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Research Article

Antagonistic activity of cowshed *Bacillus* sp. bacteria against aflatoxigenic and sclerotic *Aspergillus flavus*

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ABSTRACT: Mycotoxins produced by many food spoilage fungi cause serious damage to human as well as to livestock. Aflatoxins are one such group of mycotoxins produced by *Aspergillus flavus* in many agricultural and food products including rice. The fungus can virtually grow in any environment and also produces resistive structures such as sclerotia to overcome unfavourable environmental conditions. Hence, the need of the time is to control this toxigenic and sclerotic fungus through an eco-friendly approach. In the current study four biocontrol bacteria belonging to *Bacillus* species were isolated from Indian cow shed environment and these bacteria could efficiently control not only the mycelia growth of *A. flavus* but also the germination and growth of sclerotia. Both active bacterial culture broth and cell free culture filtrate could limit the growth of the fungus up to more than 90%. Older culture broth and filtrate lost their inhibition efficiency. Unlike many similar studies the current investigation emphasizes the importance of cowshed environment instead of cow dung and the control of sclerotia instead of fungal spores.

KEY WORDS: Aflatoxins, *Aspergillus flavus, Bacillus,* cowdung, mycotoxins, sclerotia

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INTRODUCTION

Food spoilage is often associated with the infection of fungi which produce toxic secondary metabolites known as mycotoxins which are proven to have carcinogenic, teratogenic, mutagenic, immunotoxic, neurotoxic, nephrotoxic and hepatotoxic effects. The occurrence of mycotoxigenic moulds in foods constitutes a high risk for human and animal health (Blagojev *et al*., 2012). *Aspergillus flavus* is one of such mycotoxin producing fungi which infects and subsequently produces aflatoxins in cereal grains, oil seeds, fermented beverages made from grains, milk, cheese, meat, nut products, fruit juice and a wide range of other agricultural commodities (Zhang *et al*., 2008; Bennett and Klich, 2003; Li *et al*., 2011, Makun *et al*., 2007; Nayak *et al*., 2014). The fungus mainly produces aflatoxin B1 and B2 where aflatoxin B1 is classified as Group 1A human carcinogen by the World Health Organisation-International Agency of Research on Cancer in 1993 (IARC, 1993), hence that is highly regulated in almost 77 countries (FAO, 2004). This ubiquitous fungus can grow on any organic substrate (both living and dead plant tissues) and are superbly adapted to a wide range of environmental conditions which also produces resistant structures known as sclerotia. Sclerotia are pigmented, compacted aggregates of hyphae, which resist unfavorable environmental conditions and capable of remaining dormant for long periods which is often associated with aflatoxin production (Cotty, 1988; Wicklow and Shotwell, 1983; Rollins and Dickman, 1998).

To control and prevent the occurrence of *A. flavus*, it is necessary to replace chemical pesticides or fungicides to avoid environmental pollution, many health problems and destruction of non-targeted beneficial organisms. An important alternative is the use of antifungal properties produced by microbial Biological Control Agents (BCA) (Chitarra *et al*., 2003; Gheorghe *et al*., 2008). *Bacillus* species are good biological control agents (BCA) for their ability to produce different types of antimicrobial compounds, such as

antibiotics e.g., bacilysin, iturin, mycosubtilin (Afsharmanesh *et al*., 2014; Mushtaq *et al*., 2010; Moyne *et al*., 2001). Among terrestrial bacterial strains, the genus of *Bacillus* has been well studied due to its ability to produce various types of inhibitory compounds (Stein, 2005). Both bacterial cell suspension and cell free culture filtrate have been found to be effective (Lim *et al.,* 2008; Moshafi *et al.,* 2011; Kong *et al.,* 2010). The efficiency of *Bacillus* species to inhibit *A. flavus* and other post-harvest disease causing fungi has been discussed broadly in many reports (Kong *et al.,* 2010; Pusey and Wilson, 1984). Several species such as *Bacillus subtilis*, *B. amyloliquefaciens*, *B. vallismortis* inhibited the growth of *Aspergillus flavus* (Das *et al*., 2013; Thakaew and Niamsup, 2013; Palumbo *et al*., 2007; Bhusan *et al*., 2013; Ranjbariyan*et al*., 2011). Cow dung served as a source for the presence of antifungal biocontrol agents and *Bacillus* species. Many *Bacillus* species isolated from cow dung have also been reported to possess antagonistic activity against many plant pathogens *viz*. *Fusarium oxysporum*, *Botryodiplodia theobromae*, *Sclerotium rolfsii*, *Pythium aphanidermatum*, *Helminthosporium maydis* and *Rhizoctonia solani* (Swain *et al*., 2008; Swain and Ray, 2009; Teo and Teoh, 2011). These bacteria are supposed to prevail in the indoor air of cowshed where cow-dung remains until it is cleaned by the residents. Hence cowshed environment also constitutes a source of antagonistic *Bacillus* bacteria.

Despite such importance, *Bacillus* bacteria present in cowshed environment have not been exploited for control of aflatoxigenic *Aspergillus flavus*. Hence, in the current investigation we have attempted to isolate bacteria belonging to *Bacillus* species from cowshed indoor air. Evaluation of these bacteria was carried out to observe their efficacy to inhibit mycelial growth and sclerotial germination under *in vitro*.

MATERIALS AND METHODS

Bacteria strains

Antagonistic bacteria were isolated by exposing Potato Dextrose Nutrient Agar media (PDNA) plates to cattle shed air for one minute and then incubating at 30°C for 24 hours. The bacteria colony appeared in the plate were then isolated and maintained as pure culture at Crop Protection Division of Central Rice Research Institute, India. Bacteria were identified by molecular method and the ITS sequences were submitted to NCBI GenBank. Four efficient bacteria designated as BC1, BC2, BC5 and BC6 (Table 1) belonging to *Bacillus* species were included in the current study for the evaluation of antagonistic activity.

Aspergillus flavus isolates

The two *Aspergillus flavus* isolates A2 and A28, used in the current study were obtained from Crop Protection Division of Central Rice Research Institute, India. A2 was aflatoxigenic strain where as A28 was aflatoxigenic as well sclerotia producing strain.

Evaluation of antagonistic activity

Bacterial isolates BC1, BC2, BC5 and BC6 were cultured in 50ml LB Broth for six hours and 24 hours in shaker at 250 rpm at 37°C. This culture broth was mixed properly with luke warm PDNA@ 7ml/L of media. After the media solidified, fungal isolates (A28 and A2) were inoculated. Untreated control and four replicates of each treatment maintained throughout the experiment. The plates were incubated at 30°C and periodic growth in terms of colony diameter was recorded.

The inhibition percentage was determined as follows:

$$\frac{\text{Colony area of untreated control} \quad \text{colony area of treatment}}{\text{Colony area of control}} \times 100$$

Colony area $= \varpi r^2$

Inhibition of sclerotia germination

Sclerotia of isolate A28 were harvested by growing the fungus on PDA media and then collecting mature sclerotia with a sterilised brush. Sclerotia were then washed with sterilised water and dried properly before subjecting for experimentation.

To observe inhibition of sclerotia germination, 25µl of six hours and 24 hours culture broth of bacteria (BC1, BC2, BC5 and BC6) was put in separate cavity slides and then sclerotia were kept in it. For untreated control, sclerotia were kept in LB broth. Hundred sclerotia were taken for each treatment and control. The cavity slides were then incubated at ambient temperature inside humid chamber to prevent evaporation. The germination of sclerotia and formation of germ tubes were observed under 4X objective 15X eyepiece of an Inverted tissue culture microscope (Radical). Microscopic photography was done with a digital camera (Sony Cyber shot).

Evaluation of germination or growth of sclerotia

Germination and growth of individual sclerotia was observed and scored with 1 to 5 evaluation scale as: 1- Zero (no growth), 2- Poor, 3- Moderate, 4-Good and 5- Very good. Average mode was taken into consideration for the evaluation

of sclerotia germination for each treatment and control.

RESULTS AND DISCUSSION

Inhibition of mycelia growth

Four bacteria belonging to *Bacillus* species were evaluated for antagonistic activity against aflatoxigenic and sclerotia producing isolates of *Aspergillus flavus*. The passport data of the bacteria is presented in Table 1. BC1 and BC2 were identified as *Bacillus amyloliquefaciens* where as BC5 and BC6 were *B. vallismortis* and *B. subtilis,* respectively. All the four bacteria efficiently inhibited the mycelia growth of both the fungi though a definite inhibition pattern was lacking. Periodic growth of *A. flavus* strains A2 and A28 were significantly reduced by the four bacteria (Table 2 and Fig. 1). A2 was more susceptible. The colony diameter (cd) of untreated A2 was 37.3mm on third day of growth whereas that of A2 treated with BC1, BC2, BC5 and BC6 were 12.6mm, 12mm, 10.3mm and 11.6mm respectively. Similarly, the cd of untreated A28 on third day was 40.3mm whereas that of A28 treated with BC1, BC2, BC5 and BC6 were 15.3mm, 14.6mm, 14.3mm and 15.6mm respectively. Higher inhibition was exhibited by *B. vallismortis* (BC5). In terms of area of mycelia colony, BC5 showed 92.3% inhibition towards A2 followed by *B. subtilis* (BC6) which showed 90% inhibition (Fig. 2). BC1 and BC2 (*B. amyloliquefaciens)* showed 88.6% and 89.3% inhibition to A2 respectively. Higher reduction in colony area of A28 was shown by BC5 with 87.3% inhibition followed by BC2 which showed 86.7% inhibition. BC1 and BC6 exhibited 85.7% and 85% inhibition.

Table 1. Passport data of *Bacillus* bacteria isolated from cowshed atmosphere

*Isolate ID	NCBI GenBank Accession no.	Identified Organism
BC1	JF304104	*Bacillus amyloliquefaciens*
BC2	JF304105	*Bacillus amyloliquefaciens*
BC5	JQ753710	*Bacillus vallismortis*
BC6	JF304107	*Bacillus subtilis*

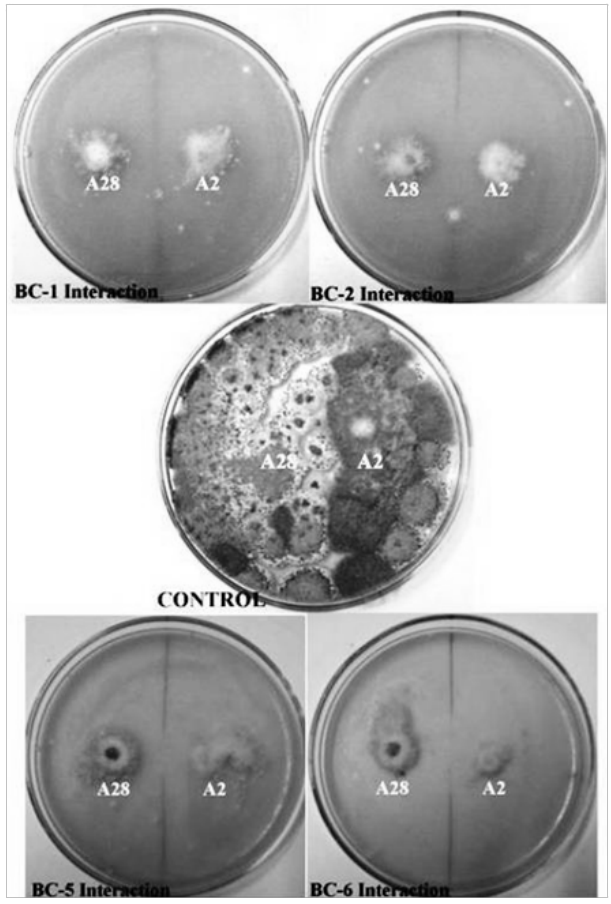


Fig. 1. Inhibition of *Aspergillus flavus* (A2 and A28) by culture broth of antagonistic bacteria isolated from cowshed environment

Efficiency of bacterial culture broth and culture filtrate to inhibit sclerotia germination and growth was evaluated (Table 3; Fig. 3 and 4). Formation of germ tubes were scored in a 1 to 5 scale range and average mode was taken into consideration for each treatment and control. In control, where sclerotia were kept in LB broth, very good growth (5-average mode) was observed. Contrary to this no growth (100% inhibition) was observed in more than 90% cases where sclerotia were kept in six hours culture broth of all bacteria. In these cases only 5% showed poor germination. The cell free culture

Table 2. Effects of culture broth of cowshed bacteria on colony diameter of aflatoxigenic *Aspergillus flavus* isolate A2 and A28

Bacteria ID	Periodic observation on Colony diameter(mm) of *A. flavus* isolates					
	A2			A28		
	Day I	Day II	Day III	Day I	Day II	Day III
BC1	4.3 ± 0.3	5.3 ± 0.3	12.6 ± 0.0	5.0 ± 0.0	10.6 ± 0.7	15.3 ± 0.3
BC2	4.3 ± 0.3	6.3 ± 0.3	12 ± 0.0	4.6 ± 0.3	10.0 ± 0.6	14.6 ± 0.9
BC5	4.3 ± 0.3	6.3 ± 0.7	10.3 ± 0.3	5.3 ± 0.3	9.0 ± 0.6	14.3 ± 0.3
BC6	5.6 ± 0.3	6.6 ± 0.3	11.6 ± 0.3	7.3 ± 0.7	10.3 ± 0.7	15.6 ± 0.3
Untreated Control	19 ± 0.6	28.3 ± 0.3	37.3 ± 0.3	25.6 ± 0.3	35.0 ± 0.0	40.3 ± 0.3

LSD at $p<0.05$ is 1.05 LSD at $p<0.05$ is 1.39
LSD at $p<0.01$ is 1.42 LSD at $p<0.01$ is 1.89

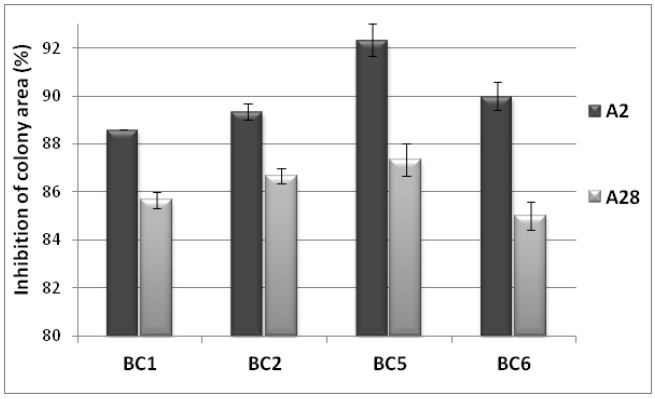


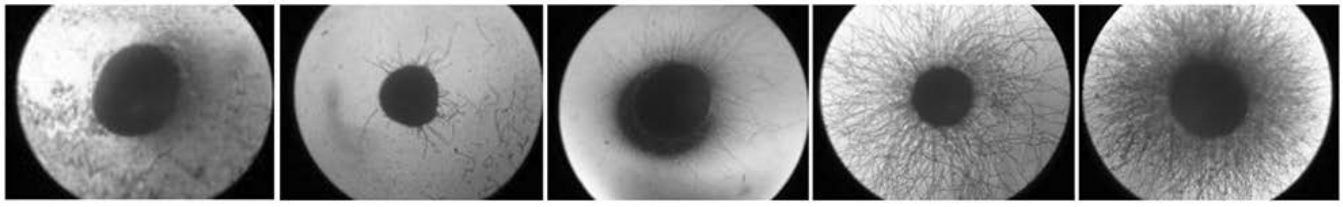
Fig. 2. Inhibition of colony area of aflatoxigenic *Aspergillus flavus* A2 and A28 by *Bacillus* species. Inhibition of sclerotia germination and growth

filtrate (ccf) of all bacteria was also able to minimise the sclerotia germination where moderate germination/growth (3-average mode) was observed. However, in case of BC1 and BC6 the scale range went up to 4 i.e. good growth in some observations. In comparison to bacterial culture broth grown for six hours which completely inhibited sclerotia growth, poor germination/growth (2-average mode) was observed in sclerotia kept in 24 hours old culture broth. The range of growth scale also ranged from 2 (poor growth) and increased to 3 i.e., moderate growth. Contrary to bacterial cell suspension the ccf of 24-hour old culture showed lower inhibition where moderate growth was observed (3-average mode). The ccf (24hours) of *B. vallismortis* (BC5) showed relatively better inhibition as the maximum growth scale was 3 whereas the ccf for other bacteria tested was increased up to 4 (Table 3). From the above observations it is clear that six hours old bacterial culture broth was the most effective to control sclerotia germination/growth than 24 hours old.

Antagonism is the relation between two species of opposite organisms, in which one affects the life of the other, inhibiting partially or totally its growth or even killing it. The mechanisms of antagonism observed in nature are competition, antibiosis, predation and hyperparasitism. Antibiosis is the inhibitory effect of one microorganism against the physiological processes of another (Luna-Romero *et al*., 2007). Bacteria belonging to *Bacillus* sp. are considered to be good agents of antibiosis and their efficiency to inhibit many crop pathogens including aflatoxigenic *A. flavus* have been well documented. Crude extract of *B. amyloliquefaciens* could inhibit *A. flavus* with more than 10mm inhibition zone (Das *et al*., 2013). An antagonist *B. subtilis* BCC 6327 was shown to inhibit the growth and spore germination of aflatoxigenic fungus. The cell free supernatant from 12, 24 and 36h of incubation could inhibit the growth and mycelium production with inhibition percentages of 92.1, 89.6 and 90.1%, respectively (Thakaew and Niamsup, 2013). Hai

Table 3. Effect of antagonistic cowshed bacteria on germination and growth of *Aspergillus flavus* sclerotia

Sl. No.	Test material	Treatments (antagonistic bacteria/control)	*In 1-5 scale evaluation of germination / growth of sclerotia (average- mode)	*In 1-5 scale range	Remarks
1.	Culture Broth (6 hrs old)	BC1	1	1-2	Sclerotia did not germinate in ≥90% cases but in about 5% sclerotia poor germination observed
2.		BC2	1	1-2	
3.		BC5	1	1-2	
4.		BC6	1	1-2	
5.	Culturefiltrate of (6 hrs old)	BC1	3	1-4	Moderate growth
6.		BC2	3	3	Moderate growth
7.		BC5	3	3	Moderate growth
8.		BC6	3	2-4	Moderate growth
9.	Culture Broth (24 hrs old)	BC1	2	2-3	In ≤90% poor germination
10.		BC2	2	2-3	In≤90% poor germination
11.		BC5	2	2-3	In≤90% poor germination
12.		BC6	2	2-3	In ≤90% poor germination
13.	Culturefiltrate of (24 hrs old)	BC1	3	2-4	In ≤80% moderate growth
14.		BC2	3	2-4	In≤80% moderate growth
15.		BC5	3	3	Moderate growth
16.		BC6	3	3-4	In ≤70% moderate growth
17.	LB Broth	Control	5	5	Very good germination and growth



1-No growth 2-Poor 3-Moderate 4-Good 5-Very good

* In 1-5 scale, evaluation of germination/ growth of *A. flavus* sclerotia

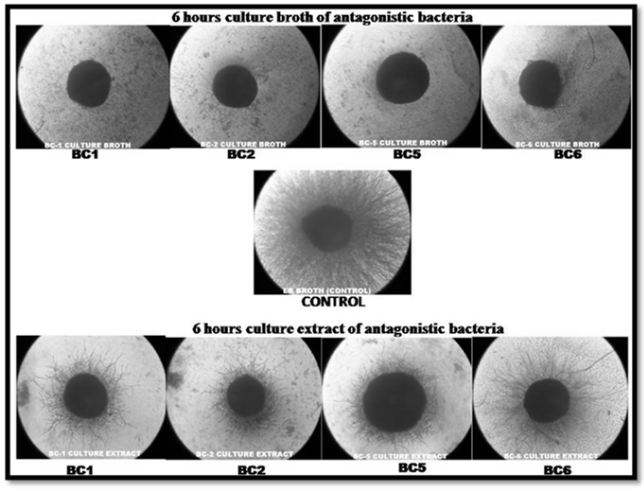


Fig. 3. Inhibition of sclerotia germination of *Aspergillus flavus* A28 by six hours culture broth of antagonistic bacteria

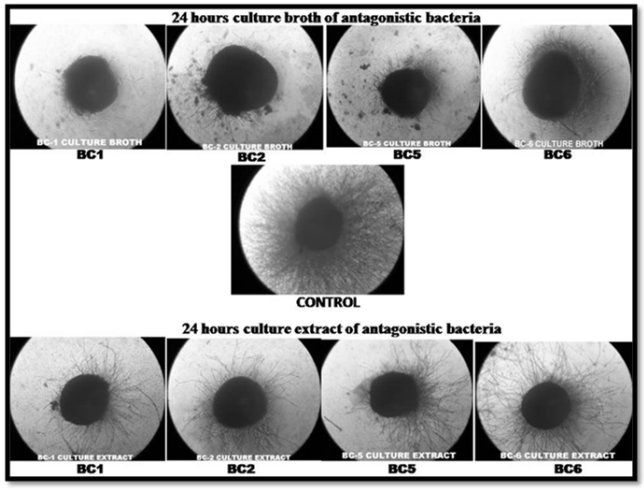


Fig. 4. Inhibition of sclerotia germination of *Aspergillus flavus* A28 by twenty four hours culture broth of *Bacillus* species

(2006) reported that *B. subtilis* metabolites inhibited both spore germination and hypha elongation, causing the decrease of fungal development. *Bacillus subtilis* also exhibited more antagonistic effect than *Pseudomonas* to seed-borne mycoflora *A. flavus* (Bhushan *et al*., 2013). Palumbo *et al*. (2007) selected *Bacillus* strains for the quantitative antifungal assays which significantly inhibited *A. flavus* in different media. Ranjbariyan *et al*. (2011) tested strains of *B. subtilis*, *B. amyloliquefaciens* and *B. vallismortis* and observed 49%, 60.1% and 60% inhibition, respectively to *A. flavus*.

Lim *et al.* (2008) found bacterial culture broth and Cell-free Cultural Filtrate (CCF) to be both effective and ineffective against mycelia and undiluted culture broth was more useful. Bacterial cell suspension effectively inhibited aflatoxin biosynthesis as reported by Kong *et al.* (2010). He observed that when the incubation time of *B. megaterium* was 60-h, the rate of decay declined to 41.67%+/-2.89%. In a study by Moshafi *et al.* (2011) CCF of soil *Bacillus* sp. inhibited *Aspergillus*. In present study, the four Bacteria of *Bacillus* species *viz. B. amyloliquefaciens* (BC1)*, B. amyloliquefaciens* (BC2), *B. vallismortis* (BC5) and *B. subtilis* (BC6) which were isolated from cowshed indoor air, were found to be effective in controlling the mycelial growth or hyphal elongation as well as sclerotia germination or growth under *in vitro*. In earlier reports *Bacillus* sp. bacteria isolated from cow dung could control crop pathogens. *Bacillus cereus* and *Bacillus subtilis* isolated from cow dung could reduce the growth of *Sclerotium rolfsii*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Helminthosporium maydis* and *Rhizoctonia solani* with inhibitory zones of up to 58% (Teo and Teoh, 2011). The *B. subtilis* strains from cowdung inhibited the growth of fungi *in vitro*, *F. oxysporum* (25–34%) and *Botryodiplodia theobromae* (100%) (Swain *et al*., 2008; Swain and Ray, 2009).

Cow dung micro flora contains abundant number of Bacilli. There are several evidences to show that fresh cow dung and cow urine are antifungal and antiseptic in nature which might be due to secretion of antimicrobial metabolites by cow dung micro flora (Sharma and Singh, 2015). Hence the indoor air of cowshed may constitute a sphere of antagonistic bacteria. Cow dung is traditionally used as organic fertilizer in Indian sub-continental farming for centuries. People in Indian villages regularly coat their huts with cow dung slurry and usually cowshed remains at close proximity to their living area. It is a general observation that those people rarely face any fungal allergic reactions like sick building syndrome. The presence of these antagonistic

bacteria may be involved in suppression of the fungi. However this needs more comprehensive study.No research has been carried out on *Bacillus* species from cowshed environment to evaluate antagonistic activity against aflatoxigenic and sclerotia producing *A. flavus*. Sclerotia form an important structure of *A. flavus* aiding in its survival under unfavourable conditions as well as aflatoxin production. Sclerotia present in infected food materials and agricultural commodities may cross geographical boundaries during transportation and thereby may spread infection as well as aflatoxin contamination. However, no report was found regarding the inhibition of sclerotial germination or growth of this important aflatoxigenic fungus. In the current study, active cell suspension (six hours old culture broth) of all *Bacillus* bacteria completely inhibited the sclerotia germination. Bacterial ccf and older cell suspension (24 hours culture broth) was also found to be effective in inhibiting germ tube formation though the efficiency was lower than that of active cell suspension.These bacteria might have inhibited fungi by producing antifungal enzymes and proteins which might degrade fungal cell wall or toxins which interfered with fungal metabolism. Those compounds might be heat labile or stable. Further study may confirm about the physical and chemical property of antifungal compounds produced by these useful bacteria.

CONCLUSION

Presence of aflatoxins in many agricultural products is a serious concern. The causal organism *Aspergillus flavus* produce sclerotia to perpetuate during unfavorable conditions. Cowshed bacteria belonging to *Bacillus* species were found to be very effective against aflatoxigenic and sclerotic isolates of *A. flavus*. Fresh bacterial cultures were more efficient to control the germination and growth of sclerotia.

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