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Research Article

Assessment of antifungal potential of biocontrol agents and fungicides against *Fusarium* wilt of cotton

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ABSTRACT

Fusarium wilt disease is a most common disease of cotton in Pakistan. Antifungal potential of fungal biocontrol agents, PGPRs and fungicides was assessed for the management of *Fusarium oxysporum* f. sp. *vasinfectum*, cause of cotton wilt disease. The antifungal properties of three species of *Trichoderma*, i.e., *Trichoderma harzianum*, *Trichoderma viride*, and *Trichoderma aureoviride* were investigated by employing the dual culture method. *T. aureoviride* showed maximum inhibition (81.17%) followed by *T. viride* (69.41%) and *T. harzianum* (38.21%) after 9 days. Four PGPRs were evaluated against *F. oxysporum* alone and in different combinations. In case of PGPRs, the best inhibition (68.13%) was observed when the *Bacillus subtilis*, Rhizobacter strains, *Pseudomonas putida* and Enterobacter strains were applied in combination after 9 days with minimum colony diameter (1.98cm). Six different fungicides were tested i.e. Score® 250EC (Difenoconazole), Amistar Top 325 SC (Azoxystrobin + Difenoconazole), Nanok® 25% SC (Azoxystrobin + Flutriafol), Nativo® 75% WG (Tebuconazole + Trifloxystrobin), Vangard® 25% EC (Triadimenol) and Triger® 25% EC (Tebuconazole) at 40, 50, and 60ppm concentrations against *F. oxysporum*. Score exhibited maximum pathogen inhibition (40.95%) followed by Nanok (40.63%) at 60ppm after a period of 9 days. Similarly, minimum colony diameter (3.83cm) and (3.87cm) of *F. oxysporum* f. sp. *vasinfectum* was recorded in Score and Nanok treated plates, respectively.

Keywords: *Fusarium* wilt, Soil-borne diseases, Biological control, *Trichoderma* spp., Fungicides, Cotton, Integrated pest management.



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INTRODUCTION

Fusarium wilt, is a soil-borne fungus (Mokhtari *et al.*, 2023) that infests the cotton plant at all growth stages. Favorable environmental parameters like temperature, pH, light and substrate all influence *fusarium* growth (Mohsen *et al.*, 2015). *Fusarium* is a soil borne fungus and grows well at optimum temperature of 35 °C and its growth restricts when the soil temperature reaches below 17 °C (Nelson, 1991). Pathogen first attacks in older plants showing yellowing of leaves starts from lower portion and spreads upward. Longitudinal section of infected roots and stem shows brown discoloration of internal tissues. Traditionally, *fusarium* wilt was controlled using delinted seed, resistant cultivars, field sanitation, removal of alternate host, reducing inoculum density, soil treatment, managing nematodes and applying chemicals (Amanda and Davis, 2015). By adopting all these practices farmers are unable to manage this disease effectively. Biocontrol agents are considered as important source for the management of plant diseases as they are environmentally safe and have no health hazards. Keeping n view the importance of BCAs, different species of *Trichoderma* and PGPRs like *Bacillus subtilis*,

Rhizobacter strains, *Pseudomonas putida* and Enterobacter strains and different commercial and new chemistry fungicides were tested alone and in combination for the management of cotton wilt disease.

To achieve the maximum yield and high-quality crop at acceptable level to food security concerns, biological control is an effective approach that can be helpful in reducing plant diseases (Zadoks and Schein, 1979). Recognition of *Trichoderma* species as effective biocontrol tool is well documented since 1969 (Rifai, 1969; Samuels, 2006). It is saprophytic in nature and mostly found in rhizosphere and work antagonistically against many soils borne as well as aerial pathogens. (Dubey *et al.*, 2007). Being a saprophytic fungi *Trichoderma* spp. decompose organic matter by utilizing cellulose, multiply in the rhizosphere and act as inducer of resistance against pathogens (Harman, 2000; Jaklitsch, 2009 and Kubicek *et al.*, 2009). In early days, a few of *Trichoderma* spp. were isolated from marine mussels and shellfish (Sreerama and Veerabhadrapa, 1993; Sallenave *et al.*, 1999) and cockroaches (Yoder *et al.*, 2008). It competes with other plant pathogenic fungus for nutrition and also releases such metabolites that induce resistance in plants against diseases (Vinalae *et al.*, 2014). *Trichoderma* is a powerful biological control agent that reduces plant diseases under natural environmental conditions (Calvet *et al.*, 1990). These fungi produce toxic metabolites that have larvicidal as well as ovicidal properties and lethal to nematodes present in soil (Singh *et al.*, 1988; Lumsden *et al.*, 1992; Devi and Bora, 2018; Ali *et al.*, 2022).

MATERIAL AND METHODS

Collection of diseased samples

Farmers' fields were surveyed in cotton growing areas of Punjab, Pakistan in the cropping seasons of 2019 to 2021. Six locations i.e., Jhang, Multan, Vehari, Mianwali, Rahim Yar Khan and Bahawalpur were visited for collection of disease samples. Diseased samples showing typical wilt (Prasad and Padwick, 1939) were collected and made a composite sample for each field. In each district, five fields were selected and ten samples were taken from each field randomly. The infected samples were collected in paper bags, labelled properly and preserved at 4 °C for further investigation.

Isolation and identification of *Fusarium oxysporum* f. sp. *vasinfectum*

Diseased specimen of cotton (Roots and Stems) was processed for the existence of *Fusarium oxysporum*. The infected roots and stems were cut into small pieces (approximately 2-3 cm) followed by surface sterilization with 1% sodium hypochlorite (NAOCL) solution and subsequent washing with sterilized water (Rangaswami, 1972). These pieces were placed on PDA plates and then incubated at 28-30 °C for 4 to 7 days for colony development (Ricker and Ricker, 1936). Petri plates were observed regularly for the appearance of the fungal colonies from the infected tissues. Later the desired fungus was picked and purified by using hyphal tip technique (Rangaswami, 1972) and pure culture was preserved on PDA slants.

F. oxysporum f. sp. *vasinfectum* was identified based on their colony and morphological characters under the dissecting microscope. Colony of *fusarium* was white to pale violet in color with cottony or ropey texture. After observation, it was observed that colony produced oval to kidney-shaped microconidia, sickle-shaped, thin-walled and delicate macroconidia, a single, terminal chlamyospore. Microconidia produced in false heads on short monophialides. These characteristics were cross checked with literature (Devika Rani and Niak, 2008).

Isolation of *Trichoderma* Species

Soil samples were collected from cotton fields of six locations i.e., Jhang, Multan, Vehari, Mianwali, Rahim Yar Khan and Bahawalpur during the growing season of 2019-2021. The dilution plate method (Iqbal *et al.*, 2017) was used for the isolation of fungi from soil samples. Later on, three different species of desired fungus (*Trichoderma aureoviride*, *Trichoderma viride* and *Trichoderma harzianum*) were isolated and identified on the basis of morphological and culture characters described by Barnett and Hunter (1972) and Ramirez (1982). Pure cultures of three *Trichoderma* spp. were multiplied on PDA and preserved.

Interaction between *F. oxysporum* and antagonistic fungi (Dual culture method)

By dual culture technique (Dennis and Webster, 1971), the antagonistic effect of *T. harzianum*, *T. viride* and *T. aureoviride* against pathogenic fungi was checked. Mycelial disc of 5-mm diameter of *F. oxysporum* was seeded at one end of each petri plate (9cm). And then a mycelial disk of 5 mm in diameter of *Trichoderma* taken from the edge of an actively growing culture plate and inoculated opposite to the pathogenic fungi in the same plate. Control plates were inoculated only with the *Fusarium* culture. These plates were then incubated for 9 days at 28 ± 2 °C. The data on radial colony growth of *Fusarium oxysporum* and inhibition zone percentage in control as well as in treated plates was measured using the formula (Rehman *et al.*, 2013):

$$I = (A-B) / A \times 100$$

Where A is colony diameter in control plates and B is the colony diameter in treatment plates.

Interaction of *F. oxysporum* f. sp. *vasinfectum* with PGPRs (Disk diffusion method)

Four different PGPRs was evaluated against *Fusarium* by disk diffusion method on the basis of inhibition zone. Four different bacteria (*Bacillus subtilis*, Rhizobacter strains, *Pseudomonas putida* and Enterobacter strains) were grown on nutrient agar at 25 °C to prepare active cultures. Then the bacterial suspension was prepared at 5×10^8 colony forming units (CFU) / mL. In the middle of a fresh NA plate, a disc (1-cm) of *Fusarium* was inoculated. Then eight-millimeter filter paper discs were cut, soaked in bacterial suspension and placed at two opposite sides in the same plates having discs of *Fusarium*. The distance between the filter paper pieces and the fungal mycelial disc in the plate was 3-cm. Plates having *Fusarium* discs without bacteria disks used as the control and incubated at 28 ± 2 °C for 9 days. Then the data on inhibition zone (%), between PGPR and the fungal pathogens was measured in centimeters.

Antimicrobial activity of fungicides against *F. oxysporum* (Food poison technique)

Inhibitory action of six fungicides i.e., Nanok® 25% SC (Azoxystrobin + Flutriafol), Amistar Top® 325 SC (Azoxystrobin + Difenconazole), Nativo® 75% WG (Tebuconazole + Trifloxystrobin), Vangard® 25% EC (Triadimenol), Triger® 25% EC (Tebuconazole), and Score® 250EC (Difenconazole) against *F. oxysporum* f. sp. *vasinfectum* was assessed using (40, 50, and 60ppm) concentrations following the food poison technique (Nene and Thapliyal, 2000). PDA medium was amended with fungicide concentrations. No fungicide was added in control plates. After solidification, 5-mm-diameter block of the pathogen was placed in the center of each plate following incubation at 28 ± 2 °C. The diameter of the colony growth was measured after 9 days. The following formula of Vincent (1927) was used to calculate the percent inhibition

$$I = (A-B)/A \times 100$$

A = Colony diameter in control plates

B = Colony diameter in treatment plates

Analytical statistics

Completely randomized design (CRD) was used to perform the experiments under lab conditions. Statistical analysis was performed on the data using M-Stat (Ver. 2.3). Tukey's HSD and the Least Significant Difference (LSD) tests were used to separate the treatment means.

RESULTS

Assessment of antagonistic activity of *Trichoderma* species

Results of dual culture method showed that colony diameter of *Fusarium* was minimum (1.6cm) in plates treated with *T. aureoviride* as compared to plates treated with *T. viride* (2.6cm) and *T. harzianum* (4.1cm) (Figure. 2) On the other hand, *T. aureoviride* showed the greatest inhibition (86.01%) followed by *T. harzianum* (58.34%) and *T. vitride* (38.21%) after 9 days. (Figure 1).

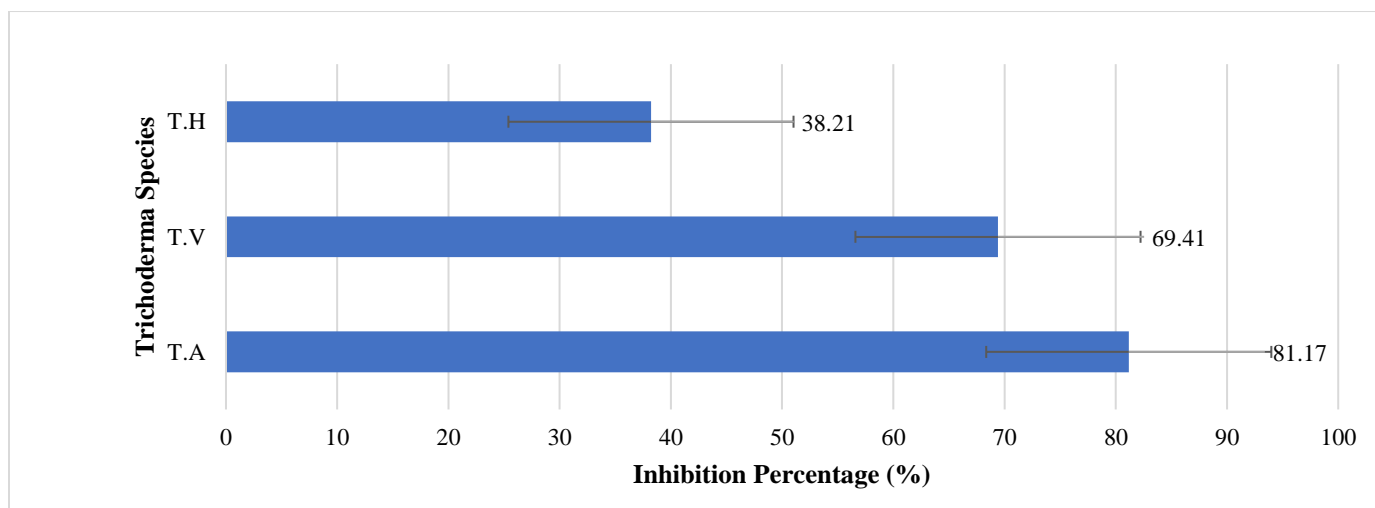


Figure 1. *Trichoderma*'s effect on inhibition of *F. oxysporum* f. sp. *Vasinfectum*.

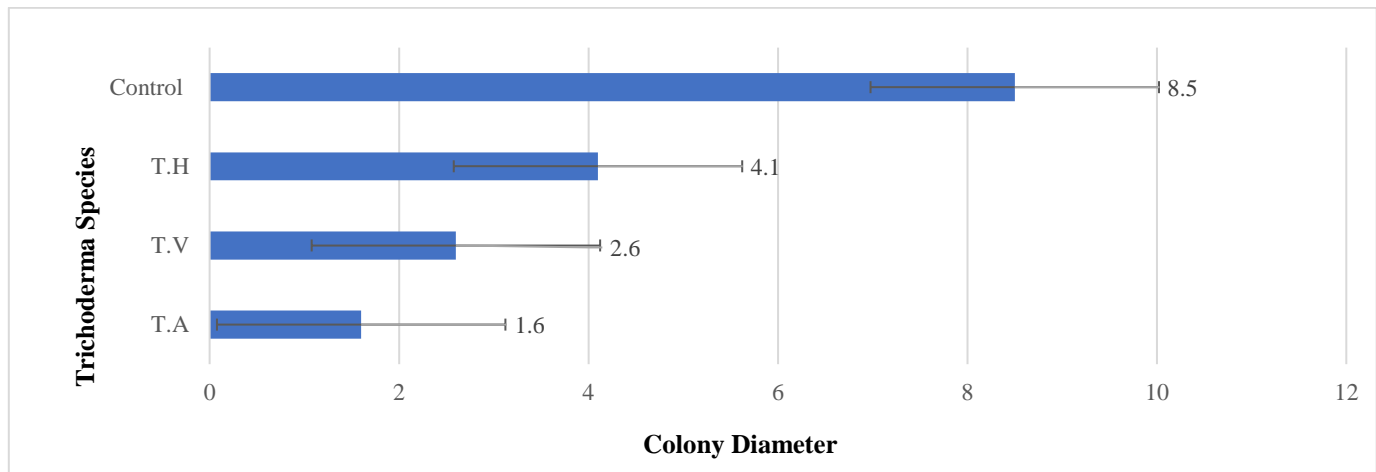


Figure 2. *Trichoderma*'s effect on colony growth diameter of *F. oxysporum* f. sp. *Vasinfectum*.

Evaluation of fungicides against *F. oxysporum*

The food poison technique was used to assess the inhibitory action of various fungicides, such as Score® 250EC (Difenoconazole), Nanok® 25% SC (Azoxystrobin + Flutriafol), Nativo® 75% WG (Tebuconazole + Trifloxystrobin), Amistar Top® 325 SC (Azoxystrobin + Difenoconazole), Vangard® 25% EC (Triadimenol) and Triger® 25% EC (Tebuconazole) at 40, 50, and 60ppm concentrations against *F. oxysporum*. It was observed that Score and Nanok performed best in controlling the colony growth of *Fusarium* as compare to other fungicides at all concentrations. Maximum pathogen inhibition (40.95%) was recorded in plates treated with Score followed by Nanok (40.63%) at 60ppm after 9 days. Similarly, minimum colony diameter (3.83cm) of pathogenic fungi was observed in case of Score amended plates, table 1.

Table 1. Fungicide effects on colony growth diameter and inhibition of *F. oxysporum* f. sp. *vasinfectum* at 40, 50 and 60ppm.

Treatment	Concentration (ppm)	Colony Diameter (cm)	Inhibition (%)
		Day 9	Day 9
Score	40	4.41e-k	30.21r-x
Nanok	40	4.43e-k	30.17r-x
Nativo	40	4.93de	19.77y-a
Amistar Top	40	4.90d-f	21.07x-a
Vangard	40	6.46b	12.89d
Triger	40	4.43e-k	22.78w-a
Control	40	7.50a	0bc
Score	50	4.07j-l	37.45m-s
Nanok	50	4.13i-l	36.75n-t
Nativo	50	4.70e-j	28.06s-y
Amistar Top	50	4.80e-h	26.42u-y
Vangard	50	6.33b	13.31bc
Triger	50	4.36e-k	31.71q-w
Control	50	6.53b	0bc
Score	60	3.83kl	40.95k-q
Nanok	60	3.87kl	40.63k-q
Nativo	60	4.43e-k	34.14o-u
Amistar Top	60	4.36e-k	34.52n-u
Vangard	60	6.23b	14.46a
Triger	60	4.26f-l	40.86k-q
Control	60	5.53cd	0bc

Antimicrobial efficacy of PGPRs

In lab four different PGPRs (*Bacillus subtilis*, Rhizobacter strains, *Pseudomonas putida* and Enterobacter strains) were evaluated for their inhibitory action against *F. oxysporum* by agar well diffusion test (Magaldi *et al.*, 2004) alone and in combinations of two, three and four. Results showed that the minimum colony growth (1.98cm) was observed in plates treated with combinations of all four bacteria (RB+BS+PS+EN) (Figure. 3). Similarly, best inhibition (68.13%) of *Fusarium* was observed by combination of four bacteria after 9 days (Figure 4). The analysis of variance for the effect of PGPR indicates that treatments had significant effect on the colony diameter of fungus and inhibition zone.

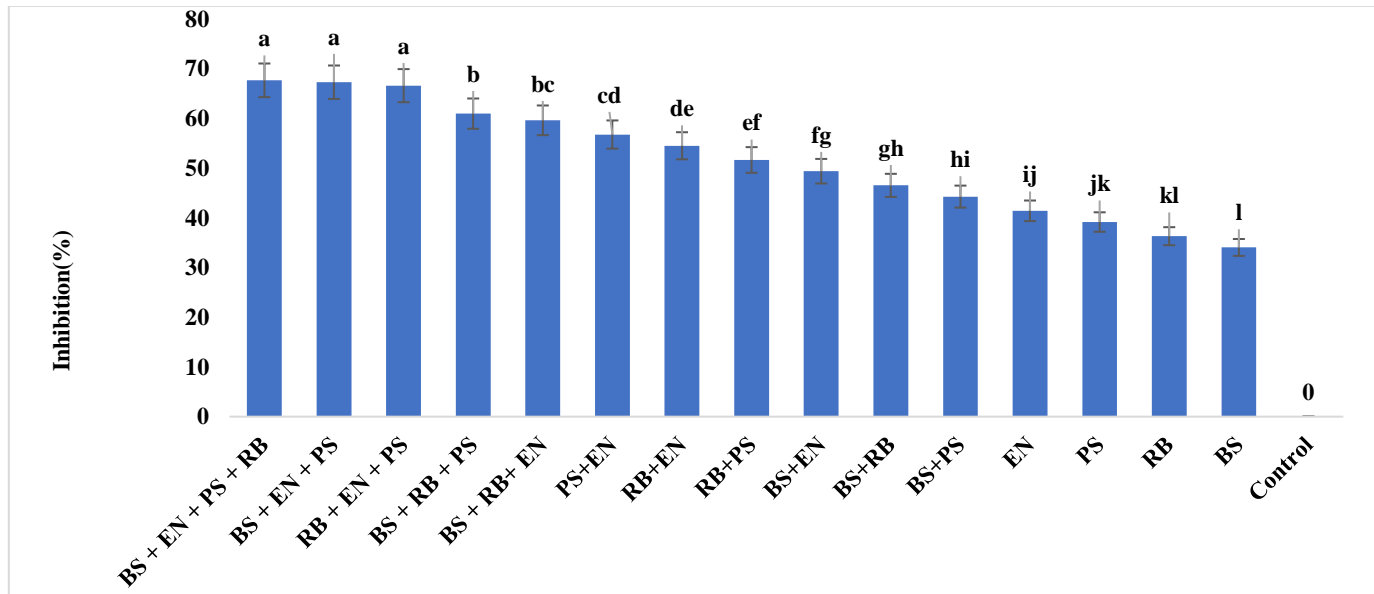


Figure 3. Effect of PGPR on colony growth diameter of *Fusarium oxysporum* f. sp. Vasinfectum.

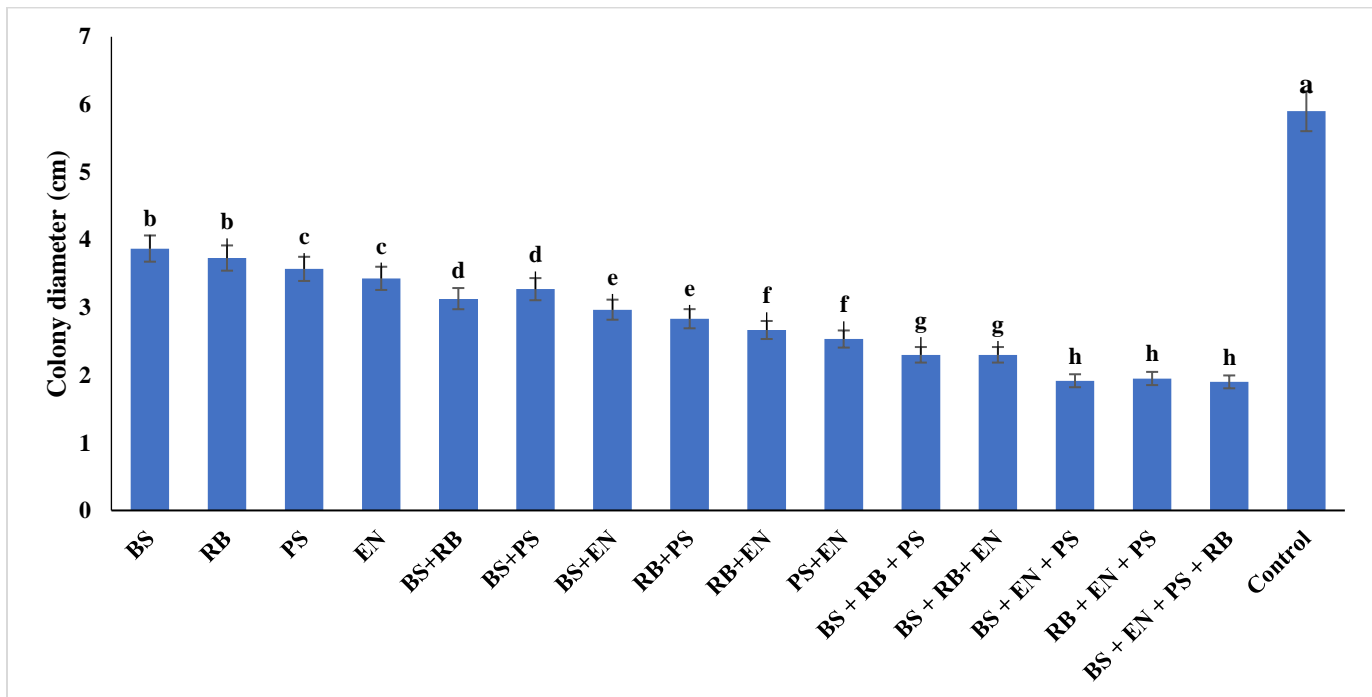


Figure 4. Effect of PGPRs on inhibition of *F. oxysporum* f.s p. vasinfectum.

DISCUSSION

Use of *Trichoderma* species is an alternative, safer approach to include in the disease management program for early potato blight. The current results are consistent with a number of earlier findings (Sabalaprad *et al.*, 2009, Devi *et al.*, 2012, Javaid *et al.*, 2014, Moosa *et al.*, 2017). Different isolates of *Trichoderma* have different repressive ability, which

could be attributed to their genetic makeup (Moosa *et al.*, 2017). *Trichoderma* restricts the ability of pathogen to spread as it has ability of rapid growth and competition with the pathogen for food and space (Devi *et al.*, 2012). By mycoparasitism, *Trichoderma* inhibits the pathogen (Doley and Jite, 2012). *T. harzianum* showed the strongest mycoparasitism in the current investigation. By producing both volatile and non-volatile compounds, *Trichoderma* also inhibits the pathogen (Tapwal *et al.*, 2011; Sumana and Devaki, 2012). *Trichoderma* produces a variety of antibiotics, including trichodermol, sesquiterpene, dermadin, harzianolide and harzianum A (Kucuk and Kyvanc 2008; Nakkeeran *et al.*, 2002). Additionally, *Trichoderma* causes host resistance by inactivating the pathogen's ability to produce enzymes (Ozbay and Newman, 2004).

Use of fungicides is also an effective to control the diseases. In this study, growth of *Fusarium* was also inhibited by fungicides. The same results were also observed in different past studies. The growth of *R. solani*, which is linked to rice sheath blight, is significantly inhibited by Tebuconazole, as noted by Moosa *et al.* (2016), which validates the repressing ability of Triger (Tebuconazole) in the current results. Xiu-Rong (2011), Horsfield *et al.* (2010), and Koley *et al.* (2016) in different trials found that Tebuconazole greatly inhibits the development of *R. solani* and their results support the current finding.

Use of bacteria as a biocontrol agent is also becoming effective way in controlling different diseases. A large number of species have been determined as biological control agents for management of tomato *fusarium* wilt including *T. harzianum*, *Pseudomonas* spp. and *Bacillus subtilis* (Tao *et al.*, 2020). In tomato the antagonistic activity of the *Pseudomonas putida* PSIRB15 (29%), *P. fluorescens* PSRB19 (31%), *B. amyloliquefaciens* IRB36 (40%) and *P. fluorescens* IRB26 (43%) was proved against *F. oxysporum* (Hariprasad *et al.*, 2009).

CONCLUSIONS

On the basis of results of current study, it was concluded that *fusarium* wilt of cotton can be best controlled by integrated disease management (IDM) strategy in combined application of PGPRs and *Trichoderma* isolates to lower fungicide doses to a safer range.

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AUTHOR CONTRIBUTIONS

Miss Maha Sarfraz planned, designed, conducted and analyzed all trials. For data collection, the trials were examined regularly. Mr. Sanwal Bakhsh, Miss Mubushra Sarwar and Miss Iqra Mubin helped in planning and data collection of all trials. Later, the data was analyzed under the supervision of Madam Saira Mehboob. Dr. Sajid Aleem Khan and Dr. Javed Ahmad assisted our team for surveying different areas of Punjab during sample collection. Special Thanks to Dr. Sajid Aleem Khan (Associate Professor, UAF) and Madam Saira Mehboob, (Principal Scientist, Plant Pathology, AARI) for providing their consistent support in this study. This research was self-funded.

COMPETING OF INTEREST

No conflicts of interest have been disclosed by the authors.

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