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## Exploration of multitrait antagonistic microbes against *Fusarium oxysporum* f.sp. *lycopersici*

### Prachi Singh

Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221005 (Uttar Pradesh), India

### Jyoti Singh

Department of Botany, Institute of Science, Banaras Hindu University, Varanasi- 221005 (Uttar Pradesh), India

### Rahul Singh Rajput

Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221005 (Uttar Pradesh), India

### Anukool Vaishnav

Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221005 (Uttar Pradesh), India

### Shatrupa Ray

Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221005 (Uttar Pradesh), India

### R.K. Singh

Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221005 (Uttar Pradesh), India

### H.B Singh\*

Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi- 221005 (Uttar Pradesh), India

\*Corresponding author. E-mail: [hbs1@rediffmail.com](mailto:hbs1@rediffmail.com)

### Abstract

*Fusarium wilt* is one of the major diseases of tomato causing extensive loss of production. Exploration of agriculturally important microbes (AIMs) for management of the tomato wilt is an ecofriendly and cost effective approach. In the present study, a total 30 *Trichoderma* and 30 bacterial isolates were screened in the laboratory for their biocontrol activity against *Fusarium oxysporum* f.sp. *lycopersici* (FOL). Out of all the isolates tested, *Trichoderma asperellum* BHU P-1 and *Ochrobactrum* sp. BHU PB-1 were found to show maximum inhibition of FOL in dual culture assay. Both the microbes also exhibited plant growth promoting activities such as phosphate solubilisation, production of siderophore, hydrogen cyanide (HCN), indole acetic acid (IAA) and protease activity. These microbes could be evaluated further in greenhouse and field studies for their potential use in management of *Fusarium wilt* of tomato.

**Keywords:** *Fusarium oxysporum* f.sp. *lycopersici*, *Trichoderma*, *Ochrobactrum*, Tomato

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## INTRODUCTION

Vegetables are essential source of macro and micronutrients, antioxidants, minerals and dietary fibres in human diet (Wargovich, 2000; Schreinemachers *et al.* 2018). Among vegetables, tomato with its high nutritional values (source of Vitamin C, Biotin, Vitamin A, Vitamin K, potassium, phosphorus and dietary fibres) plays a crucial role in our daily diet. Although tomato production is hampered by several diseases such as *Fusarium wilt*, early blight, damping off, tomato spotted wilt, bacterial wilt etc. *Fusarium oxysporum* f.sp. *lycopersici*

(FOL), but wilt pathogen causes profound loss to tomato production worldwide. FOL, a soil-borne pathogen, persists in soil for about 8-10 years in the form of chlamydospores as resting structure (Katan 1971). Management of *Fusarium oxysporum* is done by chemical pesticides such as pentachloronitrobenzene (PCNB) and soil fumigants as vapam, chloropicrin and methyl bromide. These agrochemicals are associated with several issues including phytotoxicity, pesticide residue, health hazards and increased cost (Stevens *et al.* 2003). Escalated use of chemical fertilizers and pesticides has aggravated the soil and water con-

ditions due to chemical residue accumulation. The chemical residue enters the food chain imposing serious hazards to human health and thus there is need to look for safer alternative to chemical fertilizers and pesticides. In this context, the use of agriculturally important microbes (AIMs) have emerged as an environmental friendly approach to boost up agricultural production by strengthening plant defense and promoting plant growth.

AIMs comprising of soil/rhizospheric fungi and bacteria such as *Trichoderma*, *Pseudomonas*, *Bacillus*, *Azospirillum* etc. have antagonistic activity against soil-borne fungal pathogens and potentiality to enhance plant growth (Choudhary *et al.* 2016). AIMs involve various mechanisms for antagonism such as hyperparasitism, production of antibiotics, hydrolytic enzymes, toxins, competition for nutrients and induce systemic resistance in plants (Vinale *et al.* 2008; Vaishnav *et al.* 2014; Singh 2014a; Bisen *et al.* 2016). AIMs possess several plant growth promoting properties like: phosphate solubilisation, production of siderophore, phytohormones and ACC-deaminase activity. These growth promoting properties, augment plant growth and development through increase nutrient/ water uptake efficiency, root/ shoot length, number of root/shoot, phytohormone signalling and antioxidant activities (Perrig *et al.* 2007; Vaishnav *et al.* 2016; Kumar *et al.* 2017).

In the present study, antagonistic microbes including fungal spp. and bacterial spp. were isolated from agriculture field soil samples collected from different location of Varanasi district, Uttar Pradesh, India and evaluated against the *Fusarium* wilt pathogen of tomato. These were also evaluated for their plant growth promotion activities such as phosphate solubilisation, siderophore production, IAA production, HCN production and protease activity with an objective of their exploitation as biofertilizer and biopesticides after field testing and assessment.

## MATERIALS AND METHODS

**Collection of soil samples:** Soil samples were collected from agricultural field of five different location of Varanasi district – BHU agricultural farm, Jakhini, Adalpura, Laskariya and Ramnagar from which about 25 different fungal isolates and 25 different bacterial isolates were recovered. In addition, five *Trichoderma* and five bacterial strains each were taken from Biological control laboratory, Department of Mycology and Plant Pathology, Banaras Hindu University, Varanasi, India.

**Isolation and purification of agriculturally important microbes:** Isolation of AIMs from soil was done by serial dilution upto  $10^{-7}$  dilution in sterile distilled water followed by plating on *Trichoderma* selective medium for isolation of *Trichoderma* and plating on Nutrient agar medium for isolation of

bacterial isolates. Single colonies of fungi and bacteria were transferred on fresh culture media.

### **In vitro screening of agriculturally important microbes**

**On the basis of antagonistic potential:** Isolated microbes were screened for their antagonistic potential against *Fusarium oxysporum* f.sp. *lycopersici* by dual culture assay. A 2 mm of *Fusarium* mycelia plug grown on agar plate was placed at a distance of 2 cm from the edge of the PDA agar plate and challenged on the other end of the plate at 2 cm from edge with a 2 mm plug of *Trichoderma* as shown in Fig. 2. Streaking was done in case of bacterial isolates. Petriplates were kept for incubation at  $28 \pm 2^{\circ}\text{C}$  and after 7 days of incubation, percent (%) inhibition of the FOL against each isolate was calculated using the following formula:

$$I = (C - T) \times 100 / C$$

Where, I = % inhibition in mycelia growth; C = growth of pathogen in control plates; T = growth of pathogen in dual culture plates.

**On the basis of plant growth promoting (PGP) traits:** All the isolates were assessed for plant growth promotion attributes including phosphate solubilisation and production of siderophore, HCN, IAA and protease activity.

**Phosphate solubilisation assay:** Screening for phosphate solubilisation was done on NBRI-BPB agar plate supplemented with 0.8 mM calcium triphosphate and 0.5 mM glucose and activity was evaluated according to method devised by Mehta and Nautiyal (2001) and King (1932).

**Siderophore production assay:** The isolates were screened for siderophore production according to Schwyn and Neilands (1987) by inoculation on chromo azurol Sulphonate medium agar plates followed by incubation at  $28 \pm 2^{\circ}\text{C}$  for 72 hr. Discolouration of medium around culture indicated positive result.

**Indole acetic acid (IAA) assay:** The isolates were screened for indole acetic acid production according to Loper and Scroth (1986). The fungal isolates were inoculated in PDB and bacterial in NB medium amended with tryptophan (5mM) in glass vials and incubated at  $28 \pm 2^{\circ}\text{C}$  for 72 hr at 120 rpm. After incubation, culture was centrifuged at 10,000 rpm for 10 min. at  $4^{\circ}\text{C}$ , 2 ml of supernatant was taken in separate test tube and 4 ml of Salkowski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.5 M ferric chloride) was added (Gordon and Weber 1951). Above mixture was incubated at room temperature for 30 min and development of pink red colour was recorded as positive.

**HCN production assay:** The isolates were screened for HCN production according to Bakker and Schipper (1987). The fungal isolates were inoculated in PDB and bacterial in NB medium in glass vials amended with 4.4g glycine/l. Flasks of

whatman filter paper, dipped in 2% sodium carbonate prepared in 0.5% picric acid was placed at edge of the vials and were incubated at  $28\pm 2^{\circ}\text{C}$  for 72 hr. The change in colour of flags from yellow to dark brown indicated positive results.

**Protease production assay:** Protease production assay was performed according to Liu *et al.* (2009) with slight modification. Isolates inoculated on skimmed milk agar plates were incubated at  $28\pm 2^{\circ}\text{C}$  for 72 hr. Positive test was indicated by appearance of clear zone around colonies.

**Identification of potential isolates:** One fungal and one bacterial isolates possessing all PGP traits and antagonistic potential were selected for molecular identification by ITS and 16S rRNA gene sequencing. For molecular identification, DNA isolation of both the isolates was done by CTAB method, followed by PCR amplification of rRNA genes with their respective ITS and 16S primer.

**Statistical analysis:** Experiments were repeated using completely randomized design. The data are expressed as the mean of three independent replications  $\pm$  standard deviations. Means were compared by Duncan's multiple range tests ( $p \leq 0.05$ ) using SPSS version 20.

## RESULTS

**Isolation of AIMs isolates from different location of Varanasi district:** A total of 25 isolates were recovered each of fungi and bacteria from five different locations of Varanasi district. In detail, 5 isolates of each fungi and bacteria from Jakhini, 4 isolates of fungi and 6 isolates of bacteria from Adalpura, 7 isolates of fungi and 6 isolates of bacteria from BHU agricultural farm, 4 isolates of fungi and 4 isolates of bacteria from Laskariya and 5 isolates of fungi and 4 isolates of bacteria from Ramnagar.

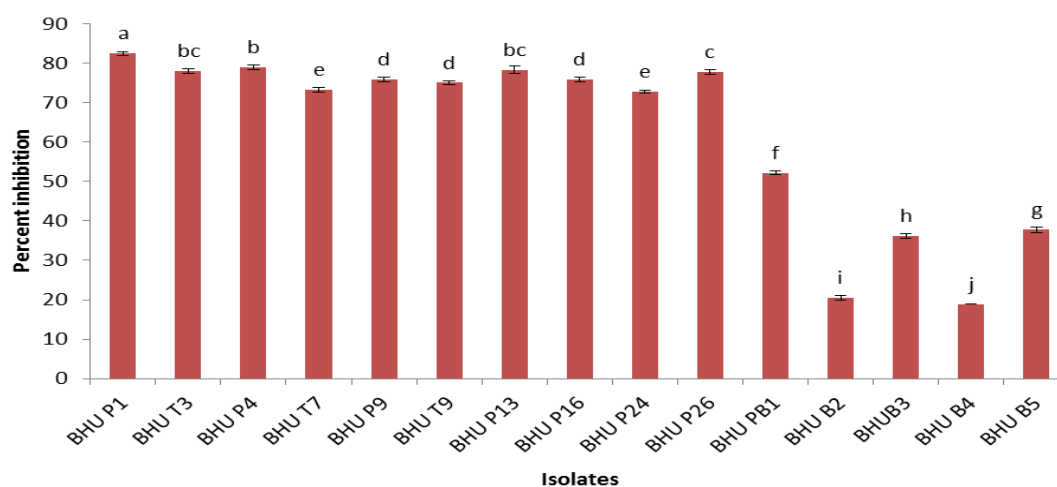
**Screening of isolates against *Fusarium oxysporum* f.sp. *lycopersici*:** Out of total, 30 fungi and 30 bacterial isolates, 10 of fungi and 5 bacterial isolates showed significant reduction in colony diameter of FOL over control. Fungal isolate BHU P1 showed maximum percent inhibition ( $82.49\pm 0.46^{\text{a}}$ ) and minimum percent inhibition was exhibited by BHU P24 ( $72.84\pm 0.39^{\text{e}}$ ) (Fig 1, 2). Whereas among bacterial isolate BHU PB1 showed maximum reduction in colony diameter ( $52.21\pm 0.54^{\text{f}}$ ) and minimum reduction was recorded by BHU B4 ( $18.86\pm 0^{\text{j}}$ ) (Fig 1, 2).

**Assessment of the plant growth promotion ability of isolates:** Out of all isolates, three fungal isolate BHU P1, BHU P4, BHU P13 and three bacterial isolate BHU PB1, BHU B3, BHU B5 with antagonistic potential against FOL also showed plant growth promotion activity. Fungal isolates BHU P1 and bacterial isolate BHU PB1 exhibited high level of all the tested PGP activities (phosphate solubilisation, siderophore production, IAA production, HCN production, protease activity) (Table 1 and Fig 3).

**Identification of the most potential fungal and bacterial isolate:** The fungal isolate BHU P1 and bacterial isolate BHU PB1 with highest antagonistic potential against FOL and all the PGP activities (phosphate solubilisation, siderophore production, IAA production, HCN production, protease activity) were identified as *Trichoderma asperellum* BHU P-1 (MK 256334) (Fig 4) and *Ochrobactrum* sp. BHU PB-1 (MK587668) (Fig 5) respectively. Sequences were submitted in GenBank database, NCBI.

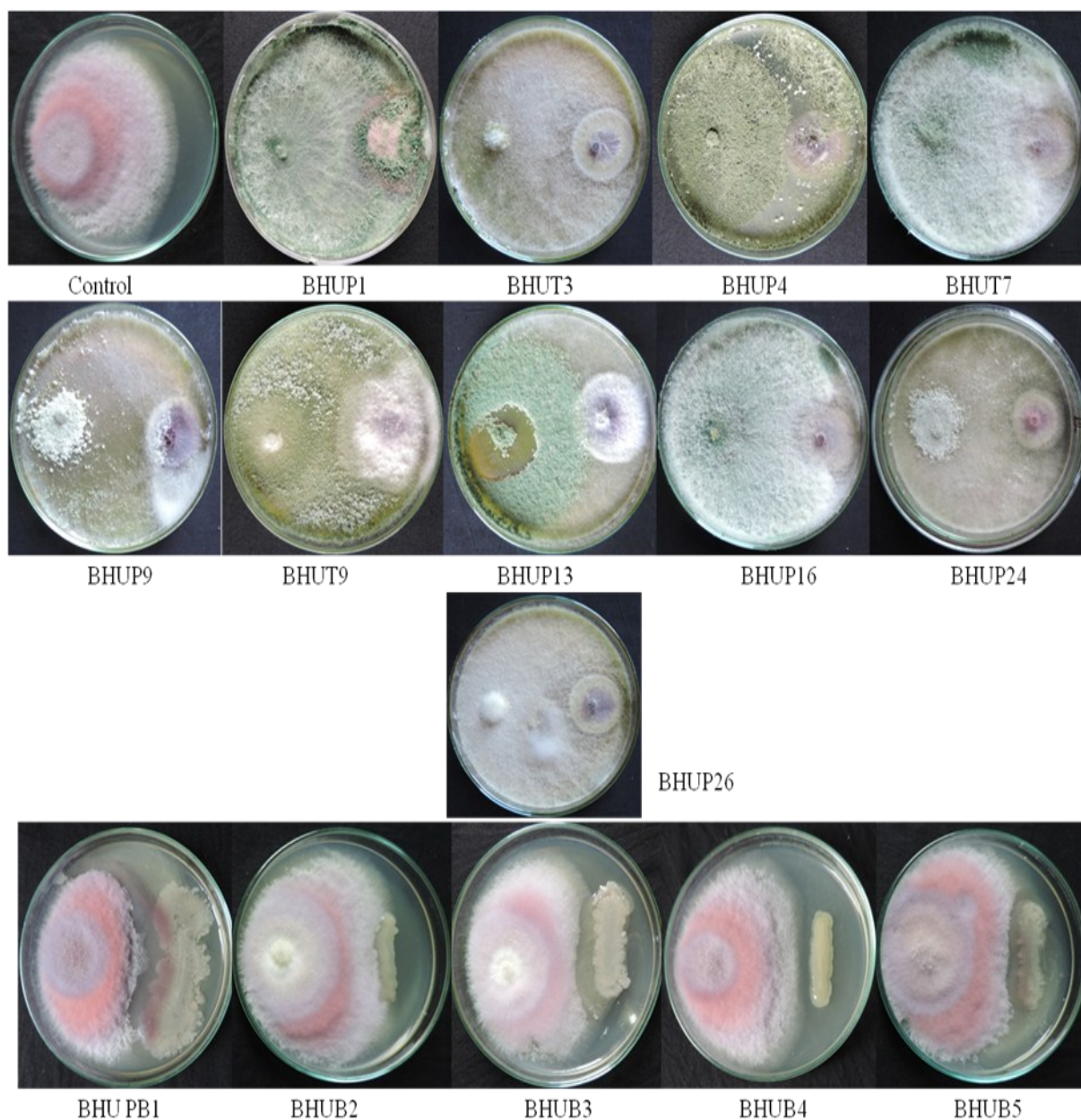
## DISCUSSION

In this study, we have isolated about 25 fungal and 25 bacterial isolates from agricultural field soil of Varanasi district. In addition, 5 each fungal and



**Fig. 1.** Percent inhibition of FOL by fungal and bacterial isolates. Results are expressed as means of three replication and vertical bar indicate the standard deviation of the means. Different letters indicate significant differences among treatments according to Duncan's multiple range tests at  $p \leq 0.05$ .



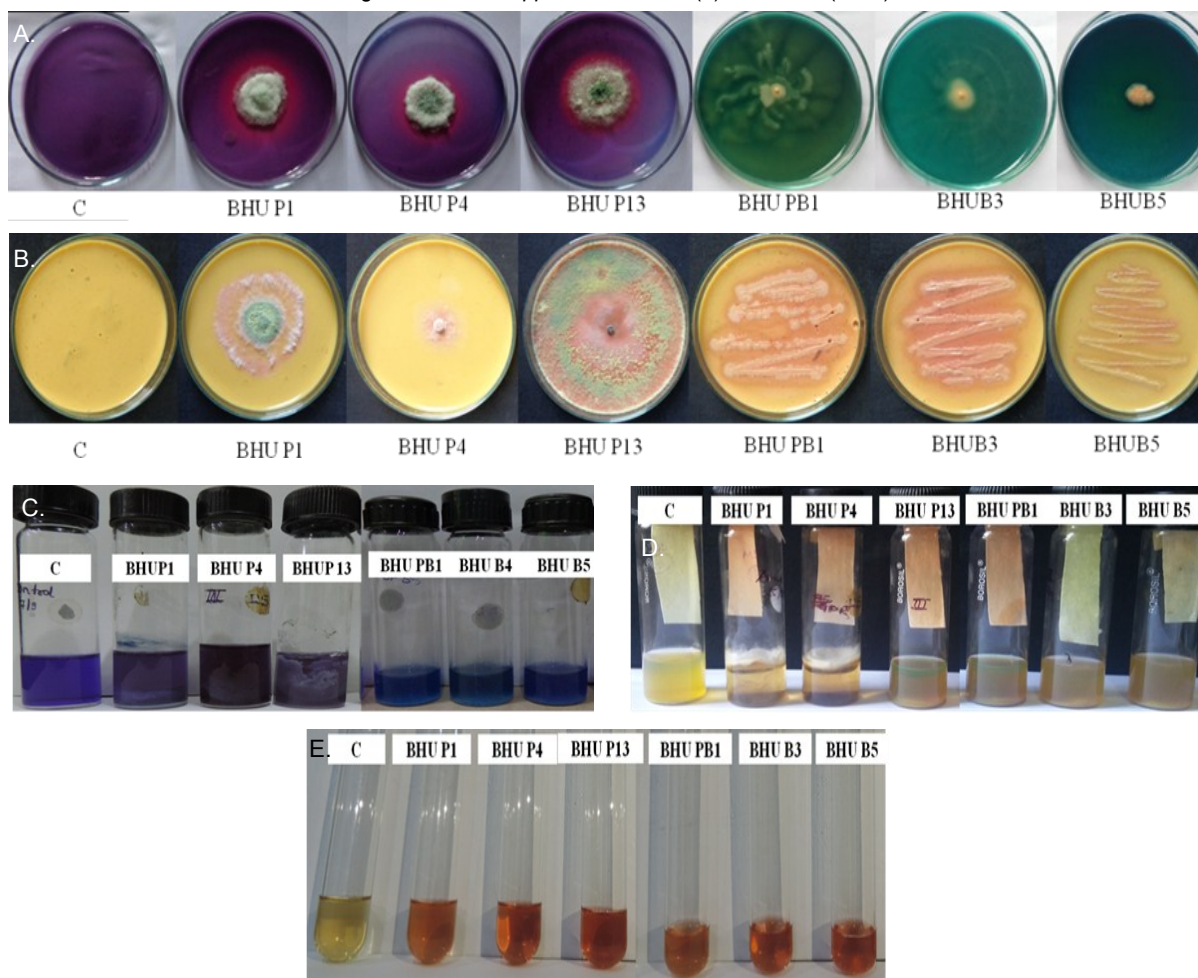


**Fig. 2.** Dual culture assay of Fungal and bacterial isolates against FOL.

**Table 1.** List of fungal and bacterial isolates showing PGP activities tested.

Isolates	Phosphate solubilization	Siderophore production	IAA production	HCN production	Protease activity
Fungi					
BHU P1	+++	+++	+++	+++	++
BHU P4	++	++	++	++	+
BHU P13	+	++	++	++	++
Bacteria					
BHU PB1	+++	+++	+++	++	+++
BHU B3	+	++	++	-	+++
BHU B5	-	+	++	-	+

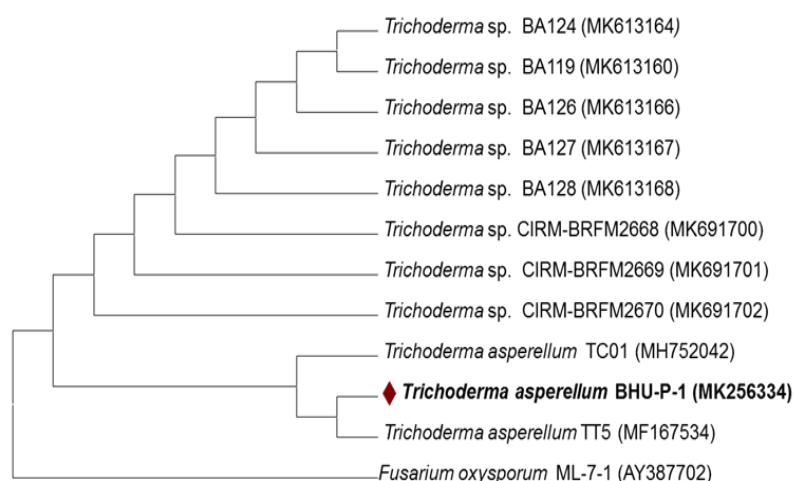
+++ = High; ++ = Moderate; + =Low and; - =absence



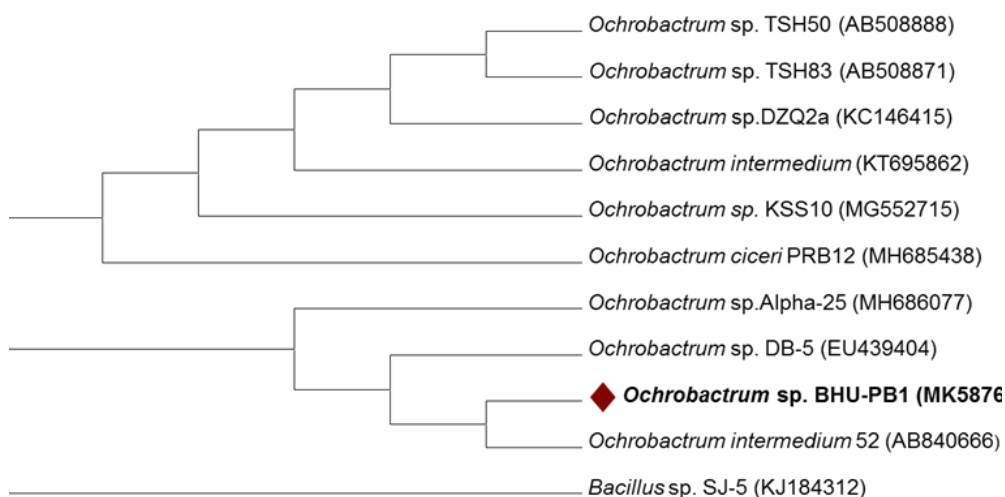
**Fig 3.** Plant growth promotion activity assays. A- siderophore production assay, B- protease activity assay, C- Phosphate solubilisation, D- HCN production, E- IAA production assay of fungal and bacterial isolates.

bacterial isolates were collected from Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, BHU, Varanasi, India. These isolates were further screened for antagonistic activity against FOL and their plant growth promotion attributes as indicated by phosphate solubilisation, IAA production, siderophore production, HCN production and protease activity in order to select, most promising isolates for further study. Out of all tested isolates, *Trichoderma asperellum* BHU-P1 and *Ochrobactrum* sp. BHU-PB1 exhibited highest antagonistic potential against FOL and possessed all PGP activities tested. Protease and HCN activities have been reported to control growth of phytopathogens (Elad 2000, Rijavec and Lapanje 2016). Phosphate solubilisation activity solubilises inorganic phosphate in soil and enhances phosphate uptake by plant (Richardson et al., 2009). Siderophores are iron chelating agents, which increases iron uptake efficiency in plants whereas IAA is a plant hormone responsible for root development (Datta and Basu, 2000;

Sharma and Johri 2003). Microbial mediate IAA production in rhizosphere could enhance root length and lateral root formation leading to more uptakes of water and nutrients in plants (Vacheron et al. 2013). *Trichoderma* spp. and bacterial genera as *Pseudomonas*, *Bacillus*, *Ochrobactrum*, *Azospirillum*, *Achromobacter* and *Flavobacterium* have previously been isolated from soil which showed beneficial effects on plant growth and development (Felici et al. 2008; Adesina et al. 2007; Forchetti et al. 2007; Swain and Ray 2009; Kumari et al. 2015). In addition, several reports further established that the plant growth promotion and diseases suppression ability of AIMs was due to multiple PGP factors (Grondona et al. 1997; Howell 2003; Antoun and Prévost 2005; Bashan and de-Bashan 2010; Jain et al. 2014). Single AIMs have been often reported to exhibit multiple mechanism of action including biological control (Howell 2003; Antoun and Prévost 2005; Singh et al. 2014b). Our results in the laboratory suggested that *Trichoderma asperellum* BHU-P1 and *Ochro-*



**Fig. 4.** Phylogenetic tree of *Trichoderma asperellum* strain BHU P-1 based on ITS gene sequence. Bootstrap values are based on 1000 replications. The isolate whose sequences have been submitted in NCBI GenBank are highlighted with brown colour symbol with their respective Accession number.



**Fig. 5.** Phylogenetic tree of *Ochrobactrum* sp. strain BHU- PB1 based on 16SrRNA gene sequence. Bootstrap values are based on 1000 replications. The isolate whose sequences have been submitted in NCBI GenBank are highlighted with brown colour symbol with their respective Accession number.

*bactrum* sp. BHU-PB1 have potential for management of *Fusarium* wilt of tomato and enhancement of growth and productivity. These microbes need to be evaluated further in greenhouse and multiple field trials. If found suitable these can subsequently be considered for developing suitable formulations, and commercialization.

### Conclusion

The above laboratory study reports two most promising AIMS viz. *Trichoderma asperellum* BHU P1 and *Ochrobactrum* sp. BHU PB-1 based on their antagonistic potential against FOL and PGP activities. These isolates could further be evaluat-

ed for greenhouse and field studies, to validate their biological control and plant growth promotion ability on different crop plants.

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