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Synergistic Effect of *Trichoderma* spp. and Plant Growth Promoting Rhizobacteria (PGPRs) in Chickpea Seed Biopriming against Fusarium wilt and Dry root rot diseases

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Abstract

The antagonistic potential of 15 biocontrol agents was evaluated against *Fusarium oxysporum* f. sp. ciceris and *Rhizoctonia bataticola* causing Fusarium wilt and Dry root rot diseases in chickpea. Fifteen potential bio antagonists, ten belonging to *Trichoderma* spp. and five to bacterial genera (*Bacillus* spp.) showed significant growth inhibition of both most important soilborne pathogen. In vitro screening for antagonistic functionality traits showed significant difference between production of volatile and diffusible antifungal metabolites. Maximum fungal inhibition due to diffusible metabolites was exhibited by isolate IIPR-75 (34.05%) followed by IIPRMUCK-1 (30%) greenhouse experiments on JG-62 variety of chickpea showed that seed treatment with biocontrol agents (IIPRTh-31) had an advantage on disease control and plant growth promotion as compared to control, isolate reduced the wilt incidence to 0 % which was at par to fungicide treatment where the observed wilt incidence in the pathogen control was 30%.Inoculation of biocontrol agents enhanced the shoot length up to 21 cm and 22 cm as compared to fungicide treatment 12.4 cm, thus showing a plant growth stimulation effect of potential bio antagonists. These results highlight the dual role of selected microbial antagonists in suppressing Fusarium wilt and dry root rot diseases and promoting chickpea plant growth, indicating their potential as sustainable alternatives to chemical control.

Key words : Biocontrol, *Trichoderma*, PGPR, Wilt, Dry root Rot.

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop worldwide, being an excellent source of protein. It is cultivated on area of 14.84 million hectares, a production of 15.08 million tons, and an average yield of 1.01 t/ha in 2020. It occupies important position in semi-arid farming system both for human nutrition and restoring the soil fertility (Singh and Sirohi, 2003). The reasons of low yield are so many apart from other reasons, the main cause of low yield of this crop is the heavy incidence of root rot diseases. India is the world leader in chickpea production. The grain losses due to chickpea wilt and root rot has been estimated around 10 per cent, which amounts to approximately 520 thousand tons annually. Wilt and dry root rot are the common and frequently occurring diseases of chickpea and causes considerable yield loss (Haware *et al*., 1996; Kaur and Mukhopadhyay, 1992). *Fusarium oxysporum* f. sp. ciceri (Padwick) Synd. and Hans. is considered to be the primary cause of wilt disease in chickpea (Chattopadhyay and Sen Gupta, 1967). In India, the first report on chickpea dry root rot (M. phaseolina) was given by Mitra in 1931 and Dastur in 1935. In central India Nene and Reddy, 1987 carried out some studies on the disease. Chickpea wilt and root rot cause complete losses in grain yield, if the diseases occur in the vegetative and reproductive stages of the crop (Haware and Nene, 1980). Researches have shown that commonly grown cultivars of chickpea in India may suffer from 9-41% seed yield loss due to wilt, depending on the cultivar and disease severity (Khan *et al*., 2004).

Biological control is one of the best low-cost and ecologically sustainable methods for managing plant diseases caused by soil-borne

pathogens like *Fusarium* and *Rhizoctonia*. Among various biocontrol agents (BCAs) evaluated against the plant pathogenic fungi, Trichoderma spp. have been found to possess biocontrol ability (Abd El-Khair *et al*., 2010; Mohiddin *et al*., 2010), these fungi mycoparasitize the pathogenic fungi via hyphal coiling and enabling enzymatic lysis through 1, 3-glucanase, cellulase, chitinase, and proteinase (Mishra 2024, Jefries and Young, 1994). Trichoderma species can also combat plant pathogens by exerting antagonism in the form of antibiosis; the production of antifungal metabolites such as trichodermin, gliotoxin, or viridin (Bruckner and Przybylski, 1984; Lorito *et al*., 1993). PGPR act as biocontrol agents to reduce disease severity, or these stimulate other beneficial symbioses, or, protect the plant by degrading xenobiotics in contaminated soils (Jacobsen, 1997). More importantly, such PGPR may also induce systemic resistance in the host (ISR), thereby protecting plants against pathogen attack. The present study was undertaken to examine the performance of ten Trichoderma and five bacterial isolates to assess the effect of the bioagents in growth and yield parameters of the chickpea.

Materials and Methods

Isolation of Pathogen

Isolation of *Fusarium oxysporum* f. sp. ciceris : A random sample of an infected root was taken from the experimental plot. The root was gently warped with the bloating sheet and soil particles were removed by washing it with tap water. Root surface sterilization was accomplished using $HgCl_2$ at a concentration of 0.1%. Root segments were sliced into small pieces using a sharp knife in an aseptic environment and arranged on petri dishes that are subjected to PDA. Petri dishes that have been infected were cultured in B.O.D. Under a microscope, the pathogen's mycelium growth was observed following the suggested incubation period. *Fusarium* sp. was purified using the single spore technique, and a pure culture of the bacterium was placed on slants for additional examination.

Isolation of *Rhizoctonia bataticola* : Naturally infected chickpea plants exhibiting the classic signs of dry root rot in standing chickpea crop fields were collected and transported to the lab where they were thoroughly cleaned with sterile distilled water, blot dried, and chopped into small (5 mm) pieces using a sharp, sterilized blade before being subjected to tissue isolation (Tuite, 1969) on PDA. The blackish mycelia mat formed after two or three days of incubation, and the microsclerotia in the plates began to grow after seven to eight days. The test pathogen was isolated aseptically on PDA medium using the hyphal tip and/or single spore/sclerotial isolation technique. It was then sub-cultured, and the pure cultures of the test isolates obtained were kept separately on PDA slant test tubes in the refrigerator for additional research. Following a week of incubation, the pure culture was once more transferred.

Isolation of bioagent

Collection and isolation of native PGPR strains : Soil samples from healthy pulse rhizosphere were collected during rabi, 2020 for isolating PGPR. Seven plant growth promoting rhizobacteria were obtained by serial dilution technique (Waksman, 1922) from different pulse growing regions (Table 1).

Isolation of *Trichoderma* spp. : *Trichoderma* and bacterial isolates were procured from the department of plant protection ICAR-IIPR Kanpur (Table 1).

2.3. Dual culture test : The pathogen and Trichoderma isolates were arranged side by side on a single Petri dish that had hardened PDA. For every isolate, there were three replications,

Table 1. Details of the isolates used in the study

Isolate Name	Isolate species	Acc. no. (ITS/16 S)	Crop rhizosphere	Location
IIPRTh-31	*T. asperellum*	MK968811	CHICKPEA	ANGRAU
IIPRTh-33	*T. afroharzianum*	MN186847	CHICKPEA	Orcha
IIPR-10	*T. asperellum*	OP783881	CHICKPEA	ANGRAU
IIPRMUCK-1	*T. harzianum*	OP783859	CHICKPEA	Kanker
IIPRPPF1-2	*T. harzianum*	MK855325	PIGEONPEA	Varanasi
IIPRPPR1-4	*T. longibrachiatum*	MN416776	PIGEONPEA	Meerut
IIPRMLKP-1	*T. asperellum*	OP783871	CHICKPEA	Sulatanpur
IIPRMGKP-5	*T. harzianum*	OP783879	MUNGBEAN	Kanker
IIPRPPKP-2	*T. asperellum*	OP783877	PIGEONPEA	Gujarat
IIPR-75	*T. asperellum*	MN416781	CHICKPEA	Dharwad
IIPRCDCP-4	*Bacillus australimaris*	MT436407.1	CHICKPEA	Lalitpur,
IIPRAJCP-6	*Bacillus amyloiquefaciens*	MK841518	CHICKPEA	Jhansi
IIPRAMCP-1	*Bacillus safensis*	MK863579	CHICKPEA	Chhatarpur
IIPRSHEP-6	*Bacillus subtiles*	ON872223	CHICKPEA	Mahoba
IIPRMKCP-8	*Bacillus megaterium*	MT436399	CHICKPEA	Mahoba

with one control each of the pathogen and the bio-control agent. They grew for seven days at 25°C in the incubator. Two measurements of the pathogen's diameter were made, and the average was computed. In accordance with Naik *et al*. (2009), the test pathogen's percent suppression of growth was computed. Three iterations of the in vitro assessment were conducted.

The percentage of inhibition was estimated using the following formula:

I=C-T/C x 100

Where; I = percentage of inhibition; C = radial growth of the pathogen in control and T = radial growth of pathogen in treatment.

2.4 Pot experiment : The pot experiment was conducted in a controlled temperature room with constant temperature at 21±1°C. Preliminary tests of Trichoderma isolates against Fusarium wilt and dry root rot chickpea was done under greenhouse condition. In order to prepare Rb and Foc inocula, proper Erlenmeyer's containing 100 g of chickpea (Var. Durum) and 100 ml of sterilized water were autoclaved at 121°C for 1 h for three successive days. After cooling, about 5-7 small plugs of seven-day-old culture of Rb and Foc were dropped into each Erlenmeyer under sterilized condition. Erlenmeyers were kept at 25±1oC for 4 weeks. Colonized chickpea grains were then transferred into the paper pockets, and were dried and grinded. Then 14 g of the prepared powder was used to infest 1 kg of soil (Frommel *et al*. 1991).

Results and Discussion

The colony growth of *Fusarium oxysporum* f. sp. *ciceri* on PDA was moderately fast, reaching a diameter of 70-80 mm within seven days at a temperature of 28±1°C. The mycelia growth exhibited varying densities, appearing dense, fluffy, or sparse, and ranged in color from white to pink. The fungus produced a large quantity of both Microconidia and Macroconidia. Abundant Chlamydospores were formed 2-4 weeks after transferring the culture. The Microconidia were small, thin-walled, hyaline, and elliptical, consisting of 1-2 cells and

measuring 4-6 x 2-4µm. On the other hand, the Macroconidia were long, curved (fusiform or sickle-shaped), pointed at both ends, septate, and measured 25-40 x 3-4 µm (Fig. 1a). In contrast, the colony of Rhizoctonia bataticola (Rb) grew very rapidly on PDA, reaching a diameter of 90 mm within seven days at a temperature of 28±1°C. The mycelial growth appeared as aerial fluffy, initially displaying a dirty white color when young, but later turning blackish with the presence of black microsclerotia. The hyphae branched at right angles, with a constriction of hyphal branches at their point of origin. The septum was positioned close to the point of branching and exhibited a highly irregular shape. The hyphae were connected by thick-walled mycelium, measuring 52.2-73.6 x 42.1-52.6 µm in size. (Fig.1b).

Morphological characters of the Trichoderma isolates were recorded on culture plates (Figure 2). Macro morphological characters including colony color, growth rate, colony edge, mycelial form etc. were recorded. genomic DNA was isolated using CTAB method. Seven days old mycelia of Trichoderma cultures were used for the DNA isolation. Isolated DNA was run with ITS and Tef primers for molecular identification. ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC-3') (White *et al*. 1990) and EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') (Carbone and Kohn. 1999). The PCR programme used for the amplification of ITS and Tef were 3 min at 95°C, followed by 35 cycles of 30 seconds at 94°C; hybridization at 58 °C for 30 s and an extension at 72 °C for 1 min, and a final extension step of 10 min at 72°C. Finally, a refrigeration step at 4°C. Obtained PCR products were sent for sequencing to Chromus Pvt Ltd.

Sequences of the amplified PCR products were analyzed using BLAST search programme. Phylogenetic tree were drawn using the neighbor joining method using MEGA-10 programme with 1000 bootstraps (Fig. 3 a&b).

Bacterial characterization was done on the colony morphology, gram staining (Figure 4). Genomic DNA of bacteria was isolated by using alkaline lysis method and 16S rRNA gene was amplified using the universal primer pair (27F: 5′-AGA GTT TGA TC[A/C] TGG CTC AG-3′, 1492R: 5′-G[C/T]T ACC TTGTTA CGA CTT-3′) (Kunitz M *et al* 1947).PCR conditions used for amplification were 1 min DNA denaturation at 94°C, 1 min annealing at 57°C,and1min extension at 72°C for 35 cycles. The amplified PCR products were sent for sequencing to

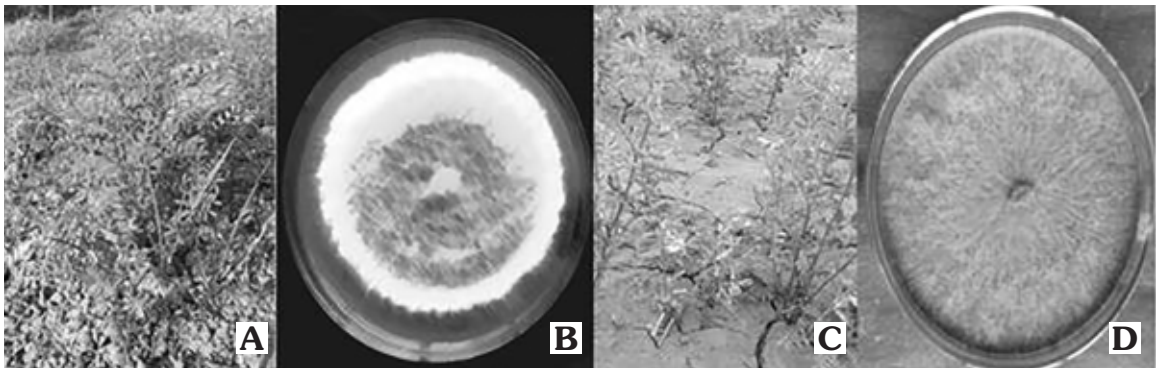

Fig. 1. (A) Wilt affected chickpea plant, (B) Pure culture of Fusarium oxysporum f. sp. ciceris and (C) Dry root rot infected chickpea plant and (D) Pure culture of Rhizoctonia bataticola on PDA plate

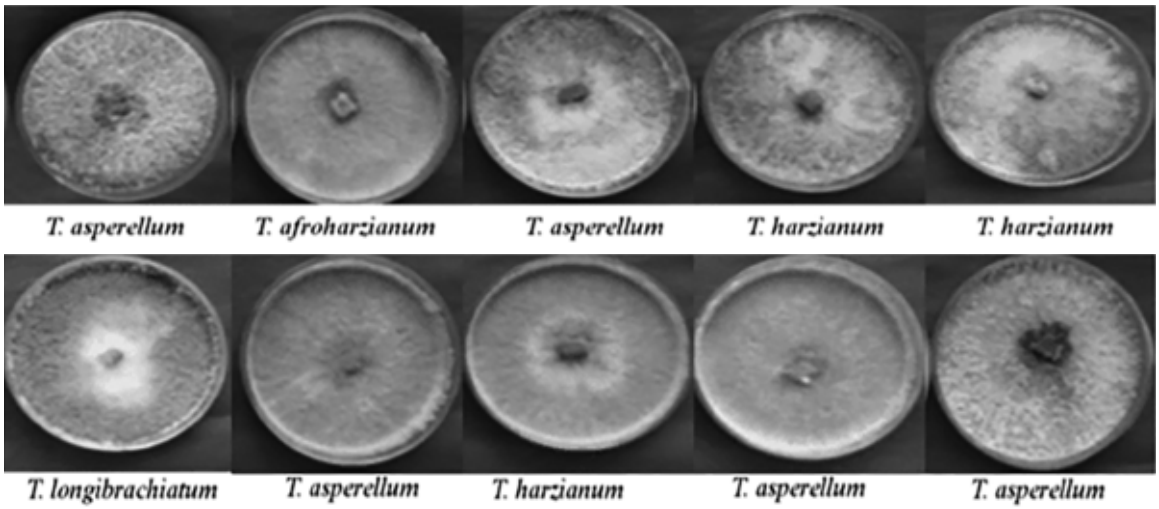

Fig. 2. Morphological characterstics of selected *Trichoderma* isolates

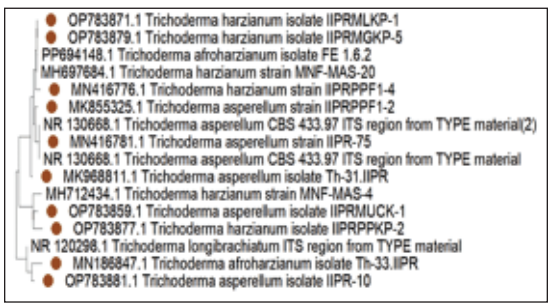

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the ITS gene sequence of Trichoderma isolates (Highlighted in red colour) and related sequences obtained from NCBI

Chromus biotech and obtained sequences were run on BLAST programme for the identification. After identification the obtained sequences were trimmed and submitted to NCBI database for accession number allotment http://www.ncbi.nlm.nih.gov/).Phylogenetic tree was constructed with the obtained 16SrRNA gene sequences and reference sequences obtained from NCBI (Fig. 5). The phylogenetic relationship among bacterial isolates and reference sequences was constructed using the Maximum Likelihood method and Tamura-Neimodel Lane DJ (1991).

In vitro antagonistic efficacy of *Trichoderma* and bacterial culture against pathogen was tested using dual culture plate technique. IIPRPP1-4 (*T. longibrachiatum*) was found to be the most effective against Foc followed by IIPRMLKP-1 (*T. asperellum*) (20%). For Rb IIPR75 (*T. asperellum*) (34.05%) followed by IIPRMUCK-1 was found to be most effective

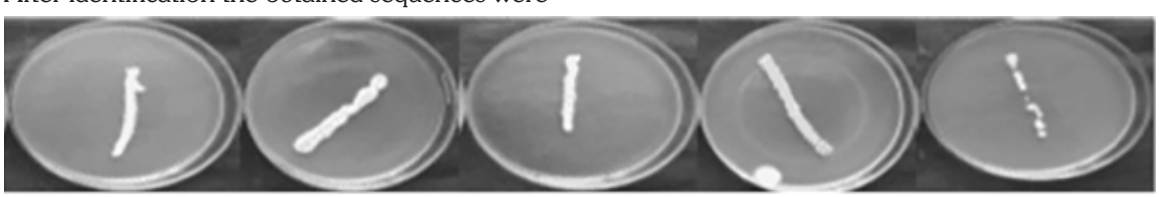

Fig. 4. Morphological characters of PGPR isolates

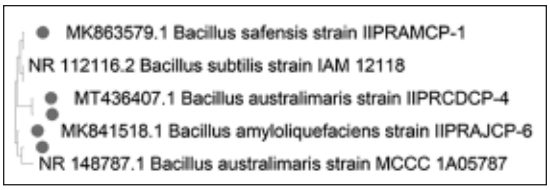

Fig. 5. Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the 16sRNA gene sequence of bacterial isolates (Highlighted in red colour) and related sequences obtained from NCBI

(Figure 6a & b). Among the bacterial isolates tested, AJCP-6 (*Bacillus amyloliquefaciens*) effective against Foc with highest mycelial inhibition showed maximum inhibition (66.52%). Followed by antagonists AMCP-1 (*Bacillus safensis*), which recorded mycelial growth (66.47%) of the tested pathogen (Figure 8 A, B & 9A, B).

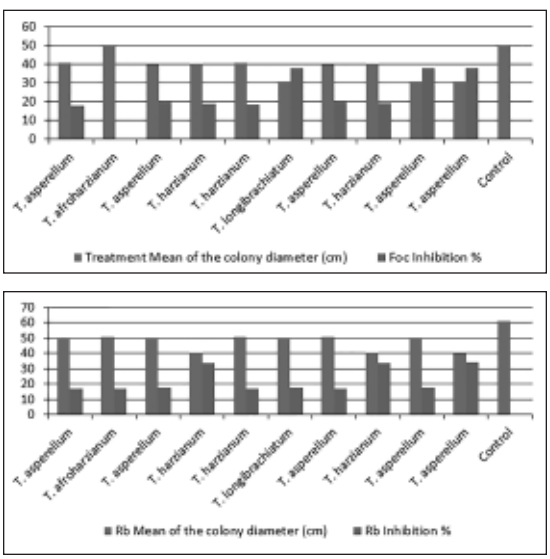

Fig. 6. Percent inhibition of *Fusarium oxysporum* f.sp. *cicero* (A) and *Rhizoctonia bataticola* (B) in presence of *Trichoderma* bioagents (Dual culture technique)

Fig. 7a. Antagonistic potential of *Trichoderma* against (Foc)

Fig. 7b. Antagonistic potential of Trichoderma against (Foc)

Trichoderma species are well known for their antagonistic activity against Foc. Efficacy of *Trichoderma harzianum* against Foc has already mentioned by many workers (Poddar *et al*., 2004; Dubey and Suresh, 2006).The inhibition mechanisms employed by *Trichoderma* against pathogens include antibiosis, lysis, competition and mycoparasitism (Cook and Baker 1983). *Trichoderma secretes* many antibiotics and volatile metabolites which aid in the inhibition. The mycoparasitic activity of *Bacillus* species is well documented (Johri *et al* 2003, Sharan and Nehra 2011) Similar conclusions have been reported by El Hassni *et al*. (2007) and Idris *et al*. (2007). They reported a modification of the fungal mycelium appearance, due to antifungal secondary metabolite production. Generally, biocontrol capacity through antagonistic bacteria involves either competition (Elad and Chet 1987) or bacterial metabolite production, such as siderophores, hydrogen cyanide, antibiotics or extracellular enzymes for antagonism towards plant pathogens (Kamilova *et al*. 2005; Sang *et al*. 2006). It has been reported that *Bacillus*

spp. contains various biocontrol characteristics including secondary metabolites, the colonizing potential, and the production of competitors (Yoshida *et al*. 2001; Schmidt *et al*. 2004).

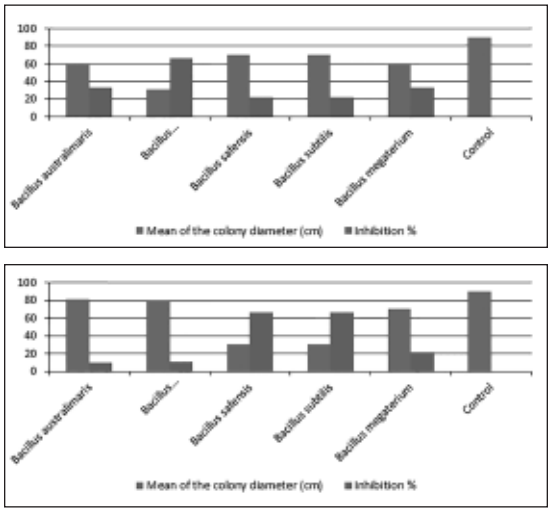

Fig. 8. Percent inhibition of *Fusarium oxysporum* f.sp. *ciceri* and *Rhizoctonia bataticola* in presence of PGPRs (Dual culture technique)

The results of the experiments conducted under greenhouse conditions revealed the supremacy of *Trichoderma* and Bacteria over control. This treatment significantly reduced the pre emergence and post emergence wilt disease on chickpea when compared to control. Among various treatments tested as seed treatment, the treatment IIPRMLKP-1 significantly reduced the wilt incidence to 0 percent respectively as compared to control. Seed treatment with *Trichoderma* and bacteria showed significantly superior results over control. Untreated control (seed treatment with pathogen) recorded the maximum wilt incidence 30% respectively. With regard to the germination and seedling growth parameters, the treatment IIPRTh-33 recorded the highest germination percentage (96%), shoot length (18 cm), root length (25.5cm). This was followed by the treatment IIPRTh-31 (Fig. 10A &B).

For instance, the results of Abd-El-Khair *et al*. (2010) focused on *T. hamatum* as the best species in inhibiting *F. solani* of beans among isolates of several species and T. harzianum had the least control potential. In another study, under laboratory conditions the inhibitory effect of *T. longibrachiatum*, *T. harzianum* and *T. atroviride* against *F. solani*, causal agents of wheat head scab were 92%, 81% and 90%, respectively (Boureghda and Renane 2011,Mishra 2023 a,b,c). Boureghda and Bouznad (2009) reported that an isolate of T. atroviride (Ta.13), caused a 65.64% reduction in colony diameter of F. solani of chick pea and was better than *T. harzianum* and T.

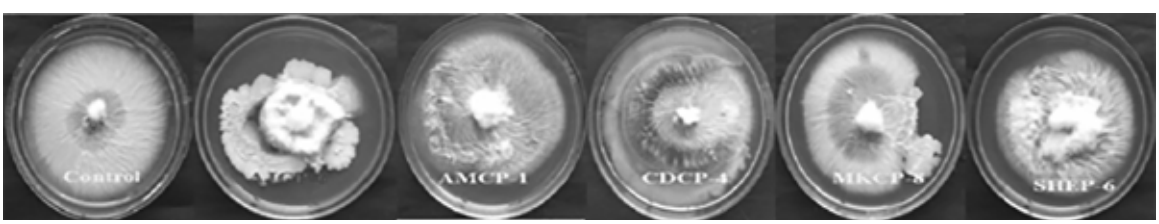

Fig. 9a. Antagonistic potential of PGPRs against *Fusarium oxysporum* f.sp. ciceri (Foc)

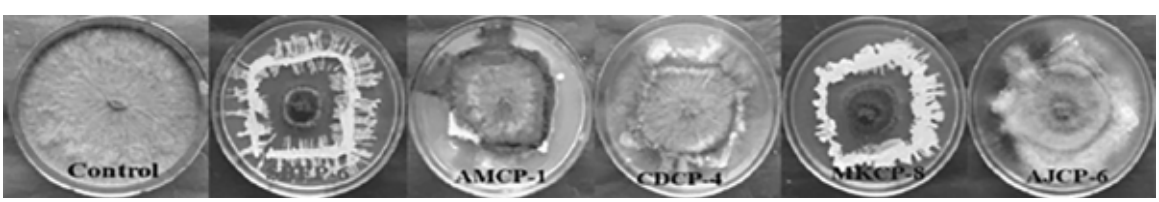

Fig. 9b. Antagonistic potential of PGPRs against *Rhizoctonia bataticola* (Rb)

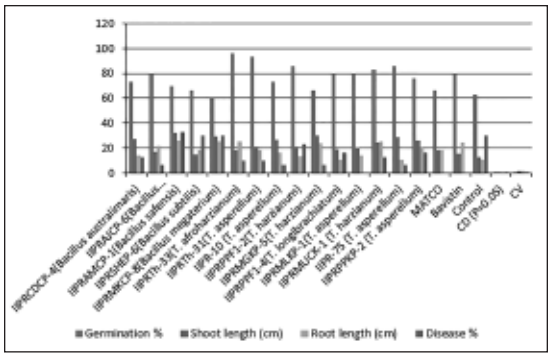

Fig. 10. Effect of *Trichoderma* and PGPRs application on chickpea plant

longibrachiatum while in our dual culture method both *T. atroviride* (T_3) and *T. harzianum* (T_{21}) were not so effective but T. *brevicompactum* (T_1) was most preventive. Under greenhouse conditions, Boureghda and Bouznad (2009) among several isolates of *T. atroviride*, *T. harzianum* and *T. longibrachiatum*, found *T. atroviride* (Ta.3, Ta.7 and Ta.13) as well as *T. harzianum* (Th.16) as the most effective isolates in protecting *Fusarium* wilt of chickpea seedlings. Soltani *et al*. (2005) reported that an isolate of *T. harzianum* was best against *Fusarium* wilt of potato. In addition, Basak and Basak (2011) reported superiority of *T. viride* over *T. harzianum* in healing the seedling disease of Shisham (*F. solani* f. sp. *dalbergiae*) while in our study *T. longibrachiatum* (T_5) had the best performance in controlling the disease. Therefore, our results might be a confirmation of the usefulness of evaluating different isolates of this antagonistic genus for their mycoparasitic properties and their biopesticide preparations. Isolates of some species namely *T. harzianum*, *T. viride* and *T. virens* (syn. Gliocladium virens) are well known and their formulations are used by many growers but there might be more effective species of Trichoderma and hence evaluating the strains of other species of this environmental-friendly genus would be beneficial. Disease suppression and plant growth promotion activity may be attributed to the multifaceted potential of bacterial strains for lytic enzymes (protease, chitinase, glucanase, and amylase) production, HCN production p-solubilization, N-fixation, and IAA production (Bashan and De-Bashan, 2010; Verma *et al*., 2014; Mukherjee *et al*., 2020, Mishra 2021 ab). Another explanation could be due to the Induced Systemic Resistance (ISR). The ISR is the ability of plants to enhance their defence by the augmentation. of different defence enzymes such as β-1,3-glucanase, peroxidase, polyphenol oxidases, phenylalanine ammonia lyase against a wide range of pathogens. The enzymes β-1,3-glucanase and chitinase both have synergistic antifungal actions (Cachinero *et al*., 2002). Several PGPR (plant growth-promoting rhizobacteria) therefore have been reported to activate the ISR in plants in response to pathogen attacks (Cachinero *et al*., 2002; Zaim *et al*., 2016; Das *et al*., 2017, Mishra 2023a). It has been reported that bacterial strains covering plant roots act as the first line of defence for plants against disease invasion and reduce the risk for early or late wilting in plants (Wei *et al*., 2015, Mishra 2020). Previously, Actinomycetes, B. subtilis, and P. aeruginosa have been reported as efficient colonizers along with the roots of chickpea, and due to their good root colonization ability reduces the wilt incidences in chickpea (Anjaiah *et al*., 2003; Gopalakrishnan *et al*., 2015a, 2015b; Sreevidya and Gopalakrishnan, 2017). Disease suppression in chickpea roots has been correlated with an increase in the growth of the root system (Akhtar and Siddiqui, 2008) and vice versa.

Conclusion

The investigation of disease-suppressive soil microbes in nature for the creation of an efficient biopesticide is a productive method that not only decreases the number of isolates but also aids in identifying a specific biocontrol agent

against a particular pathogen with multiple beneficial traits. This research highlights the utilization of this approach for the identification and selection of PGPRs/BCAs from healthy plants cultivated in soil affected by wilt disease caused by Foc. The chosen strains, possessing various capabilities such as amylase, hydrogen cyanide, protease, cellulase, chitinase, p-solubilization, nitrogen-fixing, and indole-3-acetic acid production, exhibited the potential to colonize roots and promote the growth of chickpea plants. Subsequent experiments demonstrated that these strains not only enhanced chickpea growth but also effectively suppressed the disease by stimulating the expression of several defense enzymes. The findings of this study suggest that these selected bacterial strains, especially when used collectively, are promising candidates for the development of environmentally friendly, cost-efficient, and easy-to-use biofertilizers/ biopesticides for managing chickpea wilt disease and increasing crop yield.

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