i>	-	+	-	+	-	+	+	-	-	+
<i>Asp-04</i>	+	-	-	+	-	+	+	+	-	+
<i>Asp-05</i>	+	-	+	-	-	+	+	+	-	+
<i>Asp-06</i>	+	+	-	+	-	+	+	-	+	+
<i>Asp-07</i>	+	+	+	-	-	+	+	-	+	+
<i>Asp-08</i>	-	-	+	+	-	+	+	+	+	-
<i>Asp-09</i>	-	-	-	+	+	+	-	-	-	-
<i>Asp-10</i>	-	+	+	+	-	+	-	-	+	+
<i>Asp-11</i>	-	-	+	+	-	+	+	-	-	-
<i>Asp-12</i>	-	-	+	+	-	+	+	-	+	-
<i>Asp-13</i>	-	-	-	+	-	+	+	-	-	+
<i>Asp-14</i>	-	-	+	+	-	+	+	+	-	+
Total (%)	6 (42.85)	5 (35.71)	8 (57.14)	12 (85.71)	1 (7.14)	100%	12 (85.71)	5 (35.71)	5 (35.71)	10 (71.42)

Table 5 Phosphate solubilization and organic acid production capability of soil fungal isolates

Isolates	Phosphate solubilization			Organic acid production		
	Colony diameter (mm)	Halo zone (mm)	Solubilization index (SI)	Colony diameter (mm)	Halo zone (mm)	Solubilization index (SI)
<i>Aspergillus</i> sp-01	10 ± 0.65	14 ± 0.78	2.40 ± 0.09	+	-	-
<i>Aspergillus</i> sp-02	19 ± 0.98	24 ± 1.0	2.26 ± 0.14	21 ± 1.2	25 ± 1.6	2.19 ± 0.12
<i>Aspergillus</i> sp-03	17 ± 0.74	22 ± 0.99	2.29 ± 0.16	+	-	-
<i>Aspergillus</i> sp-04	15 ± 0.68	18 ± 0.95	2.20 ± 0.10	+	-	-
<i>Aspergillus</i> sp-05	15 ± 0.71	17 ± 0.78	2.13 ± 0.08	+	-	-
<i>Aspergillus</i> sp-06	18 ± 0.89	21 ± 0.89	2.16 ± 0.07	24 ± 1.4	29 ± 1.8	2.20 ± 1.13
<i>Aspergillus</i> sp-07	26 ± 1.5	33 ± 1.9	2.26 ± 0.15	23 ± 1.3	30 ± 2.0	2.30 ± 0.15
<i>Aspergillus</i> sp-10	+	-	-	14 ± 0.80	16 ± 0.86	2.14 ± 0.10
<i>Aspergillus</i> sp-16	20 ± 0.96	23 ± 1.2	2.15 ± 0.06	17 ± 0.89	19 ± 0.96	2.11 ± 0.09
<i>Aspergillus</i> sp-17	19 ± 0.86	21 ± 1.1	2.10 ± 0.08	18 ± 0.98	21 ± 0.99	2.16 ± 0.08
<i>Alternaria</i> sp-01	12 ± 0.9	16 ± 1.1	2.33 ± 0.13	18 ± 0.94	21 ± 0.96	2.16 ± 0.07
<i>Alternaria</i> sp-02	15 ± 1.1	20 ± 1.7	2.33 ± 0.11	17 ± 0.82	23 ± 0.99	2.35 ± 0.12
<i>Curvularia</i> sp-02	19 ± 0.1.3	26 ± 2.0	2.36 ± 0.15	+	-	-
<i>Curvularia</i> sp-03	17 ± 1.0	23 ± 1.5	2.35 ± 0.10	+	-	-
<i>Curvularia</i> sp-06	21 ± 1.4	26 ± 1.8	2.23 ± 0.14	+	-	-
<i>Fusarium</i> sp-01	22 ± 1.5	27 ± 1.6	2.22 ± 0.12	16 ± 0.85	20 ± 0.88	2.25 ± 0.06
<i>Hormodendrum</i> sp-01	25 ± 1.8	33 ± 2.1	2.32 ± 0.15	+	-	-
<i>Microsporum</i> sp-01	16 ± 1.2	21 ± 1.3	2.31 ± 0.10	+	-	-
<i>Monotospora</i> sp-01	10 ± 0.8	14 ± 0.7	2.40 ± 0.16	24 ± 0.90	26 ± 1.3	1.66 ± 0.07
<i>Mycelia Sterilia</i> sp-01	+	-	-	15 ± 0.65	25 ± 1.1	2.66 ± 0.11
<i>Mucor</i> sp-01	14 ± 0.9	22 ± 1.5	2.57 ± 0.13	+	-	-
<i>Penicillium</i> sp-02	15 ± 0.8	23 ± 1.4	2.53 ± 0.13	+	-	-
<i>Penicillium</i> sp-03	13 ± 1.0	18 ± 1.1	2.38 ± 0.11	+	-	-
<i>Rhizopus</i> sp-02	14 ± 1.1	20 ± 1.3	2.42 ± 0.15	18 ± 0.78	22 ± 1.0	2.22 ± 0.08
<i>Trichoderma</i> sp-01	16 ± 1.2	20 ± 1.2	2.25 ± 0.15	26 ± 1.2	29 ± 1.5	2.11 ± 0.09
<i>Trichophyton</i> sp-01	24 ± 1.7	30 ± 2.0	2.25 ± 0.09	+	-	-
<i>Trichophyton</i> sp-02	23 ± 1.3	32 ± 2.3	2.34 ± 0.14	+	-	-
<i>Trichothecium</i> sp-01	+	-	-	15 ± 0.79	20 ± 0.96	2.33 ± 0.10
<i>Verticillium</i> sp-01	25 ± 1.5	30 ± 1.9	2.20 ± 0.10	17 ± 0.95	20 ± 0.98	2.17 ± 0.09
Unidentified sp-02	14 ± 0.8	21 ± 1.0	2.50 ± 0.08	29 ± 1.4	36 ± 2.0	2.24 ± 0.07
Unidentified sp-03	15 ± 0.7	21 ± 1.3	2.40 ± 0.07	13 ± 0.52	22 ± 1.4	2.69 ± 0.13
Unidentified sp-06	+	-	-	13 ± 0.55	15 ± 0.66	2.15 ± 0.09
Unidentified sp-07	+	-	-	18 ± 0.87	24 ± 0.98	2.33 ± 0.13
Unidentified sp-08	10 ± 0.6	16 ± 0.8	2.60 ± 0.15	14 ± 0.50	17 ± 0.78	2.21 ± 0.08
Unidentified sp-10	16 ± 1.0	23 ± 1.5	2.43 ± 0.13	+	-	-
Unidentified sp-13	+	-	-	25 ± 1.6	32 ± 2.1	2.28 ± 0.09
Unidentified sp-14	18 ± 1.2	24 ± 1.4	2.33 ± 0.11	+	-	-

+ indicates the presence of fungal growth

- indicates no solubilization zone; ± indicates SD of three replicates

Table 6 PGP typing of fungal isolates under study

PGP types	Plant growth-promoting activities				Isolate designation	Total number
	Phosphate solubilization	IAA	Siderophore	Ammonia		
I	+	+	+	++	<i>Aspergillus</i> sp-01 and sp-03; <i>Alternaria</i> sp-01; <i>Curvularia</i> sp-02; <i>Fusarium</i> sp-01; <i>Mucor</i> sp-01; <i>Penicillium</i> sp-03; <i>Rhizopus</i> sp-02; <i>Trichoderma</i> sp-01; Unidentified sp-02, sp-10, and sp-14	12
II	-	+	+	+	<i>Aspergillus</i> sp-14; <i>Chlamydozpora</i> sp-01; <i>Curvularia</i> sp-04; <i>Mycelia sterillia</i> sp-01; <i>Trichothecium</i> sp-01; Unidentified sp-04, sp-05, and sp-15	8
III	+	-	+	++	<i>Alternaria</i> sp-02, <i>Monotospora</i> sp-01	2
IV	-	-	+	++	<i>Aspergillus</i> sp-08, sp-11, sp-12, and sp-18, <i>Curvularia</i> sp-07 and sp-09; <i>Monotospora</i> sp-02; <i>Mycelia sterillia</i> sp-02; <i>Penicillium</i> sp-01; <i>Trichoderma</i> sp-02	10
V	-	-	-	+	<i>Aspergillus</i> sp-09; <i>Curvularia</i> sp-01 and sp-08; <i>Monilia</i> sp-01; Unidentified sp-06 and sp-07	6
VI	+	-	-	++	<i>Aspergillus</i> sp-16 and sp-17, <i>Curvularia</i> sp-03 and sp-06, <i>Monilia</i> sp-02	5
VII	-	-	+	-	<i>Curvularia</i> sp-05, Unidentified sp-12	2
VIII	+	+	-	++	<i>Aspergillus</i> sp-04, sp-02, and sp-06; <i>Hormodendrum</i> sp-01; <i>Microsporium</i> sp-01; <i>Penicillium</i> sp-02; <i>Trichophyton</i> sp-01 and sp-02; Unidentified sp-03; <i>Verticillium</i> sp-01	10
IX	-	+	-	+	<i>Aspergillus</i> sp-10 and sp-13; <i>Microsprum</i> sp-02; <i>Mycellia sterillia</i> sp-03, sp-04, and sp-05; <i>Nigrospora</i> sp-01; <i>Rhizopus</i> sp-01; Unidentified sp-01, sp-09, sp-11, and sp-13	12
X	+	+	+	-	<i>Aspergillus</i> sp-05 and sp-07; Unidentified sp-08	3

in *Alternaria* sp-01 (465 $\mu\text{g}\cdot\text{ml}^{-1}$) followed by *Fusarium* sp-01 (375 $\mu\text{g}\cdot\text{ml}^{-1}$), *Rhizopus* sp-02 (320 $\mu\text{g}\cdot\text{ml}^{-1}$), *Curvularia* sp-02 (295 $\mu\text{g}\cdot\text{ml}^{-1}$), and *Penicillium* sp-03 (263 $\mu\text{g}\cdot\text{ml}^{-1}$) (Table 7).

During the phosphate solubilization, the biomass production of *Aspergillus* isolates ranged between 0.53 and 0.28 $\text{g}\cdot 100\text{ ml}^{-1}$. *Aspergillus* sp-01 produced maximum (0.53 $\text{g}\cdot 100\text{ ml}^{-1}$) biomass followed by *Aspergillus* sp-02 (0.46 $\text{g}\cdot 100\text{ ml}^{-1}$) after 1 week of growth (Table 7). The growth of other fungal isolates remained in the range of 0.2 to 0.43 $\text{g}\cdot 100\text{ ml}^{-1}$ as compared with *Aspergillus*

isolates. The pH of the liquid medium was recorded in the acidic range for all the tested fungal isolates (Table 7).

Production of indole acetic acid and biomass

The production of indole acetic acid was estimated in the presence of a fixed concentration of tryptophan (500 $\mu\text{g}\cdot\text{ml}^{-1}$) in liquid media. *Aspergillus* sp-05 produced the maximum amount (31.80 $\mu\text{g}\cdot\text{ml}^{-1}$) of IAA among the isolates of *Aspergillus* genera after 1 week of growth (Table 8). The isolates of other fungal genera also produced a significant amount of

Table 7 Quantification of phosphate solubilization by selected fungi

Isolates	Phosphate ($\mu\text{g}\cdot\text{ml}^{-1}$)	Dry biomass ($\text{g}\cdot 100\text{ ml}^{-1}$)	pH
<i>Aspergillus</i> sp-01	188 \pm 4	0.53 \pm 0.04	5.1 \pm 0.05
<i>Aspergillus</i> sp-02	240 \pm 6	0.46 \pm 0.02	4.5 \pm 0.15
<i>Aspergillus</i> sp-03	258 \pm 5.5	0.44 \pm 0.03	5.5 \pm 0.10
<i>Aspergillus</i> sp-04	230 \pm 5.1	0.28 \pm 0.025	5.6 \pm 0.11
<i>Aspergillus</i> sp-05	245 \pm 2	0.29 \pm 0.023	4.8 \pm 0.20
<i>Aspergillus</i> sp-06	322 \pm 4	0.37 \pm 0.035	4.3 \pm 0.10
<i>Aspergillus</i> sp-07	435 \pm 7.0	0.35 \pm 0.04	5.0 \pm 0.2
<i>Aspergillus</i> sp-16	283 \pm 4.1	0.33 \pm 0.02	5.4 \pm 0.21
<i>Aspergillus</i> sp-17	306 \pm 2.6	0.38 \pm 0.031	5.0 \pm 0.11
<i>Alternaria</i> sp-01	465 \pm 26.1	0.43 \pm 0.030	5.0 \pm 0.20
<i>Curvularia</i> sp-02	295 \pm 24.18	0.21 \pm 0.017	5.7 \pm 0.26
<i>Fusarium</i> sp-01	375 \pm 23.2	0.33 \pm 0.028	5.0 \pm 0.20
<i>Penicillium</i> sp-03	263 \pm 23.51	0.20 \pm 0.018	5.4 \pm 0.21
<i>Rhizopus</i> sp-02	320 \pm 25.3	0.28 \pm 0.023	5.5 \pm 0.31

Table 8 Quantification of IAA production by selected fungi

Isolates	IAA ($\mu\text{g}\cdot\text{ml}^{-1}$)	Dry biomass ($\text{g}\cdot 100\text{ ml}^{-1}$)
<i>Aspergillus</i> sp-01	21.53 \pm 0.25	0.59 \pm 0.04
<i>Aspergillus</i> sp-02	15.40 \pm 0.15	0.51 \pm 0.02
<i>Aspergillus</i> sp-03	26.60 \pm 0.26	0.49 \pm 0.026
<i>Aspergillus</i> sp-04	13.60 \pm 0.1	0.36 \pm 0.025
<i>Aspergillus</i> sp-05	31.80 \pm 0.3	0.30 \pm 0.037
<i>Aspergillus</i> sp-06	19.50 \pm 0.17	0.43 \pm 0.05
<i>Aspergillus</i> sp-07	29.56 \pm 1.24	0.59 \pm 0.05
<i>Aspergillus</i> sp-10	14.40 \pm 0.2	0.25 \pm 0.015
<i>Aspergillus</i> sp-13	24.63 \pm 0.25	0.19 \pm 0.01
<i>Aspergillus</i> sp-14	17.40 \pm 0.26	0.31 \pm 0.03
<i>Aspergillus</i> sp-15	11.50 \pm 0.24	0.23 \pm 0.02
<i>Aspergillus</i> sp-19	22.63 \pm 0.2	0.54 \pm 0.03
<i>Aspergillus</i> sp-20	12.80 \pm 0.41	0.18 \pm 0.022
<i>Alternaria</i> sp-01	24.16 \pm 1.48	0.49 \pm 0.024
<i>Curvularia</i> sp-02	19.17 \pm 1.05	0.37 \pm 0.022
<i>Fusarium</i> sp-01	42.41 \pm 2.82	0.55 \pm 0.041
<i>Penicillium</i> sp-03	37.26 \pm 2.14	0.67 \pm 0.052
<i>Rhizopus</i> sp-02	35.27 \pm 2.02	0.59 \pm 0.046

\pm indicates SD of three replicates

IAA. *Fusarium* sp-01 produced a maximum (42.41 $\mu\text{g}\cdot\text{ml}^{-1}$) amount of IAA followed by *Penicillium* sp-03 (37.26 $\mu\text{g}\cdot\text{ml}^{-1}$), and *Rhizopus* sp-02 (35.27 $\mu\text{g}\cdot\text{ml}^{-1}$) (Table 8).

The fungal isolates exhibited different trends of IAA production. *Aspergillus* sp-01 and *Aspergillus* sp-07 isolates produced 0.59 g·100 ml⁻¹ biomass after 1 week of

the incubation period. The maximum biomass among the isolates of other genera was produced by *Penicillium* sp-03 (0.67 g·100 ml⁻¹), whereas *Curvularia* sp-02 (0.37 g·100 ml⁻¹) produced the minimum biomass (Table 8).

Inoculation effects of selected fungal isolates on wheat growth and yield

Shoot length Wheat seeds inoculated with plant growth-promoting fungi (*Aspergillus niger*, *Penicillium* sp-03, and *Rhizopus oryzae*) demonstrated a variable plant shoot growth when grown in unfertilized and fertilized clay loam soil. The composite inoculation of microbial cultures resulted in significantly different shoot lengths of wheat at 50, 70, and 145 DAS (Tables 9 and 10). The single inoculation of *Aspergillus niger* significantly increased the shoot length by 32.8, 44.2, and 86.7 cm at 50, 70, and 145 DAS, respectively as compared with the uninoculated and unfertilized control. A similar trend was also observed in single inoculations of *Penicillium* sp-03 and *Rhizopus oryzae*. Among dual culture inoculations, the combination of *Aspergillus niger* + *Penicillium* sp-03 significantly ($P < 0.05$) increased the shoot growth by 35.7, 52.2, and 87.9 cm at 50, 70, and 145 DAS, respectively as compared with the control. Generally, the shoot length consistently increased in all treatments up to the harvesting stage. The combination of NPK+ *Aspergillus niger* showed a better response in comparison to NPK+ *Penicillium* sp-03 and NPK+ *Rhizopus oryzae* inoculants. Triple inoculation treatment (*Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae*) produced better results than single

Table 9 Effect of fungal inoculation on different parameters of wheat plant parts at 50 and 70 days after sowing

Treatments	Shoot length (cm)		Shoot weight (g)		Shoot weight dry (g)		Leaf number	
	50 days	70 days	50 days	70 days	50 days	70 days	50 days	70 days
Control	14.5 ^{g*}	24.9 ^h	12.6 ⁱ	20.5 ⁱ	6.9 ^g	11.4 ^g	15 ^g	24 ^f
NPK	28.5 ^{bc}	35.8 ^{fg}	16.8 ^{ef}	24.8 ^{gh}	12.3 ^e	16.4 ^e	22 ^d	26 ^{ef}
NPK + <i>Aspergillus niger</i> (Asp-07)	40.6 ^{ab}	56.4 ^b	24.3 ^b	38.9 ^b	24.8 ^b	24.7 ^b	27 ^b	36 ^{bc}
NPK + <i>Penicillium</i> sp-03	35.2 ^{cd}	49.5 ^c	20.5 ^c	34.5 ^{cd}	18.6 ^c	20.3 ^c	24 ^c	31 ^d
NPK + <i>Rhizopus oryzae</i> (Rzp-02)	31.4 ^{de}	45.6 ^d	18.1 ^{de}	30.6 ^e	15.9 ^d	17.9 ^d	20 ^e	28 ^e
<i>Aspergillus niger</i>	32.8 ^d	44.2 ^d	16.4 ^{fg}	30.8 ^e	14.8 ^d	16.5 ^{de}	24 ^c	32 ^d
<i>Penicillium</i> sp-03	25.6 ^{ef}	38.1 ^{ef}	15.1 ^{gh}	26.2 ^g	11.7 ^e	14.9 ^f	20 ^e	27 ^e
<i>Rhizopus oryzae</i>	20.3 ^{fg}	33.7 ^g	13.9 ^{hi}	23.1 ^h	8.5 ^f	11.6 ^g	17 ^f	25 ^f
<i>Aspergillus niger</i> + <i>Penicillium</i> sp-03	35.7 ^b	52.2 ^c	21.5 ^c	35.5 ^c	18.7 ^c	21.5 ^c	27 ^b	38 ^b
<i>Aspergillus niger</i> + <i>Rhizopus oryzae</i>	25.3 ^c	45.6 ^d	18.4 ^d	32.3 ^d	15.7 ^d	17.4 ^d	25 ^c	35 ^c
<i>Penicillium</i> sp-03 + <i>Rhizopus oryzae</i>	22.6 ^e	39.8 ^e	15.1 ^g	27.4 ^f	12.8 ^e	13.9 ^f	22 ^d	28 ^e
<i>Aspergillus niger</i> + <i>Penicillium</i> sp-03 + <i>Rhizopus oryzae</i>	43.5 ^a	60.9 ^a	27.8 ^a	43.5 ^a	27.3 ^a	29.4 ^a	29 ^a	41 ^a
LSD at 5%	1.35	3.43	1.46	2.93	1.29	1.45	1.78	2.40

* Means in columns followed by different superscript letters are significantly different at $P < 0.05$ as determined by Duncan's multiple range test

Table 10 Effect of fungal inoculation on the yield attributes of wheat

Treatments	Shoot weight Fresh (g)	Shoot weight Dry (g)	Shoot length (cm)	Leaf no.	Spikelet number/ Ear	Ear number	Grain no./ Ear	1000 seeds weight (g)	Grain yield/ plant	Protein content (%)
Control	12.9 ^{h*}	5.2 ^g	71.9 ^e	27 ^f	49 ^e	4 ^c	32 ^g	33.5 ^f	7.8 ^j	10.8 ^g
NPK	26.4 ^{cd}	8.9 ^b	86.8 ^b	34 ^d	67 ^c	6 ^a	53 ^{cd}	53.5 ^b	12.8 ^{de}	15.5 ^{bc}
NPK + <i>Aspergillus niger</i>	30.2 ^b	9.4 ^b	92.2 ^{ab}	38 ^b	74 ^{ab}	6 ^a	59 ^b	60.3 ^a	17.4 ^b	16.8 ^{ab}
NPK + <i>Penicillium</i> sp-03.	25.5 ^{de}	8.3 ^{cd}	85.8 ^{bc}	35 ^{cd}	68 ^c	6 ^a	51 ^{de}	54.6 ^b	13.1 ^d	14.6 ^{cd}
NPK + <i>Rhizopus oryzae</i>	20.2 ^f	7.6 ^e	82.5 ^{cd}	34 ^d	63 ^d	6 ^a	48 ^{ef}	51.3 ^{bc}	10.7 ^{gh}	13.9 ^{de}
<i>Aspergillus niger</i>	24.1 ^e	8.0 ^d	86.7 ^b	35 ^c	65 ^c	5 ^b	52 ^d	52.6 ^b	12.6 ^{ef}	14.1 ^d
<i>Penicillium</i> sp-03.	19.5 ^f	6.9 ^f	78.3 ^{de}	33 ^{de}	61 ^d	5 ^b	47 ^f	47.8 ^{cd}	10.2 ^h	12.7 ^{ef}
<i>Rhizopus oryzae</i>	15.8 ^g	5.8 ^g	73.3 ^e	32 ^e	59 ^d	6 ^a	46 ^f	41.6 ^e	8.9 ^j	11.6 ^{fg}
<i>Aspergillus niger</i> + <i>Penicillium</i> sp-03.	28.2 ^c	8.8 ^{bc}	87.9 ^b	37 ^{bc}	69 ^{bc}	6 ^a	56 ^{bc}	54.9 ^b	15.3 ^c	15.9 ^b
<i>Aspergillus niger</i> + <i>Rhizopus oryzae</i>	24.3 ^e	7.7 ^{de}	82.6 ^c	34 ^d	64 ^{cd}	5 ^b	52 ^d	51.6 ^b	13.8 ^d	14.9 ^c
<i>Penicillium</i> sp-03 + <i>Rhizopus oryzae</i>	19.5 ^f	7.1 ^{ef}	78.9 ^d	34 ^d	62 ^d	4 ^c	49 ^e	46.5 ^d	11.6 ^{fg}	12.7 ^e
<i>Aspergillus niger</i> + <i>Penicillium</i> sp-03 + <i>Rhizopus oryzae</i>	34.2 ^a	10.7 ^a	97.5 ^a	42 ^e	79 ^a	6 ^a	66 ^a	64.3 ^a	19.7 ^a	17.9 ^a
LSD at 5%	1.90	0.626	6.49	2.67	5.05	0.424	3.98	4.00	1.03	1.35

* Means in columns followed by different superscript letters are significantly different at $P < 0.05$ as determined by Duncan's multiple range test

and dual inoculation treatments. The composite application of *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* significantly ($P < 0.05$) enhanced the shoot length by 43.5, 60.9, and 97.5 cm during all three stages of plant growth.

Fresh shoot biomass The fresh shoot mass of plants uprooted at 60, 90, and 145 DAS varied significantly (Tables 9 and 10). *Aspergillus niger* significantly ($P < 0.05$) increased the fresh shoot biomass by 50.24% at 70 DAS as compared with unfertilized treatment. Generally, the single and dual cultures of plant growth-promoting fungi demonstrated better performance than the control. The combination of *Aspergillus niger* + *Penicillium* sp-03 was found to be superior among dual inoculation treatments and significantly ($P < 0.05$) increased the fresh mass of shoots at 70 DAS. The combination of NPK + *Aspergillus niger* enhanced the shoot weight by 24.3, 38.9, and 30.2 g at 50, 70, and 145 DAS, respectively, in comparison to NPK + *Penicillium* sp-03 and NPK + *Rhizopus oryzae* inoculants. The tripartite combination of fungi *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* exhibited a more profound effect as compared with single, dual, and other inoculation treatments. The consortium of three inoculant cultures significantly ($P < 0.05$) increased the fresh shoot biomass by 27.8, 43.5, and 34.2 g at 50, 70, and 145 DAS respectively, as compared with the controls.

Dry shoot biomass The dry matter accumulation in the top parts of the wheat crop differed among treatments at 50, 70, and 145 DAS (Tables 9 and 10). The co-inoculation of *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* remained superior to all other treatments and significantly enhanced the shoot dry biomass by 27.3, 29.4, and 10.7 g at 50, 70, and 145 DAS, respectively relative to the control. In comparison, the dual inoculation treatment (*Aspergillus niger* + *Penicillium* sp-03) significantly ($P < 0.05$) increased the dry matter accumulation in shoots by 18.7, 21.5, and 8.8 g at the three stages (50, 70, and 145 DAS) of plant growth, respectively as compared with the control. In general, this dual combination performed better than other dual inoculation treatments. The dual inoculation of NPK with *Aspergillus niger* significantly increased the shoot biomass by 24.8, 24.7, and 9.4 g at 50, 70, and 145 DAS as compared with NPK + *Penicillium* sp-03 and NPK + *Rhizopus oryzae* inoculations. The single inoculation treatments of *Aspergillus niger* also significantly increased the shoot dry biomass by 14.8, 16.5, and 8.0 g as compared with the unfertilized control treatment at 50, 70, and 145 DAS respectively.

Leaf number Generally, the single inoculation treatments of *Aspergillus niger*, *Penicillium* sp-03, and *Rhizopus oryzae* significantly ($P < 0.05$) increased the leaf number by 29.62%, 22.22%, 18.51%, respectively than

controls at 145 DAS in 2007. The combination of NPK + *Aspergillus niger* significantly ($P < 0.05$) increased the leaf number by 80%, 50%, 40.74%/Ear at 50, 70, and 145 DAS, respectively than the control treatment (Tables 9 and 10). The results indicated that the inoculation of *Aspergillus niger* in combination with plant growth-promoting *Penicillium* sp-03 significantly ($P < 0.05$) increased the leaf number by 58.33% and 37.03% at 70 and 145 DAS, respectively as compared with the inoculation with unfertilized treatment or control. The combination of three cultures (*Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae*) produced better results than dual and single inoculation treatments (*Aspergillus niger* + *Penicillium* sp-03, NPK + *Aspergillus niger*, *Aspergillus niger*, and *Penicillium* sp-03) and significantly ($P < 0.05$) increased the leaf numbers of wheat plants (Tables 9 and 10).

Spikelet number The results presented in Table 10 indicate that the inoculation with a combination of *Aspergillus niger* and plant growth-promoting *Penicillium* sp-03 significantly ($P < 0.05$) increased the spikelet numbers as compared with the inoculation with unfertilized treatment or control. Generally, the combination of three cultures (*Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae*) produced better results than dual and single inoculation treatments (*Aspergillus niger* + *Penicillium* sp-03, NPK + *Aspergillus niger*, *Aspergillus niger*, and *Penicillium* sp-03) and significantly ($P < 0.05$) increased the spikelet numbers of wheat plants. The combination of NPK + *Aspergillus niger* significantly ($P < 0.05$) increased the number of spikelets by 51.02%/Ear at harvesting time as compared with all other treatments. The single inoculation treatment of *Aspergillus niger*, *Penicillium* sp-03, and *Rhizopus oryzae* also significantly ($P < 0.05$) increased the spikelet numbers by 32.65%, 24.48%, and 20.40%, respectively in comparison with the control.

Ear number The treatments of NPK and *Aspergillus niger*, *Penicillium* sp-03 and *Rhizopus oryzae*, *Penicillium* sp-03 + *Rhizopus oryzae*, and *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* depicted a significant ($P < 0.05$) 50% increase as compared with the unfertilized and uninoculated control. The combination of *Penicillium* sp-03 + *Rhizopus oryzae* also showed a similar effect on the ear numbers of the wheat plant. A single inoculation of *Penicillium* sp-03 and *Rhizopus oryzae* significantly ($P < 0.05$) increased the ear numbers (25%) than control (Table 10).

Grain number The results presented in Table 10 indicate that inoculation of *Aspergillus niger* in combination with plant growth-promoting *Penicillium* sp-03 significantly ($P < 0.05$) increased the grain number by 75%/Ear

as compared with inoculation with unfertilized treatment or control. The combination of three cultures (*Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae*) proved significantly ($P < 0.05$) better than dual and single inoculation treatments (*Aspergillus niger* + *Penicillium* sp-03, NPK + *Aspergillus niger*, *Aspergillus niger*, and *Penicillium* sp-03) and increased the grain numbers by 106%/Ear of the wheat plants. The combination of NPK + *Aspergillus niger* increased the number of grains by 84.37%/Ear at harvesting time as compared with the control. The single inoculation treatment of *Aspergillus niger*, *Penicillium* sp-03, and *Rhizopus oryzae* also significantly ($P < 0.05$) increased the number of the grains by 62.5%, 46.87%, and 43.75%/Ear, respectively, in comparison to the control (Table 10).

Seed yield The seed yield was expressed in terms of both weight of 1000 seeds (g) and seed weight ($\text{g}\cdot\text{plant}^{-1}$) after single and combined applications of beneficial rhizosphere microorganisms, as presented in Table 10. All treatments significantly increased the grain production during the wheat crop experiments. Single inoculation with *Aspergillus niger* significantly ($P < 0.05$) increased the grain yield (weight of 1000 seeds) by 57.01% as compared with the control. The dual inoculation treatments comparatively performed better than the single inoculation, fertilizer treatment, and control. However, inoculation with *Aspergillus niger* + *Penicillium* sp-03 was found to be the most effective than other single and dual inoculation treatments. The highest grain yield of $64.3 \text{ g}\cdot 1000 \text{ seeds}^{-1}$ was obtained in *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* treatment, which was even greater than the best performing combination of NPK + *Aspergillus niger* (Table 10).

The single inoculation of *Aspergillus niger* significantly ($P < 0.05$) increased the seed yield by 61.53% than control. The co-inoculation of *Aspergillus niger* + *Penicillium* sp-03 was found better than other dual culture treatments. Triple inoculation of *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* enhanced the seed yield by $19.7 \text{ g}\cdot\text{plant}^{-1}$ as compared with the control. The triple inoculation factor had a significantly greater effect on seed yield as compared with dual culture treatments. The application of NPK + *Aspergillus niger* was noted to be significantly superior to other combined treatments of fertilizers and cultures and increased the seed yield by $17.4 \text{ g}\cdot\text{plant}^{-1}$ as compared with the control ($7.80 \text{ g}\cdot\text{plant}^{-1}$).

Seed protein The effect of microbial inoculation on seed protein (SP) was variable (Table 10). The maximum average seed protein (17.9%) was observed with the inoculation of *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* in 2007. Among the dual inoculation

treatments, the combination of *Aspergillus niger* + *Penicillium* sp-03 significantly enhanced the SP (5.10%) as compared with the control. The single inoculation of *Aspergillus niger* produced 14.1% SP as compared with the uninoculated and unfertilized control (10.80%). The application of NPK + *Aspergillus niger* was significantly superior to other combined treatments with fertilizers and demonstrated 16.8% seed protein as compared with 10.80% in the control. The comparison of SP mean values revealed that it decreased in the following order: triple < dual < single inoculation.

Discussion

Resident soil fungi and bacteria are crucial for maintaining soil fertility and crop productivity. The role of plant growth-promoting rhizobacteria and mycorrhizal fungi is well known. However, the rich diversity of free-living rhizospheric fungi has not been systematically screened and evaluated as plant growth-promoting fungi (PGPF). Since the majority of free-living soil fungi is not host-specific; therefore, promising isolates can be developed as broad host range bio-inoculants for crops. During this study, common free-living soil fungi were isolated and evaluated as potential PGPF bio-inoculants. The screening was carried out *in vitro* and soil-plant system.

The extracellular enzymes of fungal origin have various industrial and biotechnological applications in food processing, manufacturing of detergents, textiles, pharmaceutical products, medical therapy, and molecular biology (Wösten 2019). Different extracellular fungal enzymes are known to degrade complex carbonaceous and nitrogenous substances of soil to synthesize humus that enhances soil fertility and nutrient availability for other organisms including plants (Frąc et al. 2018). These enzymes have been comprehensively studied and utilized in industrial applications. The fungal extracellular enzymes can effectively degrade organic matter and facilitate nutrient recycling. The isolates with efficient enzyme production can better survive in the rhizosphere and may also be indirectly beneficial for plant growth through nutrient turnover. Therefore, fungal extracellular enzymes with potential PGP traits were evaluated.

Lipase activity was noted to be the highest (95.52%) followed by amylase (61.11%), cellulase (41.79%), and chitinase (35.82%) among extracellular enzymes isolated from fungal strains. The production of these enzymes varied among different genera and even among the isolates of the same genus such as *Aspergillus*, *Curvularia*, and *Mycelia Sterillia* group. The differences in the genetic makeup, nature and regulatory mechanisms involved in enzyme synthesis might be the reasons behind varying enzyme production (Crueger et al. 1990). Lipases act upon the organic substrates in the rhizosphere to release

the minerals, which are available for other microorganisms and plants (Costa and Peralta 1999; Wadia and Jain 2017). Amylase activity was next to lipase in most of the genera except for some isolates such as *Fusarium*, *Chlamydozpora*, *Mucor*, *Rhizopus*, and *Verticillium*. The production of amylases is widely distributed among filamentous fungi (Rekha et al. 2013; Ogbonna et al. 2014; Verma and Verma 2018). Such fungi solubilize and utilize starch to co-mineralize nitrogenous compounds for better availability to plants in the rhizosphere. Similarly, cellulolytic activities were observed among certain groups of fungi like *Aspergillus*, *Curvularia*, *Fusarium*, *Monotospora*, *Tricophyton*, *Trichoderma*, *Mycelia sterillia*, *Monillia*, and some other isolates belonging to unidentified fungal strains. Fungi with cellulolytic activity play an important role in the enrichment and acceleration of the composting process leading to increased mineralization in the rhizosphere and might also modulate the microbial activity in the rhizosphere (Ram et al. 2014; Reddy et al. 2014; Saxena et al. 2015). Chitin is one of the most important structural polysaccharides that provide mechanical strength to organisms. Chitin is synthesized by plants, animals, and certain microbes except for bacteria and actinomycetes. Chitin-degrading activity is predominant among soil actinomycetes, bacteria, and fungi (Nampally et al. 2015; Veliz et al. 2017). The chitinase production might also have a lytic effect on phytopathogens containing chitin in their cell wall constituents. However, the actual mechanisms of phytopathogen suppression by a biocontrol organism through chitinase production have not been well illustrated. It was hypothesized during a study that chitinase may directly break down the fungal cell walls (Veliz et al. 2017). However, the role of chitinases in the carbon cycle of the biosphere is well known (Rathore and Gupta 2015).

Among the tested fungi, 37.31% fungal isolates belonging to *Aspergillus*, *Curvularia*, *Alternaria*, *Trichophyton*, *Hormodendrum*, *Monotospora*, *Mucor*, *Microsporium*, *Penicillium*, *Trichoderma*, and *Verticillium* solubilized phosphate. Several unidentified isolates (15) also served as phosphate solubilizers. The production of organic acid was detected among 28.35% fungal isolates belonging to different genera such as *Aspergillus*, *Alternaria*, *Penicillium*, *Mucor*, *Fusarium*, *Trichoderma*, *Verticillium*, and many unidentified isolates. Our findings of phosphate solubilization by *Aspergillus*, *Curvularia*, *Alternaria*, *Trichophyton*, *Hormodendrum*, *Monotospora*, *Mucor*, *Microsporium*, *Penicillium*, *Trichoderma*, and *Verticillium* are in agreement with previous studies (Gaur 1990, Scervino et al. 2010; Altaf et al. 2018; Elias et al. 2016). The phosphate-solubilizing capacity of *Trichophyton*, *Hormodendrum*, *Monotospora*, and *Microsporium* species from Northern Indian soil is probably

the first report. Similarly, our findings of organic acid production by certain fungi (*Aspergillus*, *Rhizopus*, *Alternaria*, *Penicillium*, *Monotospora*, and *Verticillium*) are in line with previous reports (Scervino et al. 2010; Liaud et al. 2014). The production of plant growth regulators is considered an important PGP trait of plant-associated microbes. The rhizospheric fungi capable of producing plant growth hormones (indole acetic acid (IAA)) might directly influence plant growth promotion (Chanclud and Morel 2016; Altaf et al. 2018). Forty-four (65.67%) isolates out of a total 67 produced detectable amounts of IAA and IAA-like compounds *in vitro* in the presence of tryptophan. These findings are supported by the previous reports on IAA production by *Aspergillus*, *Curvularia*, *Alternaria*, *Trichophyton*, and *Penicillium* (Abri et al. 2015; Fu et al. 2015; Altaf et al. 2018; Shah et al. 2019). Siderophore production is considered an indirect mechanism of plant growth promotion by rhizospheric microorganisms (Altaf et al. 2018). To solubilize ferric iron, many microbes utilize an efficient system consisting of low molecular weight (≥ 1000 da) compounds with high iron affinity, known as siderophores. Siderophores have recently received much attention in agriculture as a mechanism of promoting plant health and protection of plants from other fungal pathogens by depriving them of iron (Altaf et al. 2018; Kumar et al. 2018). Organic acid produced by *Aspergillus* spp. (citric acid and oxalic acid) might serve as siderophores (Haas 2014). *Aspergillus* spp. is known to produce hydroxamate-like siderophores (Haas 2014; Patel et al. 2017). During this study, siderophore production was noted in eight *Aspergillus* isolates followed by the isolates of *Curvularia*, *Alternaria*, *Monotospora*, *Penicillium*, *Trichoderma*, *Chlamydozpora*, *Mucor*, and *Trichothecium*. Certain isolates of *Mycelia sterilia* and unidentified fungi could also produce siderophores. However, such activity was not detected among *Rhizopus*, *Trichophyton*, and *Verticillium*. Hydroxamate-type siderophore production in fungi belonging to zygomycetes, basidiomycetes, and ascomycetes has been reported, whereas the members of Mucorales produced carboxylates (Comensoli et al. 2017). Similarly, the production of ammonia by 91.04% fungi demonstrated their role in ammonification, which is the key compound for further cycling of nitrogen elements (Espenberg et al. 2018). The role of ammonia production in plant growth promotion is well known. The production of antibiotics by eight isolates of test fungi inhibited or suppressed pathogenic bacteria. However, such activity should be thoroughly investigated as it can also inhibit useful bacteria (Rani and Jain 2017). The antibiotic production by tested fungi signifies their ecological role for better survival and occupation of ecological niche in the rhizosphere.

The rhizospheric soil is rich in fungal diversity; therefore, inoculant strain must be a competitive and effective rhizosphere colonizer. These fungi have a high level of metal tolerance (data not shown) and are expected to have competitive advantages for survival and effective colonization in the rhizosphere. The consortium used in this study was based on the compatibility of the isolates and expressed multiple biochemical and PGP traits. The three most promising fungal isolates were selected and used as inoculants in single and combined treatments to assess their inoculation effect on the growth and yield of wheat under field conditions.

Inoculation response of single-tested fungi and in combinations of two or three species showed stimulatory effect on plant growth and yield attributes of wheat crop like shoot length as well as weight, spikelet, ear and grain number, seed yield, and protein content. Generally, the inoculation of three cultures (*Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae*) together was observed most effective as compared with dual and single inoculation treatments and showed a significant increase in yield attributes of the wheat plants. Triple inoculation of *Aspergillus niger* + *Penicillium* sp-03 + *Rhizopus oryzae* enhanced the seed as compared with control. This triple inoculation factor had significantly greater effect on seed yield as compared with dual culture treatments. The combined effects of PFPG inoculation is expected due to the probability of expression of desired plant growth-promoting traits of these fungi which might have influenced the plant growth both by direct and indirect mechanisms. Inoculation of *Aspergillus niger* combination with plant growth-promoting *Penicillium* sp-03 significantly increased the grain number 75%/Ear as compared with the inoculation with unfertilized treatment or control. The phosphate-solubilizing capabilities of filamentous fungi and bacteria have been studied. Wahid and Mehana (2000) have reported the consortium effect of three phosphate-solubilizing fungal isolates (*Aspergillus niger*, *A. fumigatus*, and *Penicillium pinophilum*) on the faba bean and wheat. They observed that the yield of wheat and faba bean plants increased in response to the soil inoculation with three fungal species, and *Penicillium pinophilum* played the most effective role. The yield of wheat grains increased by 28.9 and 32.8% in the soil treated with rock phosphate and superphosphate, respectively. Similarly, the same treatments increased the production of faba bean seeds by 14.7 and 29.4%. The phosphorus uptake of both crops significantly increased after the inoculation of soil with tested fungi.

The bacterial isolate *Pseudomonas* sp. BR2, a mycorrhiza helper bacterium, significantly enhanced the early emergence of wheat seedlings (5 days after planting) under field conditions and increased the root dry matter

yield by 128%. The two TPR-solubilizing fungal isolates *Aspergillus awamori* Nakazawa C1 and *Penicillium chrysogenum* Thom C13 also increased the root dry matter yields by 60 and 44%, respectively (Babana and Antoun 2006). Wakelin et al. (2007) tested three phosphate-solubilizing fungi (*Penicillium radicum*, *Penicillium bilaiae*, and *Penicillium* sp. KC6-W2) to assess their ability in increasing the growth and phosphorus (P) nutrition of wheat, medic, and lentil. *Penicillium* sp. KC6-W2 was found to be the best plant growth-promoting (PGP) strain and significantly increased the shoot growth and dry mass in seven of the nine experiments.

The effect of three phosphate-solubilizing fungi including *Penicillium expansum*, *Mucor ramosissimus*, and *Candida krissii* on the seedling growth of wheat, has been reported by Xiao et al. (2009). All the isolates promoted growth, availability of soil phosphorus, and nitrogen uptake of wheat seedlings in the soil containing rock phosphate under pot cultivation conditions. In recent years, researchers are focusing on indigenous soil fungi as PGPF. For instance, Hossain et al. (2014) have demonstrated the ability of soil *Penicillium* sp. G15-1 in promoting plant growth and providing protection against plant pests and pathogens in cucumber plants. Recently, the plant growth-promoting activities of *Alternaria* sp. A13 have been reported in the Chinese herbal plant *Salvia miltiorrhizae* through promoting root growth and accumulation of active ingredients (Zhou et al. 2018).

Conclusion

Based on the extensive screening and evaluations, this study concludes that free-living soil fungi have great potential in promoting plant growth through diverse metabolic activities. These fungi require further detailed investigations to develop bio-inoculants with broad-spectrum fitness to wheat and other crops under normal and stress conditions.

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Authors' contributions

IM conducted the experiments. HA prepared the early draft and edited the final draft of the manuscript. MM participated in data analysis. KE participated in data analysis. S participated in the language editing of the manuscript. IA designed the experiments and wrote up the manuscript. The author(s) read and approved the manuscript.

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The data sets used and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Declarations

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Not applicable.

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Competing interests

The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

Author details

¹Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 202002, India. ²Present Address: Nazz Apartment, Kalyanpur West Ring road, Lucknow, UP, India. ³Department of Biology, Faculty of Applied Science, Umm Al-Qura University, Makkah, Saudi Arabia. ⁴Research Laboratories Unit, Faculty of Applied Science, Umm Al-Qura University, Makkah, Saudi Arabia. ⁵Department of Agricultural Microbiology, Faculty of Agriculture, Fayoum University, Fayoum, Egypt.

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