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UTILIZATION OF BIOLOGICAL CONTROL AGENTS FOR THE MANAGEMENT OF POSTHARVEST PATHOGENS OF TOMATO

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Abstract

Twenty five isolates of *Trichoderma*, *Bacillus* and *Pseudomonas* spp. were obtained from rhizosphere of tomato growing fields using soil dilution technique on potato dextrose agar (PDA) and nutrient agar (NA) medium. Screening of these isolates were done against *Geotrichum candidum*, *Trichothecium roseum* and *Rhizopus oryzae*, causal agents of sour rot, pink mold rot and *Rhizopus* soft rot of tomato under the laboratory conditions. One promising isolate of each *Trichoderma harzianum*, *Bacillus* spp. and *Pseudomonas fluorescens* from the twenty five isolates were chosen and further evaluated as potential biological control agents (BCAs) against three important postharvest pathogens of tomato. Dual culture and spore concentration assay revealed that all three isolates inhibited radial growth of *G. candidum*, *T. roseum* and *R. oryzae*. Tomato fruits were inoculated with 25µl suspension of 10^8 cfu mL^{-1} for *T. harzianum* and 10^8cfu $mL^{-1}$for each *Bacillus* sp. and *P.fluorescens*. Twenty four hours later the treated fruits were inoculated with 25µl of 10^5 conidia/mL of each of three postharvest pathogens. The results showed that *P. fluorescens* provided good control (78.1%) of *G. candidum* and (82.2%) *R. oryzae*, while, *T. harzianum* proved less effective to control all three pathogens. *Bacillus* spp. was only effective (88.4%) against *T. roseum*. Hence, our results depicted that *Bacillus* spp. and *P. fluorescens* proved to be a potential antagonist of *T. roseum* and *R. oryzae* however, all the tested BCAs were not consistent in their action against three postharvest pathogens of tomato.

Key words: Biological control agents (BCAs), Pink mold rot, Sour rot, *Rhizopus* rot, Tomato, Postharvest management.

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is ranked second (Akram *et al*., 2014) among vegetables of Pakistan containing vitamin A, C and minerals (Saleem *et al*., 2013). Tomato-based products are considered as valuable source for improving our diet as they contained lycopene and flavonoids (George *et al*., 2004; Lenucci *et al*., 2006). Pre and post-harvest factors adversely affect these phenolic/antioxidant contents of tomatoes (Dumas *et al*., 2003). Many microorganisms have been reported to be the cause of postharvest diseases in fruits and vegetables (Spadaro & Gullino, 2004). Harvested fruits of tomato carries propagules of pathogens which become latent in the field and cause rotting of tomato fruits when condition become favorable during storage. Therefore, proper handling is necessary to minimizing the incidence of postharvest decay (Mahovic *et al*., 2004).

Sour rot, pink mold rot and *Rhizopus* soft rot in tomato are caused by *Geotrichum candidum*, *Trichothecium roseum* and *Rhizopus oryzae*, respectively (Fajola, 1979; Bartz *et al*., 2010; Bourret *et al*., 2013; Hamid *et al*., 2014). Various postharvest treatments like UV radiation, short-period ozone treatment have proved beneficial in enhancing antioxidant capacity of tomatoes by mitigating softening process (Liu *et al*., 2011; Obande *et al*., 2011). Still, these fungal pathogens cause severe economic losses in field and after harvest during storage and transportation (Ukeh & Chiejina, 2012). Conventionally, farmers heavily relied on synthetic chemicals to manage decay caused by postharvest pathogens (Wichitra *et al*., 2008). However, few fungicides have been banned in market because of their toxicity, hazards to human health and environment (Usall *et al*., 2000; Dilantha *et al*., 2005) further, they also changed the quality characteristics of the harvested fruits (Domínguez *et al*., 2012). Due to negative impact of application of chemical fungicides to environment, health hazard and development of resistant strains of the pathogens, prompted the scientists to search out ecofriendly alternatives for the management of tomato postharvest pathogens.

Biological control refers the application of living antagonistic microorganisms directly or their metabolic products to impede or kill undesired microorganisms (Sanae *et al*., 2007; Zhou *et al*., 2011). Since 1980s, biological control obtained by antagonistic microorganisms which, includes fungi, bacteria and yeasts are particularly promising in the absence of fungicides which can provide full and effective protection of fungal rots of various fruits and vegetables (Duraisamy *et al*., 2008).The advantages that the BCA possess as compared to chemical fungicides are there safety to environment and human health moreover, fit well in sustainable agriculture (Das *et al*., 2008). During the past two decades, several biocontrol antagonistic microorganisms have been exploited and investigated against different postharvest fungal pathogens like *Aspergillus* spp., *Monilia* spp., *Rhizopus* spp. and *Penicillium* spp. (Anna *et al*., 2003). *Trichoderma* species are well known antagonist of many plant pathogenic fungi (Mohamed & Haggag, 2006). They are

most commonly studied biocontrol agent and commercially available as bio fertilizer, bio pesticide and soil amendment (Harman *et al*., 2004). Recently, *Bacillus subtilis* has been found an efficient postharvest BCA of different diseases on tomato, orange, avocado and maize (Saligkarias *et al*., 2002; Cavaglieri *et al*., 2005; Besrat & Lise, 2006; Smilanick *et al*., 2006; Zhao *et al*., 2008; El-Katatny & Emam, 2012). *P. fluorescens* isolate 1100-6 was evaluated to control blue mold of apple (Etebarian *et al*., 2005). It is evident from the published results that antibiotics, extracellular enzymes and antifungal metabolites produced by antagonistic bacteria and fungi are responsible to control postharvest pathogens (Sharma *et al*., 2009).

This study was carried out with the objective to explore the efficacy of indigenous isolates of *T. harzianum*, *Bacillus* spp. and *P. fluorescens* collected from rhizosphere of local tomato fields in order to find sustainable management of sour rot, pink mold rot and *Rhizopus* soft rot of tomato.

Materials and Methods

Isolation and identification of pathogens: Tomato fruits showing typical symptoms of the disease were collected in plastic zip bags. They were surface sterilized with 0.1% $Hgcl_2$ (mercuric chloride) for one to two minutes and then washed three times with sterile distilled H_2O in separate Petri plates in order to remove $Hgcl_2$. These sterile symptomatic tomatoes pieces were placed on sterile potato dextrose agar (PDA; potato starch 4gm, glucose 20 gm, agar 15gm, D.W 1 L) (LAB M Limited, United Kingdom) and incubated at room temperature 24±1°C to recover the fungal pathogens. They were identified both morphologically and molecular bases using ITS regions (Hamid *et al*., 2014). Pure cultures of the isolated fungi were obtained by single spore according to Riker & Riker (1936) and maintained in potato dextrose slants. An aggressive isolate of each *G. candidum*, *T. roseum* and *R. oryzae* which had showed higher pathogenicity potential in screening tests was obtained from the Culture Bank, Department of Plant Pathology, University College of Agriculture, University of Sargodha, Pakistan for this study.

Isolation and collection of bio-control agents: Twenty five isolates of fungi and bacteria were isolated from rhizosphere soil of tomato growing fields of Sargodha District (32°5′1″N; 72°40′16″E). The isolation was done by using soil dilution technique on PDA and nutrient agar (NA; beef extract = 3g, peptone = 5g, agar = 15g, distilled H_2O = 1 L) medium. These isolates were initially screened against *G. candidum*, *T. roseum* and *R. oryzae* under the laboratory conditions. Later on, an aggressive isolate of each *T. harzianum*, *Bacillus* spp. and *Pseudomonas fluorescens* were selected and evaluated for their potential as effective biocontrol agent of sour rot, pink mold rot and *Rhizopus* soft rot of tomato.

Evaluation of biological control agents

***In vitro* growth inhibition of tested microorganisms:** The inhibitory effect of *T. harzianum*, *Bacillus* spp. and *P. fluorescens* isolates against *G. candidum*, *T. roseum* and *R. oryzae* was tested by amending 1 mL spore suspension of three concentrations of *T. harzianum*, (10^6, 10^7 and 10^8cfu mL^{-1}) into each Petri plate. Saline water was amended in PDA served as control. After that plates were poured with pre-cooled PDA. For bacterial isolates same procedure was followed and plates were amended with 1 mL aliquot of three concentrations (10^6, 10^7, and 10^8cfu mL^{-1}). When PDA solidified in plates, a 5-mm plug of *G. candidum*, *T. roseum* and *R. oryzae* was placed into center of agar plates. These Petri plates were incubated for 5 to 8 days at 24°C±1 temperature.

Dual culture method to evaluate antifungal activity bio control agents: The dual culture assay technique was utilized in order to evaluate the atagonistic effect of *Bacillus* spp., *T. harzianum* and *P. fluorescens* isolates against *G. candidum*, *T. roseum* and *R. oryzae*. A pathogen-fungal antagonist combination was examined on 20ml of PDA medium in 9cm Petri plates by placing *T. harzianum* plug (5mm diameter) on opposite side from each other. The bacterial isolates were tested on nutrients agar (NA) medium. Aliquots of 0.1 mL bacterial suspension were streaked on one side of plates. After 1 day of incubation in dark at 20°C, a 5mm diameter plug of *G. candidum*, *T. roseum* and *R. oryzae* was placed opposite side of the Petri plate. Postharvest pathogens alone in PDA plates served as control. These Petri plates were incubated at temperature of 24°C ± 1 for 5 to 8 days. Mycelial growth inhibition of individual fungus was measured 5 to 8 days post inoculation. Three replications were done with each antagonist independently each time. The radial mycelial growth of pathogen and percent reduction over control was determined by using the following formula as:

$$\text{Inhibition over control percentage (\%)} = \frac{\text{C-T}}{\text{C}} \times 100$$

where C = mycelial growth of pathogen in control, and T = mycelial growth of pathogen in dual culture.

Evaluation of biological control agents on harvested tomato fruit: Fruits of tomato were injured using a sterilized punch. 25µl suspension of 10^8 cfu $mL^{-1}$of *T. harzianum* while, 10^8 cfu $mL^{-1}$of each *Bacillus* sp., and *P. fluorescens* were placed in each wounded fruit. Control fruits were inoculated with distilled water. Later, each of the wound was inoculated with dose (25µl of 10^5cfu mL^{-1}) of *G. candidum*, *T. roseum* and *R. oryzae* within 45 minutes after inoculation of BCAs. After drying at room temperature, fruits were kept in plastic bags and were incubated at 24°C ± 1 for 5 to8 days. The experiment includes 3 fruits per treatment and each treatment was replicated thrice.

Statistical analysis: The statistical analysis was carried out using R.3.0.3-Statistical package. Two factor factorial analyses were used for the interpretation of the results. The treatment means were calculated by (DMRT) Duncan's Multiple Range Test (Gomez & Gomez, 1984).

Results

The fungal pathogens involved in postharvest diseases of tomatoes were isolated from the decay fruits showing typical fungal growth. They were collected from different markets of Sargodha, Punjab, Pakistan. The associated fungi were identified and their pathogenicity was confirmed by applying Koch's postulates.

Effect of BCAs on mycelium growth: The effectiveness of three biocontrol agents, *Bacillus* spp., *P. fluorescens* and *T. harzianum* against the mycelium growth of *G. candidum*, *T. roseum* and *R. oryzae*, at three concentrations were studies *In vitro* (Fig. 2a, b, c). The results showed that all the concentration inhibited more than 50% mycelium growth as compared to control. It was observed that all the three concentrations showed varied degree of inhibition effect on mycelium growth. The inhibition percentage of *G. candidum*, *T. roseum* and *R. oryzae* was increased with increased concentration of biocontrol agents. *B. subtilis* was the most effective, suppressing 88.4% *T. roseum* of mycelia growth with 3.44 mm colony growth (Fig. 2a, 3a). *P. fluorescens* was the next most effective, suppressing 80.3% of *T. roseum* mycelia growth while *T. harzianum* was proved least effective against the *T. roseum*. The mean inhibition percentage in dual culture assay was 73.6 (7.3mm), 78.1 (7.0mm) and 67.7% (8.4mm) for *G. candidum*; 78.0 (13.3mm), 82.2 (11.1mm) and 79.2% (12.9mm) for *R. oryzae* by the antagonistic action of *Bacillus* spp., *P. fluorescens* and *T. harzianum* (Fig. 2b, c and 3b, c).

***In vivo* control of tomato postharvest pathogens with BCAs:** In present study, tomato fruits were inoculated with BCAs and three postharvest pathogens whereas, control fruits were inoculated with water and pathogen. Fruits which were inoculated with 10^8 conidia/mL^{-1} *P.fluorescens* provided complete control (100%) of sour rot and *Rhizopus* soft rot (Table 1). *Bacillus* spp., successfully controlled *G. candidum* but was not able to provide efficient (48.3%) results against *T. roseum*. Artificially inoculated fruits with 10^8 conidia/mL^{-1} *T. harzianum* were not a potential antagonist (39.9%) on harvested tomatoes to control pink mold rot disease (Fig. 1). All the BCAs used in this study were unable to control pink mold rot caused by *T. roseum*. Among the three tested postharvest pathogens *G. candidum* proved susceptible to all tested biological antagonists while pink mold caused *T. roseum* showed resistant to biological antagonists. The antagonistic action was more evident after 72 hours after application of BCAs.

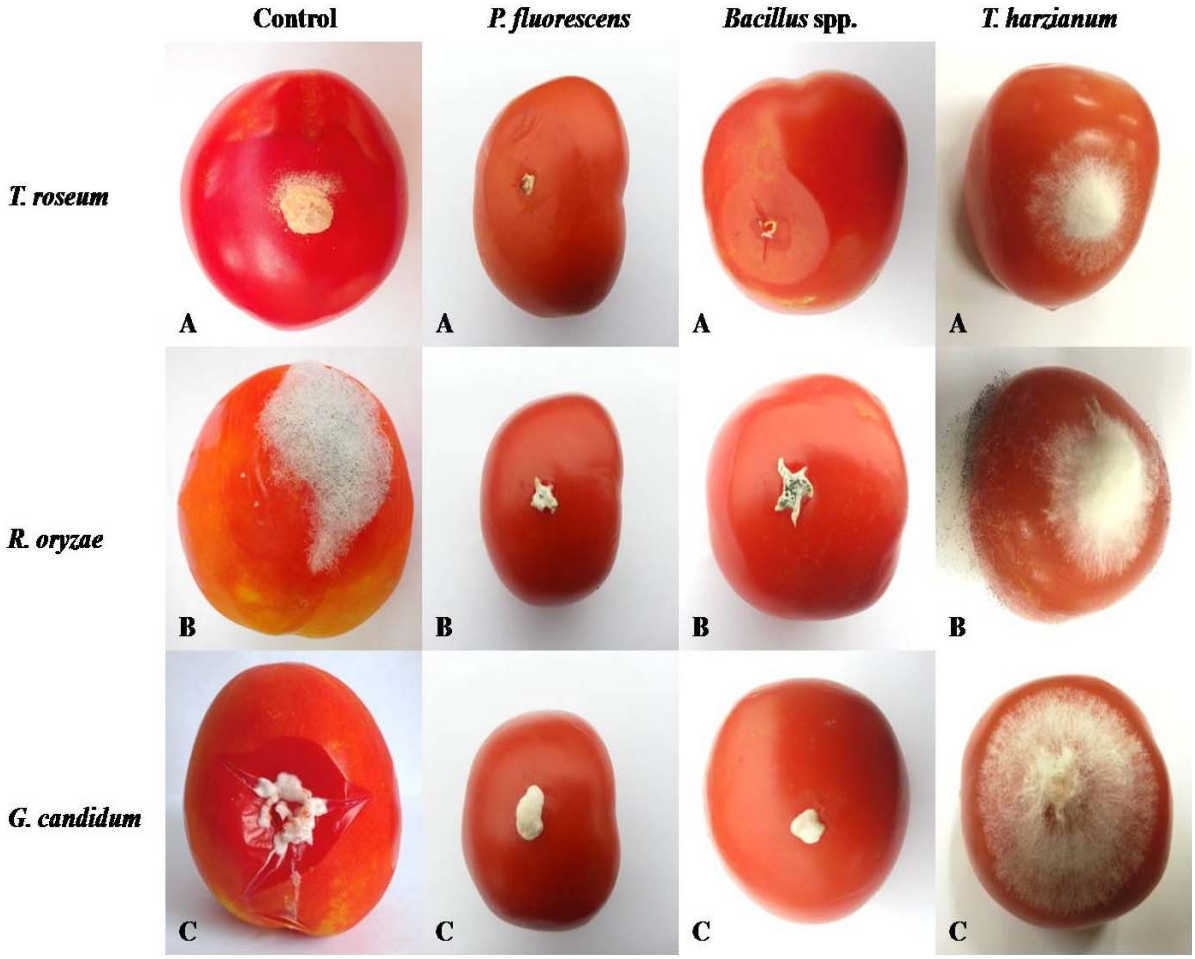

Fig. 1. Biocontrol effect of *P. fluorescens*, *Bacillus* spp., and *T. harzianum* on tomato fruits inoculated with (A) *T. roseum* (B) *R. oryzae* and (C) *G. candidum*.

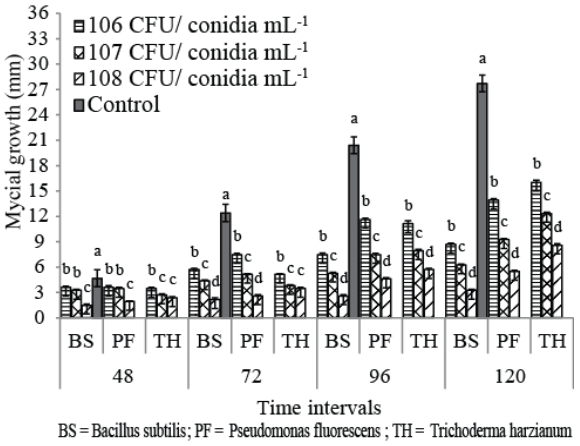

Fig. 2a. Effect of different BCAs concentrations on the mycelial growth of *T. roseum* on potato dextrose agar at 24±1°C. Significant differences (p<0.05) between means were indicated by different letters above histogram bars.

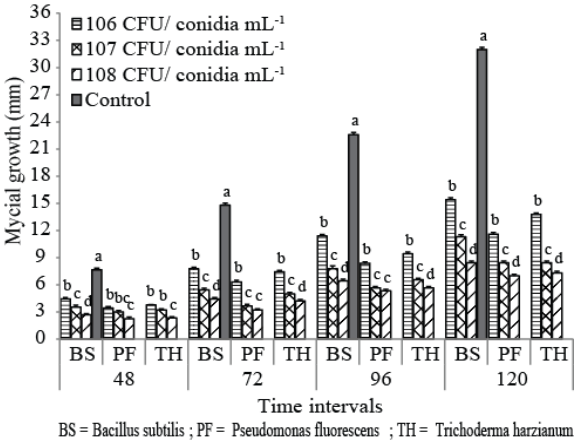

Fig. 2b. Effect of different BCAs concentrations on the mycelial growth of *G. candidum* on potato dextrose agar at 24±1°C. Significant differences (p<0.05) between means were indicated by different letters above histogram bars.

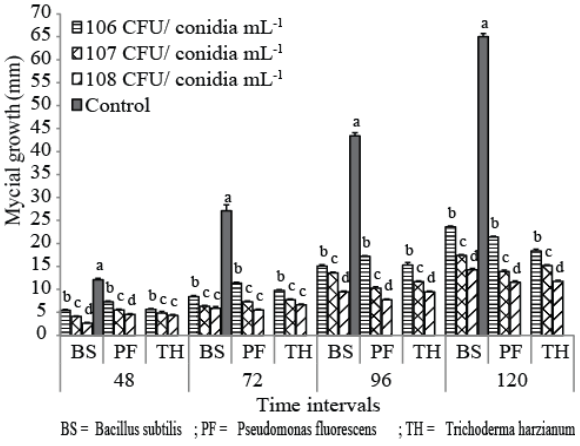

Fig. 2c. Effect of different BCAs concentrations on the mycelial growth of *R. oryzae* on potato dextrose agar at 24±1°C. Significant differences (p<0.05) between means were indicated by different letters above histogram bars.

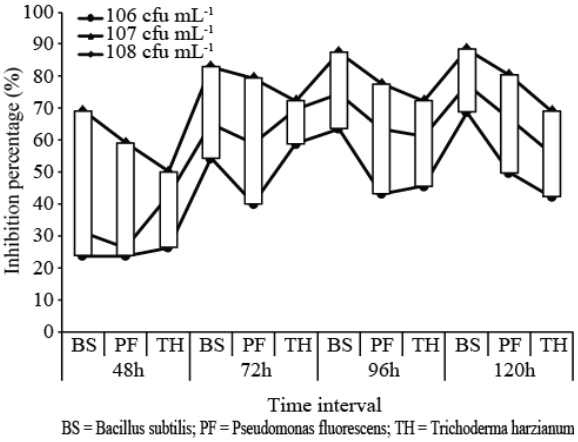

Fig. 3a. Inhibition percentage of *T. roseum* on PDA at 24±1°C influenced by different concentrations of BCAs.

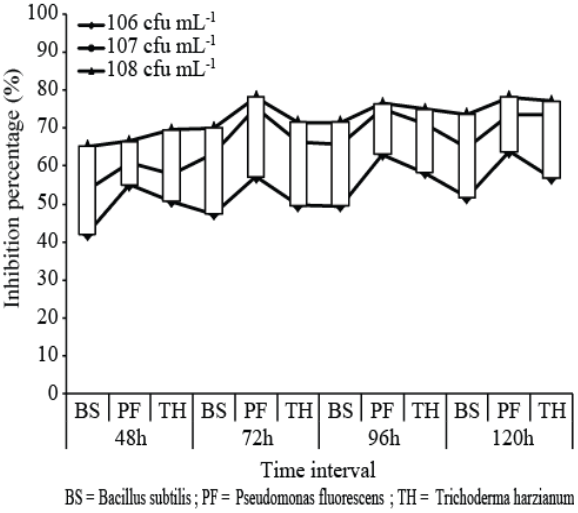

Fig. 3b. Inhibition percentage of *G. candidum* on PDA at 24±1°C influenced by different concentrations of BCAs.

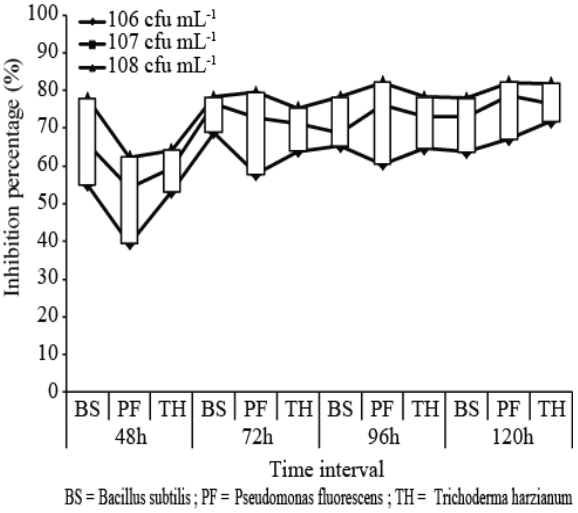

Fig. 3c. Inhibition percentage of *R. oryzae*on PDA at 24±1°C influenced by different concentrations of BCAs.

Table 1. Effects of different concentrations of BCAs on lesion diameter of tomato fruit caused by *T. roseum, G. candidum* and *R. oryzae* applied as dip application method and stored at 24±1°C.

Biocontrol agents		*B. subtilis*			*P. fluorescens*			*T. harzianum*		
Treatments		Time intervals								
		24	48	72	24	48	72	24	48	72
10^8cfu mL^{-1}	*T. roseum*	5.5 ± 0.18^{ab}	5.33 ± 0.24^{ab}	3.56 ± 0.18^{c}	11.4 ± 0.18^{b}	9.67 ± 0.29^{c}	8.44 ± 0.24^{c}	16.8 ± 0.22^{b}	15.0 ± 0.23^{b}	12.8 ± 0.22^{c}
Control			6.89 ± 0.35^{a}			15.4 ± 0.29^{a}			21.3 ± 0.53^{a}	
Inhibition percentage (%)		19.4	22.6	48.3	25.9	37.2	45.2	21.1	29.6	39.9
10^8cfu mL^{-1}	*G. candidum*	3.56 ± 0.17^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	11.0 ± 0.55^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	20.0 ± 0.52^{b} 0	0.00 ± 0.00^{c}	00 ± 0.00^{c}
Control			5.22 ± 0.22^{a}			22.8 ± 0.46^{a}			44.8 ± 0.62^{a}	
Inhibition percentage (%)		31.8	100	100	51.8	100	100	55.4	100	100
10^8cfu mL^{-1}	*R. oryzae*	12.0 ± 0.24^{b}	7.33 ± 0.16^{c}	0.00 ± 0.00^{d}	25.6 ± 0.41^{b}	16.7 ± 0.24^{c}	0.00 ± 0.00^{d}	43.6 ± 0.50^{b}	23.7 ± 0.41^{c}	0.00 ± 0.00^{d}
Control			15.4 ± 0.38^{a}			28.6 ± 0.44^{a}			49.1 ± 0.68^{a}	
Inhibition percentage (%)		22.1	52.4	100	10.5	41.6	100	11.2	51.7	100

Values followed by the same letter are not significantly different at the 5% level by DMRT

Discussion

Postharvest diseases cause 20-30% losses to perishable commodities in various countries around the globe (Senthil *et al*., 2011) and this condition is more worsen in the developing countries due to lack of transport and storage facilities (Sharma *et al*., 2009). This makes the environment more encouraging for the development of microbes and other contaminants. Postharvest disease control strategies that are being currently used relied upon new fungicides (Kanetis *et al*., 2007; Droby *et al.,* 2009), antagonistic microorganisms (Mari *et al*., 2014), and plant extracts (Ameziane *et al*., 2007; Plooy *et al*., 2009). The introduction of fungicides resistant strains of postharvest pathogens new safer and eco-friendly alternatives control methods (Mari *et al*., 2014) need to be searched out continuously in order to minimize the losses inflicted by postharvest pathogens.

Many potential fungal and bacterial antagonists with antifungal properties have been explored and employed to overcome postharvest diseases to enhance the preservation period of fruits and vegetables (Mari *et al*., 2014). Especially *Bacillus* spp., *P. fluorescens* and *T. harzianum*, ubiquitous in rhizosphere soil and also recognized as ecofriendly, safe for consumers, broad spectrum and a strong inhibitory effect on postharvest pathogens (Moretto *et al*., 2014; Quaglia *et al*., 2011; Peeran *et al*., 2014). In our previous study, we had reported *Trichothecium roseum* for the first time on tomato, orange and apple in Pakistan (Hamid *et al*., 2014). Moreover, two other important postharvest pathogens *G. candidum* and *R. oryzae* are main menace to tomato as well as other crops and causing substantial economic losses to farmers, market and house holders (Fatima *et al*., 2009). We found that *P.s fluorescens* and *Bacillus* spp. proved to be a potential antagonist in controlling *G. candidum* and *R. oryzae*. Moreover, all tested BCAs showed great potential in reducing lesion diameter of *T. roseum* but failed to completely control it on harvested tomato fruits. The results clearly exhibited that the bacterial and fungus biocontrol agent significantly inhibit mycelium growth of *G. candidum*, *T. roseum* and *R. oryzae* as compared to control treatment and inhibition zone between BCA and target pathogen was clearly observed. This inhibition zone between pathogen and BCA might be due to antimicrobial substances produced by BCAs. Similar to our results (Benizri *et al*., 1995) reported that *Pseudomonas* spp. showed antagonistic activity due to release of antibiotics, siderophores and volatile compound. The antagonistic bacteria *Bacillus cereus* produced metabolites in dual culture assay on PDA which inhibited *Fusarium roseum* var. *sambucinum* growth (Sadfi *et al*., 2001) also confirm our results. Another probability of formation of inhibition zone is nutrient depletion by BCAs, surrounding target pathogen thus inhibited the growth of *G. candidum*, *T. roseum* and *R. oryzae*. The presence of inhibition zone and its size revealed that this is due to production of antibiotic by (Crawford *et al*., 1993) bacteria and fungi. Zhou *et al*. (2011) reported that the different spore concentration of *B. subtilis* strain fmbj into PDA medium significantly inhibited, mycelium growth and sporulation of *R. stolonifer*. Fungi belong to genus *Trichoderma* have great potential as antagonist of phytopathogens and produces a wide variety of antibiotics and enzymes that degrade the cell wall of the pathogens (El-Katatny *et al*., 2011; Harman *et al*., 2004). Similar to our results with *Trichoderma* (El-Katatny *et al*., 2011) found inconsistent results against the postharvest pathogens of tomatoes when he applied two isolates of *Trichoderma* against fourteen tested fungi. The eight isolates of native and introduced *Trichoderma* spp., showed great variation (Sangeetha *et al*., 2009) against two postharvest pathogens of banana (*Musa paradisiaca*) also in line with the present findings. Various isolates of *Pseudomonas* spp. have provided effective in managing postharvest pathogens of fruits and vegetables (Janisiewicz & Jeffers, 1997; Mikani *et al*., 2008; Sharma *et al*., 2009). The *Pseudomonas* spp., used in this study clearly inhibit the mycelial growth of *G. candidum* and *R. oryzae* with maximum inhibition 72 hours post inoculation. The inhibition zone was also evident where *Pseudomonas* spp., was used which may be due to inhibitory substances (Mikani *et al*., 2008) produced. The findings of our experiments corroborate the pervious findings (Ganeshan & Kumar, 2005; Mikani *et al*., 2008; Droby *et al*., 2009)

as the authors reported the varying level of efficacies of *Pseudomonas* isolates against postharvest pathogens.

Our results from *In vivo* study showed that bacterial biocontrol agents (*Bacillus* spp. and *P. fluorescens*) provided good control of *G. candidum* and *R. oryzae* on artificially inoculated tomatoes fruits whereas, *Trichoderma* did not proved effective. The reason of difference in the efficacy of these biocontrol agents on artificially inoculated tomato fruits may be maintenance of population density of *Bacillus* and *Psudomonas* spp., in the wounded area (Zong *et al*., 2010), where they compete for nutrients and space considered as mode of action of biocontrol agents. Zhou *et al*. (2011) reported that *B. subtilis* reduced lesion diameter and hyphal growth of *R. stolonifer* in peach fruits, this support our findings as we found *Bacillus* spp., reduced lesion diameter on tomato fruits as compared to control and reduced hyphal growth was also observed. Singh & Daverall (1984) also reported that *Bacillus* spp., are good biocontrol agents of postharvest pathogens because species of *Bacillus* survive well at high and low temperature. *T. harzianum* was unable to reduced lesion diameter of *G. candidum*, *T. roseum* and *R. oryzae* on inoculated tomato fruits. El-Katatny *et al*. (2011) found that *T. harzianum* significantly suppressed hyphal growth of *Penicillium* and *Aspergillus* on tomato slices except *Rhizopus* spp. The population of *P.fluorescens* increases at the wound sites as the time progressed which helps in inhibiting population of pathogens (Etebarian *et al*., 2005). In present study *P. fluorescens* completely inhibited growth of *G. candidum* and *R. oryzae* at wounded sites of tomato and no rot lesion was observed. This could be due to good colonization of wounded part by *P. fluorescens*, competition for nutrients (Senthil *et al*., 2011), production of antibiosis against the pathogen (Sturz *et al.,* 1998). All the BCAs were unable to completely inhibited *T. roseum* on artificially inoculated fruits which may show the inconsistency of biocontrol agents (Francesco & Mari, 2014). The efficacy of BCAs could be enhanced by manipulating environment, mixture of antagonist and genetic manipulation of BCAs (Janisiewicz & Korsten, 2002). This is the first study which reported the efficacy of indigenous biocontrol agents against the postharvest pathogens of tomato in Pakistan.

Conclusions

In conclusion, experimental results showed that *Bacillus* spp., and *P. fluorescens* were effective in managing sour rot and *Rhizopus* soft rot of tomato at 24^{o}C for 3 days. *T. harzianum* was unable to control all these pathogens on harvested fruits. Future studies will be focused on investigating potential microbial antagonists and also genetic manipulation of these microbes to enhance their efficacy in range of environmental conditions and identifying mode of action by which BCAs suppressed target pathogen.

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