In vitro screening of some antagonists against *Sclerotinia sclerotiorum* (Lib.) de Bary, a causative agent of white mold disease in mustard

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ABSTRACT

An *in vitro* experiment was conducted during 2018–2020 at BARI, Bangladesh, Gazipur to screen the antagonistic effects of nine fungal and five bacterial antagonists against *Sclerotinia sclerotiorum* (Lib.) de Bary. White mold disease of mustard is one of the most important diseases of mustard caused by the phytopathogen *S. sclerotiorum*. Among the fungal antagonist tested, *Aspergillus niger* 2, *A. niger* 3, *Penicilium* sp, *Trichoderma harzianum*, *T. viride* and *T. virens* were most effective in inhibiting 89.35% to 100% mycelial growth of *S. sclerotiorum* in the dual culture method on PDA medium. Among these antagonist fungi, *Aspergillus niger* 2, *A. niger* 3, *T. harzianum*, *T. viride* and *T. virens* were capable of completely stopping the sclerotia formation of *S. sclerotiorum*. The Culture filtrates of *Trichoderma* species viz., *T. harzianum, T. viride, T. virens* completely (100%) inhibited the radial growth of the test pathogen. The highest inhibition of radial growth of *S. sclerotiorum* observed with the volatile metabolites of *Trichoderma viride* (90%) followed by *T. harzianum* (89.39%) and *T. virens* (89.35%). More than 90% of mycelial growth was inhibited by *Pseudomonas* sp. and *Bacillus subtilis* strain B20 in dual culture on PDA medium and also effective in stopping sclerotia formation. In the volatile method, all the tested antagonistic bacteria showed moderate antagonistic effects against *S. sclerotiorum*. Further study is required to develop effective formulation of those biocontrol agents for controlling this alarming pathogen in field condition in an eco-friendly manner.

Keywords: White mould, *Sclerotinia sclerotiorum*, Antagonism, Biological control

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INTRODUCTION

Mustard is an essential and leading edible oilseed crop in Bangladesh. White mold disease of mustard is caused by the devastating fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, one of the major fungal diseases and causes yield loss in every year. Commercially cultivated all of the varieties of mustard are susceptible against white mold disease in Bangladesh (Najmun *et al.,* 2018). White mold disease depends on various environmental factors which determine sclerotial survival and ascospores dissemination. Mustard plants are primarily infected by air-borne ascospores from carpogenic germination of sclerotia. The stem rot fungus *S. sclerotiorum* produces a lot of sclerotia which can survive a long time in the soil. These sclerotia give rise to fruiting body apothecia that produce a huge number of ascospores which become airborne and blow to nearby crop fields and create rot disease in plants (Smolińska and Kowalska, 2018; Grau and Hartman, 1999). The pathogen has very extensive host range and attacks more than 500 plant species of different families. It was also reported by Mondal *et al.* (2015) that there was no host specificity among the different hosts recorded from southern parts of West Bengal (India). The pathogen produced white, branched and septate mycelial growth on the infected plant parts. Black sclerotia near spherical to irregular in shape having rough and pitted surface generally were formed towards

the periphery of the culture medium (PDA) in petridish. Sclerotinia stem rot of mustard is difficult to control because of the life cycle pattern of the pathogen and its wide host range (Bolton *et al.*, 2006, Mondal *et al.,* 2016). Disease management strategies focus on cultural controls to reduce the number of viable sclerotia in soil, crop rotation, chemical and biological control and postharvest management. Plant diseases are generally controlled by the use of chemical fungicides and in certain cases by cultural practices. The widespread use of chemicals in agriculture has been a subject of public concern and scrutiny due to their potentially harmful effects on the environment. The chemical pesticides have been shown to pollute practically every part of our ecosystem, posing major dangers to both the environment and nontarget biota and ultimately affecting natural biodiversity. Pesticides have also been linked to human health hazards, ranging from short-term effects to long-term consequences, including cancer and reproductive disruption. Recently, researchers have focused their efforts on developing alternative inputs to synthetic chemicals for diseases management (Cook and Baker, 1983). Several studies have been done on the biological control of *S. sclerotiorum* by using fungal antagonists (Tiwari *et al.*, 2011, Jawadayn *et al.*, 2015) and bacterial bio control agents (Abdullah *et al.*, 2008, Fernando *et al.*, 2007, Fernando *et al.*, 2013, Xiaoning *et al.*, 2014, Tozlu *et al.*, 2016) in either laboratory or field trials. Application of chemical fungicides is commercially and successfully reported to control *Sclerotinia* spp. Most of the canola cultivars are susceptible to this disease (Bradley *et al.*, 2016, Anonymous, 2014). Search for biocontrol agents against sclerotinia stem rot in mustard is considered to be an essential part of ecofriendly disease management. In Bangladesh, there is no sufficient data on the use of biocontrol agents to manage this pathogen. Considering the widespread prevalence and destructive nature of this pathogen in mustard-growing regions of Bangladesh, along with its wide host range, long-term survival in soil through sclerotia, and limited success of conventional chemical-based control methods, there is an urgent need for eco-friendly alternatives. This study sought to assess the antagonistic potential of selected microorganisms using dual culture techniques on PDA medium, and to further evaluate the effects of culture filtrates and volatile metabolites, particularly from *Trichoderma* spp., on the mycelial growth and sclerotia formation of *S. sclerotiorum*. The goal was to identify promising microbial strains, including species of *Aspergillus*, *Penicillium*, *Trichoderma*, *Pseudomonas*, and *Bacillus subtilis*, that exhibit strong inhibitory effects on the pathogen and could be further developed into sustainable biocontrol formulations for managing white mold disease in mustard under field conditions.

MATERIALS AND METHODS

The experiment was conducted during 2018–2020 at Bangladesh Agricultural Research Institute, Bangladesh, Gazipur with a view to explore alternative and effective bio-control option against *S. sclerotiorum* causing white mold disease of mustard.

Isolation of test pathogen

S. sclerotiorum the causal organism of white mold disease was isolated from diseased infected stems of mustard following the "Tissue planting" method. The pathogenicity of the isolated fungi was tested following the "detached stem technique" (Azad and Shamsi, 2011).

Collection of antagonists

To study the antagonistic activity of nine soil fungi, viz. *Aspergillus flavus*, *A. fumigatus*, *A. niger* 1, *A. niger* 2, *A. niger* 3 and *Penicillium* sp. and five bacterial isolates such as *Bacillus subtilis* B20, *B. subtilis* PB18, *Bacillus* sp. BVC38, *B. amyloliqufaciens* Egg 25 and *Pseudomonas* sp. were obtained from Dhaka University and *Trichoderma harzianum*, *T. viride* and *T. virens* from Bangladesh Agricultural Research Institute, Bangladesh. All the isolates were grown on PDA (potato dextrose agar) and NA (Nutrient agar) medium at 25ºC and 30ºC in an incubator for 5 days

in order to obtain juvenile colonies for studying antagonism.

Colony interaction

Fifteen ml of sterilized potato dextrose agar medium was poured into sterile Petri plates and allowed to solidify. Fungal and bacterial antagonists were inoculated at one side of the Petri plate and the test pathogen was inoculated at the opposite side of the same plate by leaving a 3 cm gap and incubated at 25±1°C for 7 days. The percentage of inhibition over control was measured according to the formula given by earlier scientists (Rolim *et al.*, 2019).

Percentage inhibition of growth (PGI) = r-r1/r×100

Where

r = radial growth of fungus measured from the center of the colony towards the center of the Petri plate without antagonistic fungus,

r1 = radial growth of fungus measured from the center of the colony towards the antagonistic fungus in the center of the Petri plate.

The colony growth of the pathogen was measured at the both sides, that is, towards and opposing each other from their central loci. Intermingled and inhibition zone was also measured during the same period. Assessments of colony interaction between the test pathogen and the soil fungi were done in terms of grades which were determined by the model of Skidmore and Dickinson (1976). Antagonist was also rated for inhibitory effects using a scale by Sangoyomi (2004) as: ≤10% inhibition (not effective), >11–20% inhibition (slightly effective), >21–50% inhibition (moderately effective), >51–<80% inhibition (effective) and >81% and above inhibition (highly effective).

Growth of the pathogen

In volatile methods, selected antagonist fungi and bacteria were grown in Petri plates on PDA and NA medium at 25±1°C for 3 days. After the inoculation, the lid of each Petri plate was replaced by the same sized bottom plate, containing 15 ml PDA medium, centrally inoculated with a test pathogen. Then Petri plates were covered by Parafilm so that no volatile substances can be moved from the inside of the Petri plates. The percentage of inhibition of the test fungi was calculated after the 7th day of incubation.

Culture filtrates of the soil fungi on the growth of the pathogen

In non-volatile methods, nine fungal antagonists cultured in a conical flask on Potato Dextrose Broth (PDB) and incubated at 25±1°C for 10 days. Liquid culture filtrate was collected separately and filtered through filter paper; then, centrifuged at 3000 rpm for 20 min. Bio-efficacy of culture filtrates against test pathogen was tested by following "Poisoned Food Technique method" (Dennis and Webster, 1971). The percentage of growth inhibition was recorded by following the procedure mentioned earlier.

Statistical analyses

The data were collected as inhibition zone values in mm in each replication, and evaluated by analysis of variance (ANOVA) by using STAR v2.0.1 (2014) developed by IRRI.

RESULTS

Antagonistic effect of fungi against the test pathogen

The result of colony interaction between the test fungi and the antagonist fungi is summarizing in Fig 1 and 2. Different level of antagonistic effects of the antagonist fungi was observed against the test pathogen. Out of nine soil fungi, six fungi viz. *Aspergillus niger* 2, *A. niger* 3, *Penicilium* sp, *Trichoderma harzianum*, *T. viride* and *T. virens* exhibited strong antagonistic effects against the test fungi which were completely inhibited the radial growth of the test fungi in dual culture methods. Whereas, *A. flavus* and *A. niger* 1 exhibited moderate antagonistic effects, inhibiting radial growth by 83.75% and 83.5%, respectively, followed by *A. fumigatus* with 75.75% inhibition.

The effect of volatile metabolites of antagonistic fungi against white mold pathogen is presented in Fig. 1 and 2. Volatile substances emanating from the cultures of the soil fungi inhibited the radial growth of the test fungi to varied degrees. The maximum inhibition of radial growth of *S. sclerotiorum* was observed in *Trichoderma viride*

(90%) followed by *T. harzianum* (89.39%), and *T. virens* (89.35%) due to the volatile metabolites after 7 days of incubation. *Penicillium* sp. showed medium inhibition of radial growth followed by *Aspergillus niger* 2. The least inhibition of radial growth noticed with *A. niger* 1 was 45.46%.

The effect of non-volatile metabolites on the growth of *S. sclerotiorum* is also shown in Fig. 1. The selected antagonists showed varied degrees of growth inhibition of the pathogen. The culture filtrates of three species of *Trichoderma viz.*, *T. harzianum*, *T. viride* and *T. virens* equally inhibited the mycelial growth of *S. sclerotiorum* (100%) at 10% concentration after 5 days of incubation at 25±2˚C. Among the *Aspergillus* species tested, *Aspergillus niger* 2 inhibited the highest mycelial growth of the test fungi, followed by *A. fumigatus* (68.33%) and *A. flavus* (67.08%). *Penicillium* sp. also showed medium inhibition of radial growth. The lowest inhibition of radial growth of *S. sclerotiorum* was observed in *A. niger* 1.

Antagonistic effects of different antagonist fungi against *S. sclerotiorum*

Antagonistic effect and interaction level of the test pathogen and antagonist fungi are summarized in Table 1. Out of nine fungi, eight fungi were highly effective against *S. sclerotiorum* in the dual culture method (SD=9.20975, SE=3.06992). Three species of *Trichoderma* viz. *T. harzianum*, *T. viride* and *T. virens* were highly effective against the test pathogen in all the methods tested. Among the three methods, dual culture is more effective than volatile (SD=16.5997, SE=5.53324) and non-volatile (SD=16.6118, SE=5.53728) methods, respectively

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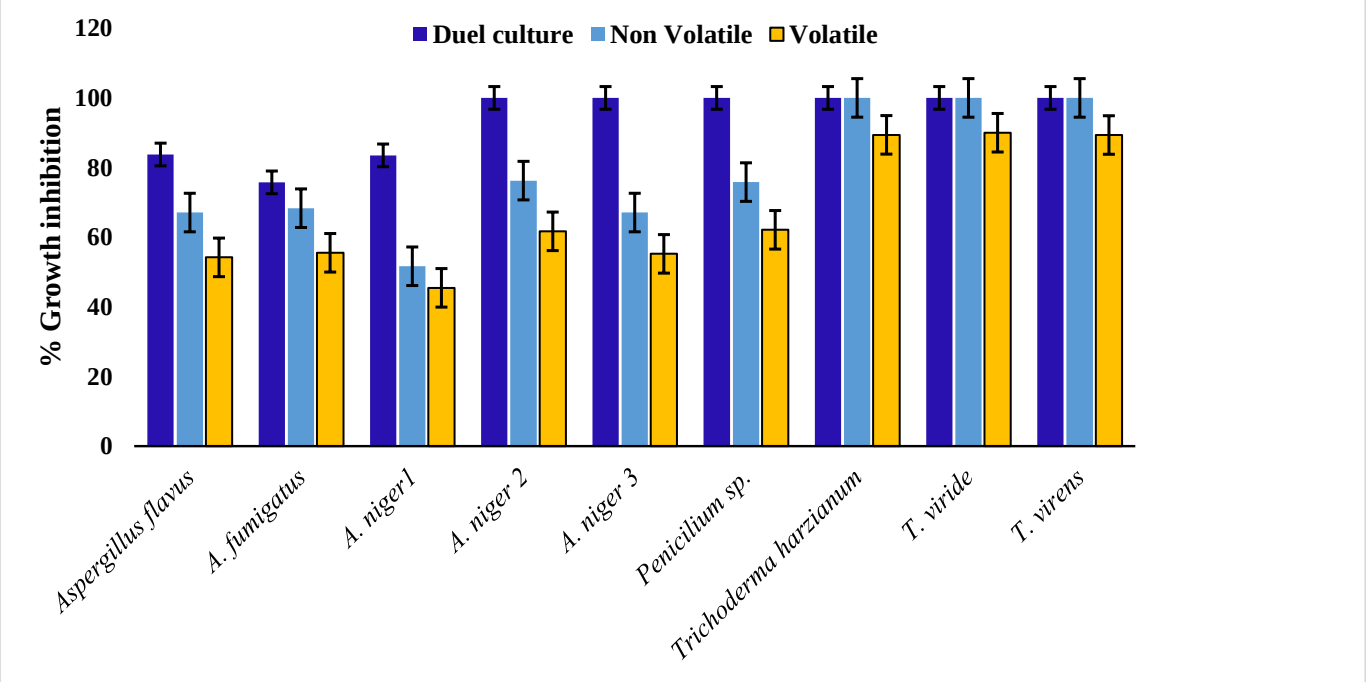

Figure 1. *In vitro* inhibition of *S. sclerotiorum* using antagonistic fungi in different methods

Table1. Antagonistic effects and grade of different soil fungi against *S. sclerotiorum*

Name of fungi	Antagonistic effects		
	Dual culture method	Volatile method	Non-volatile method
Aspergillus flavus	Highly effective	Effective	Effective
A. fumigatus	Effective	Effective	Effective
*A. niger*1	Highly effective	Effective	Moderately effective
A. niger 2	Highly effective	Effective	Effective
A. niger 3	Highly effective	Highly effective	Effective
Penicilium sp.	Highly effective	Effective	Effective
Trichoderma harzianum	Highly effective	Highly effective	Highly effective
T. viride	Highly effective	Highly effective	Highly effective
T. virens	Highly effective	Highly effective	Highly effective

Where, ≤ 10% inhibition (not effective), >11-20% inhibition (slightly effective), >21-50% inhibition (moderately effective), >51-<80% inhibition (effective) and >81% and above inhibition (highly effective).

Reduction of sclerotia formation

Among the fungi tested, five fungi such as *Aspergillus niger* 2, *A. niger* 3, *Trichoderma harzianum*, *T. viride*, and *T. virens* were capable to completely stopped the sclerotia formation of the *S. sclerotiorum*. *Aspergillus niger* 1 reduced 82.85% sclerotia formation followed by *A. flavus* (Fig. 3).

Antagonistic effects

Evaluation of the antagonistic ability of five antagonist bacteria viz. *Bacillus amyloliquefaciens* Egg 25, *B. subtilis* PB 18, *B. subtilis* strain B20, *Bacillus* sp. strain BVC 38 and *Pseudomonas* sp. against *S. sclerotiorum* also studied (Fig. 4). In dual culture methods, *Pseudomonas* sp. and *Bacillus subtilis* strain B20 exhibited strong antagonistic effects which are capable to inhibit the radial growth of *S. sclerotiorum*, and it was 94.16 and 92.91%, respectively. The case of *Bacillus* sp. strain BVC 38 and *Bacillus subtilis* strain PB 18 showed medium-inhibiting effects that inhibit the radial growth of test fungus were 67.5 and 64.58%, respectively. The lowest inhibition of radial growth was recorded with *Bacillus amyloliquefaciens* Egg 25.

In the volatile method, all of the antagonist bacteria showed poor antagonistic effects against *S. sclerotiorum.* The maximum inhibition of radial growth of *S. sclerotiorum* was showed by *Bacillus amyloliquefaciens* strain Egg 25 followed by *Pseudomonas* sp. due to the volatile metabolites after 7 days of incubation. *Bacillus subtilis* strain PB 18, *B. subtilis* strain B20 and *Bacillus* sp. strain BVC 38 showed closely inhibiting effects against test fungi that inhibited the radial growth were 22.91, 22.08 and 20.83%, respectively.

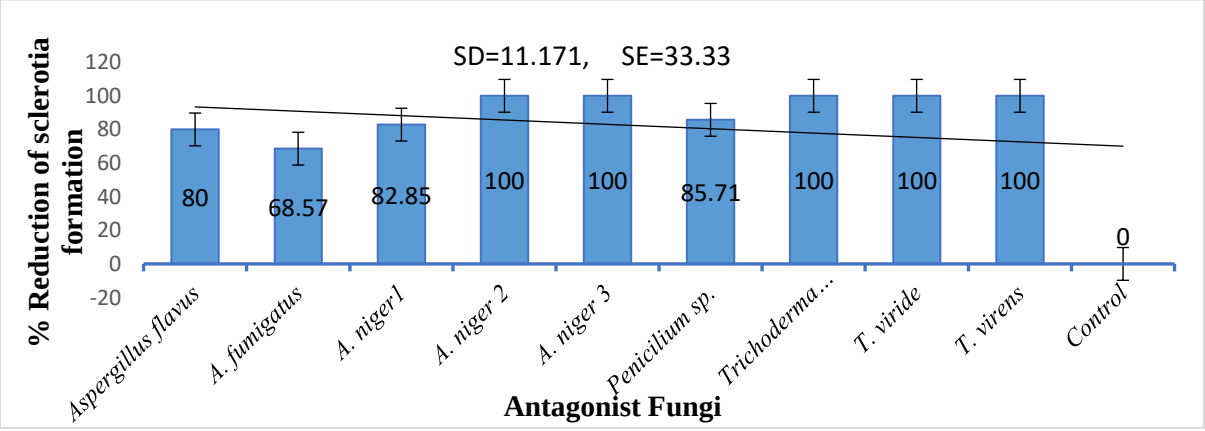

Figure 2. Reduction of sclerotia formation during interaction between antagonistic fungi and *S. sclerotiorum*.

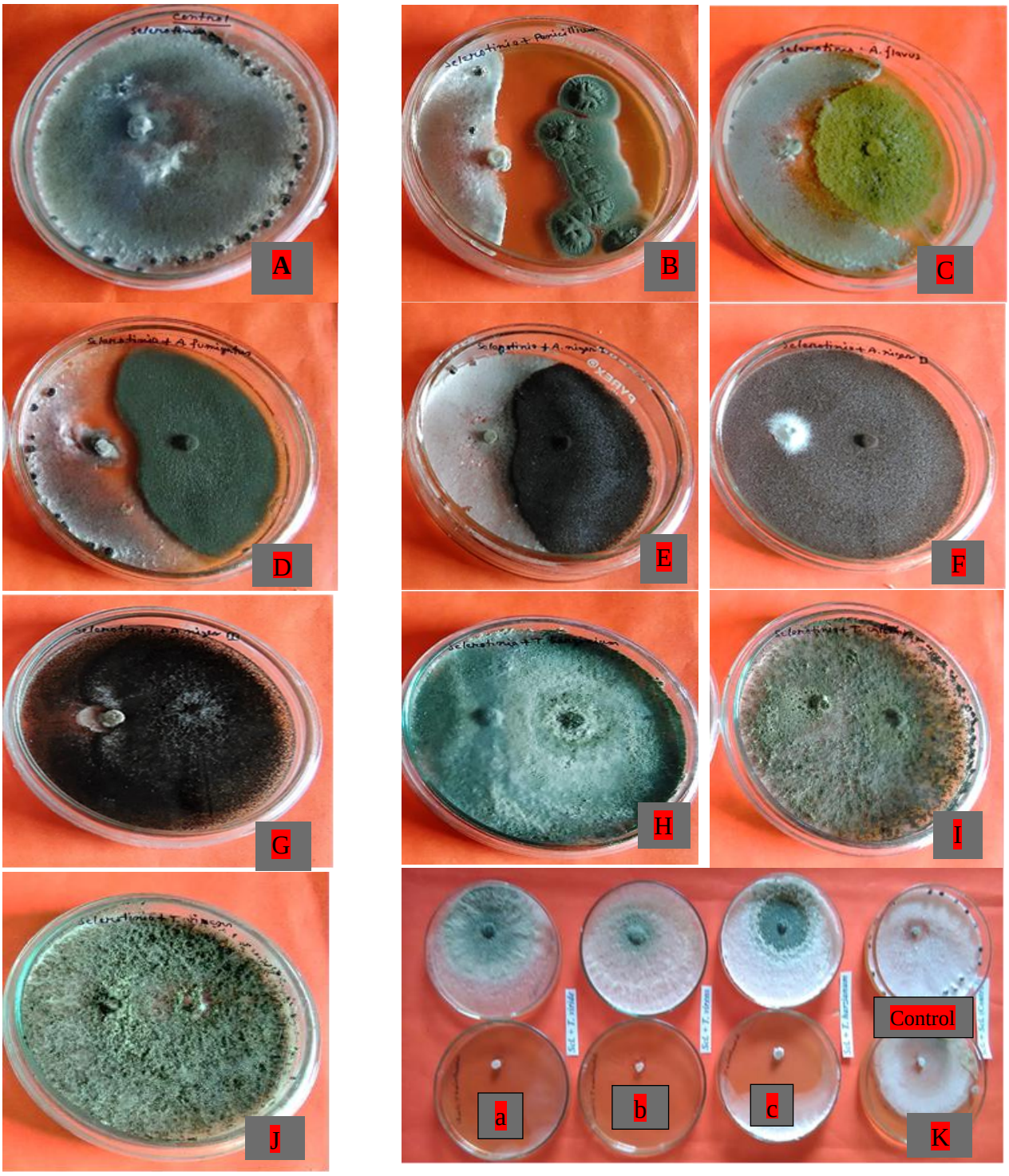

Figure 3. Colony interaction of *Sclerotinia sclerotiorum* with antagonist fungi: A) Control; B) *S. sclerotiorum* + *Penicilium* sp.; C) *S. sclerotiorum* + *Aspergillus flavus*; D) *S. sclerotiorum* + *A. fumigatus*; E) *S. sclerotiorum* + *A. niger*1; F) *S. sclerotiorum* + *A. niger*2; G) *S. sclerotiorum* + *A. niger* 3; H) *S. sclerotiorum* + *Trichoderma harzianum*; I) *S. sclerotiorum* + *T. viride*; J) *S. sclerotiorum* + *T. virens*; K)Volatile substances of *Trichoderma* spp. inhibit the radial growth of *S. sclerotiorum*: a) *S. sclerotiorum* + *T. viride*; b) *S. sclerotiorum* + *T. virens* c) *S. sclerotiorum* + *T. harzianum*.

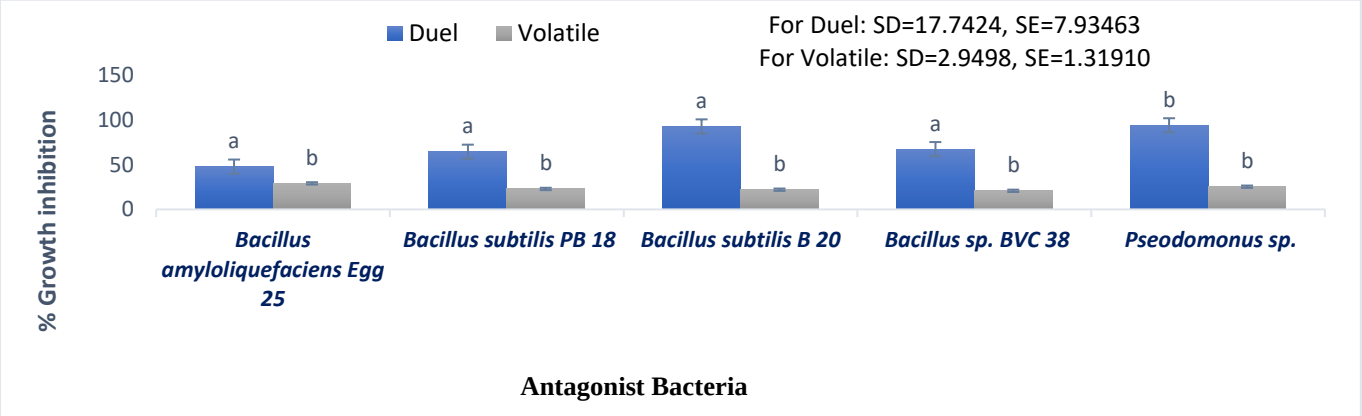

Figure 4. Interaction between antagonistic bacteria with *S. sclerotiorum*

Antagonistic effects and grades

The result of antagonistic effects and grade between the test fungi and the antagonist bacteria has presented in Table 2. Among the bacteria tested, two bacteria *Bacillus subtilis* B20 and *Pseudomonas* sp. showed highly effective against the test fungus *Sclerotinia sclerotiorum* in the dual culture method. In volatile methods, all the bacterial isolates showed a moderate level of antagonistic reaction.

Reduction of sclerotia formation of *Sclerotinia sclerotiorum*

The result of sclerotia formation of *Sclerotinia sclerotiorum* with the interaction between antagonist bacteria and stem rot fungi *S. sclerotiorum* has presented in Fig. 5. Out of five antagonist bacteria two bacteria namely *Bacillus subtilis* B20 and *Pseudomonas* sp. were capable of completely stopping the sclerotia formation. In the case of *Bacillus subtilis* PB 18 reduced 60% sclerotia formation followed by *Bacillus* sp. BVC 38. Whereas, a reduced number of sclerotia formations were recorded in *Bacillus amyloliquefaciens* Egg 25 than other antagonist bacteria (Fig. 5).

DISCUSSIONS

The percent inhibition of the test pathogen towards the soil fungi varied because of variations in nature, quality, and quantity of the inhibitory substances produced by the soil fungi. Results from colony interaction of *S. sclerotiorum* and antagonist soil fungi, *Aspergillus niger* 2, *A. niger* 3, *Trichoderma harzianum*, *T. viride*, and *T. virens* could reduce the mycelial growth and completely stop the sclerotia formation of the test fungi *S. sclerotiorum*. Tiwari *et al.* (2011) found that *Aspergillus niger* and *Trichoderma viride* were equally inhibited the radial growth of the white rot and brown rot wood decay fungi in dual culture technique. Hyphal interactions between the species of *Trichoderma* spp. and *S. sclerotiorum* were investigated in dual culture and found that *T. harzianum* hyphae grew towards and coiled across the *S. sclerotiorum* hyphae. Mari *et al.* (1996) reported the similar result and found that thick coils of hyphae of *T. harzianum* and partial degradation of the *Sclerotinia sclerotiorum* cell wall were damaged in the later stages of the parasitism. *Trichoderma* spp. *viz.*, *T. harzianum* and *T. virens* have also been shown to inhibit both sclerotial and mycelial growth of *Sclerotinia* spp. (Tu, 1980, 1998). Jawadayn *et al.* (2015) was also found 84.44% and 100% inhibition of the mycelial growth of *S. sclerotiorum* through *T. viride* TV10 and *T. harzianum* TH12. *Trichoderma* spp. prompted cell-wall degrading enzymes when the parasitic interaction occurs with pathogenic fungi (Elad *et al.*, 1983, Limon *et al.*, 2015). Antagonism is owing to the antagonistic interaction between the biocontrol agents and the pathogens. Direct antagonism also results from physical contact between two microbes where the bioagent defeats the pathogen through space occupancy and nutrient competition (Harman *et al.*, 2004).

Table 2. Antagonistic level and grade of different bacteria against *S. sclerotiorum*

Name of Bacteria	Antagonistic level	
	Dual culture method	Volatile method
Bacillus amyloliquefaciens Egg 25	Moderately effective	Moderately effective
Bacillus subtilis PB 18	Effective	Moderately effective
Bacillus subtilis B 20	Highly effective	Moderately effective
Bacillus sp. BVC 38	Effective	Slightly effective
Pseudomonas sp.	Highly effective	Moderately effective

Where, ≤ 10% inhibition (not effective), >11-20% inhibition (slightly effective), >21-50% inhibition (moderately effective), >51-<80% inhibition (effective) and >81% and above inhibition (highly effective)

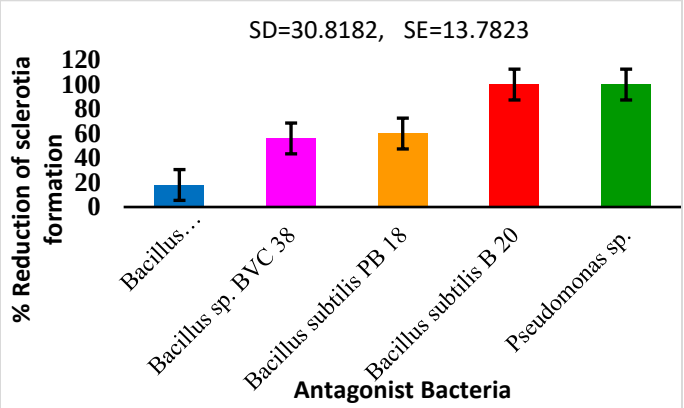

Figure 5. Sclerotia formation of *S. sclerotiorum* during interaction with antagonistic bacteria

Yang *et al.* (2008) noticed that *Penicillium oxalicum* isolated from soil and found strong antagonistic activity against plant pathogenic fungi *S. sclerotiorum*. This finding supports the present findings. It was reported that seven antagonistic soil fungi such as *Penicillium cyclopium*, *Paecilomyces lilacinus*, *Aspergillus niger*, *A. fumigatus*, *Acremonium implicatum*, *Penicillium shearii*, and *Trichothecium roseum* that have antagonistic effects against *S. sclerotiorum* (Budge and Whipps, 1991, Singh, 2008). Recently, in some studies, volatile compounds produced by antagonistic fungi and bacteria have been shown to have potential antifungal activities (Wheatley, 2002). Gaber and Derbalah (2011) reported that culture filtrates of different microbial isolates such as *Trichoderma viride* and *Bacillus pumilus* were more effective against powdery mildew in squash than the fungicide. Volatile compounds produced by *Trichoderma viride* extremely reduced the mycelial growth and sclerotial production of *S. sclerotiorum* (Faheem *et al.*, 2010). Mansour *et al.* (2008) reported that *Trichoderma harzianum* and *Bacillus amyloliquefaciens* inhibited radial growth and sclerotia formation. Similar result also observed in the present study, the maximum inhibition of radial growth of *S. sclerotiorum* was found from *Trichoderma harzianum* and *Bacillus amyloliquefaciens* strain Egg 25. In the present investigation, all the antagonist bacteria tested, exhibited poor antagonistic effects against *S. sclerotiorum* in the volatile method. The maximum inhibition of radial growth of *S. sclerotiorum* was found from *Bacillus amyloliquefaciens* strain Egg 25 (29.16%) followed by *Pseudomonas* sp. (25.41%) because of the emission of the volatile metabolites of the antagonist bacteria. All the strains of *Bacillus subtilis* showed closely inhibiting effects against test fungi that inhibited radial growth were 20.83–22.91%. All the antagonist bacteria showed low antagonistic levels against *S. sclerotiorum* in the volatile method (Table 2).Fernando *et al.* (2007) reported four bacterial strains such as *Pseudomonau chlororaphis* (PA-23), *Bacillus amyloliquefaciens* (BS6), *Pseudomonas* sp. (DF41), and *B. amyloliquefaciens* (E16) which have antagonist effects against *S. sclerotiorum* in canola from *in vitro* assays. In the dual culture method, eight strains of *Pseudomonas* sp. and different species of *Bacillus* such as *B. subtilis*, *B. megaterium*, and *B. pumilus* were capable to produce inhibition zone against mycelial growth of *S. sclerotiorum* on PDA medium in *in vitro* assay (Tozlu *et al.*, 2016). The endophytic bacterium *Bacillus subtilis* was evaluated as a

biocontrol agent against *S. sclerotiorum* on oilseed rape in a petri dish which strongly inhibited mycelium growth and also sclerotial germination of *S. sclerotiorum* (Xiaoning *et al.*, 2014). In the existing study shows that, different volatile compounds produced by antagonistic bacteria and fungi have potential antifungal activities to reduce the mycelial growth of test pathogen. In the volatile method, all of the antagonist bacteria showed poor antagonistic effects than fungal antagonists against *S. sclerotiorum*. The present study also revealed that volatile compounds produced by the antagonists inhibited the mycelial growth of the pathogen to varying degrees, which may be attributed to qualitative and quantitative differences in their volatile production. Many bacterial antagonists inhibit the growth of fungi by employing various mechanisms, like secretion of lytic enzymes, production of siderophores, production of antibiotics etc. Some bacterial antagonists may indirectly support plant growth promotion. Induced systemic resistance and competition for space, food and light are some other mechanisms for inhibiting the growth of the target pathogens. Directly inhibition of the growth and activities of the pathogens by secreting different types of cell degrading enzymes was mentioned by Neeraja *et al.* (2010). Volatile compounds released from bacterial antagonists inhibit the mycelial growth of soil-borne pathogen (Kai *et al.*, 2006, Zou *et al.*, 2007). *Bacillus subtilis* produced different types of volatile compounds that significantly inhibited the mycelial growth and completely stopped the sclerotial germination of *S. sclerotiorum* (Chen *et al.*, 2008, Fernando *et al.*, 2013, Kamal *et al.*, 2015), which also supports the present findings. Das *et al.* (2014) reported the pronounced effect of antagonistic strain *Bacillus subtilis* (S-12) on the pathogen causing citrus canker.

CONCLUSION

The present observation suggests that there were qualitative and quantitative differences in the volatile substances and culture filtrate produced by antagonistic soil fungi that exhibited different degrees of growth inhibition against *Sclerotinia sclerotiorum*. *Aspergillus niger* 2, *Trichoderma harzianum*, *Trichoderma viride* and *Trichoderma virens* were effective inhibiting mycelial growth and sclerotia formation. *Bacillus subtilis* B20 and *Pseudomonas* sp. were also exhibited strong antagonistic effects on sclerotia formation. Preparation of effective formulation of those biocontrol agents is important to manage this pathogen.

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