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Allelopathic effects of native *Bacillus* sp. against *Fusarium oxysporum* causing chickpea wilt

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ABSTRACT

Twenty-eight potential antagonistic bacteria were isolated from the chickpea (*Cicer arietenum* L.) rhizosphere soil and tested for their antifungal effects against *F. oxysporum.* The antagonistic rhizobacteria were identified as *Bacillus* sp. The allelochemicals involved in antagonistic behaviour of *Bacillus* isolates included β- 1,4 glucanase, chitinase, siderophore, hydrogen cyanide, ammonia and other biocidal and thermo-stable non-volatile antifungal metabolites, which significantly inhibited the growth of the test fungus. The allelochemical/(s) delayed, inhibited the spore germination and a reduced the germ tube length. Scanning electron microscopy of the interaction between *Bacillus* sp. and test fungus revealed intumescent hyphae with irregular cell surface morphology and sparse fungal growth in response to bacterial allelochemicals.

Key words: Allelochemicals, antagonistic rhizobacteria, antifungal metabolites, *Bacillus* sp., biocidal volatiles, chickpea wilt, *Fusarium oxysporum*, lytic enzymes, spore germination, rhizobacteria, rhizosphere soil.

INTRODUCTION

Root pathogenic microorganisms severely affect the plant health and decrease the crop yield worldwide. The modern intensive agriculture is fully dependent on agrochemicals to ensure reliable crop production, however, the non-judicious use of chemicals has led to deleterious effects on the ecosystem *viz*., development of resistance in pests to applied chemicals and the adverse effects on beneficial micro and macro-flora (9). The use of micro-organisms due to their multifunctional traits, rapid growth, easy handling and aggressive colonization of rhizosphere, has made them potential candidates for biocontrol of phytopathogens (28). Non-pathogenic soil *Bacillus* species are promising agents for disease management due to their abundance and persistence in soil owing to the formation of resistant endospores and their anti-metabolites (11). For easy handling as bacterial suspensions, these can be converted to powder formulations without bacterial mortality (14). There are several methods to suppress the disease, among these allelochemicals needs to be explored for integrated disease management. Allelopathy which involves release of bioactive metabolites known as "allelochemicals", can resolve health defects, soil and environmental pollution etc. (21). Rhizobacteria are known to produce wide array of secondary metabolites that may act as allelochemicals (13).

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Chickpea is the third most important legume cultivated globally. Several factors influence its yields, among which pathogenic microorganisms and insect attacks are the most serious. *F. oxysporum* f. sp. *ciceris* (Padwick) (Foc), a soil-borne fungus, causing fusarium wilt is most destructive pathogen of chickpea (6). It is controlled by use of fungicides, however, the rising public concern about the harmful environmental effects of fungicides has necessitated the development of alternate disease control strategies. Considering the potential of naturally occurring micro-organisms as antagonists of *F. oxysporum*, this investigation aimed to isolate the rhizobacterial antagonists and elucidate their mechanisms of inhibition.

MATERIALS AND METHODS

The soil samples were collected from the chickpea fields of 9-states in Chhattisgarh (Raipur, Dhamtari), Himachal Pardesh (Solan, Mandi), Mumbai, Punjab (12 sites: PAU Ludhiana, Bhatinda, Mavikalan, Hoshiarpur, Gurdaspur, Kartarpur, Ropar, Faridkot, Dhanera, Balbira, Moga, Talwandi), U.P. (Pantnagar) and West Bengal (Asansol).

I. ISOLATION OF RHIZOBACTERIA: Soil samples from chickpea (early reproductive stage) rhizosphere were collected from the chickpea fields in Punjab, UP, Chhattisgarh, Himachal Pradesh, Mumbai and West Bengal. The rhizosphere samples were collected carefully using shovel, and the soil loosely attached to the root was collected as rhizosphere soil. The samples were then placed into prelabelled translucent ziplock bags and maintained at ambient temperature. This collected soil was serially diluted in sterile water and spread on nutrient agar (Beef extract: 3g/l, peptone: 5g/l, NaCl: 5g/l, Agar: 20g/l, pH: 7.0) in petri plates (9 cm dia). The plates were incubated at 30°C for 24 h. The isolated colonies were selected and purified by sub-culturing and preserved on Nutrient agar slants at 4 to 5°C.

Dual plate assay: A 5-mm piece of test fungus, *F. oxysporum* (procured from Department of Plant Breeding and Genetics, of our University) from a 7 day old culture was placed at the centre of a petri plate containing PDA (Potato infusion: 250g/l, dextrose: 10g/l, Agar: 20g/l, pH: 6.5) and nutrient agar in 2:1 ratio. The rhizobacterial isolates were inoculated at opposite sides and plates were incubated for 96 h, at 28ºC. A control plate with fungus alone was used as control. The growth of pathogen was measured in terms of diameter and percent inhibition was calculated as under:

$$\% \text{ Inhibition} = (R - r)/R \times 100$$

Where, r: Radial growth of fungus in dual plate and R: Radial growth of fungus in control plate.

II. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

The bacterial isolates were characterized based on colony morphology and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology (24).

Molecular characterization as *Bacillus* sp.

(i). DNA extraction: Total genomic DNA was isolated from the bacterial isolates by chloroform-isoamyl alcohol extraction. Bacteria were cultured in 5ml Nutrient broth for 24-48 h at 28ºC and cells were harvested by centrifugation (10 min at 10,000 rpm). Pellet was suspended in 650 µl of extraction buffer (consisting of 250 mM NaCl, 100 mM Tris HCl, 100 mM EDTA, 10% SDS and 10% PVP; pH 8.0) and incubated at 65ºC for 30 min followed by addition of 100 µl of potassium acetate buffer (pH 4.5). Genomic DNA was extracted twice with approximately equal volume (0.7-0.8 ml/700-800 µl) of chloroform isoamyl alcohol (24:1). Nine hundred µl of isopropanol was added to precipitate the nucleic acids, centrifuged at 10,000 rpm for 10 min and supernatant was carefully discarded by aspirating the isopropanol. Pellet was washed by adding 0.5 ml ice cold 70% ethanol, air-dried and dissolved in 100 µl of sterile distilled water. Biophotometer was used to quantify the extracted DNA.

(ii). PCR amplification: Polymerase chain reaction (PCR) was carried out to amplify 16S rRNA using *Bacillus* genus specific primers. Reaction mixture consisted of Taq DNA polymerase 2 units; Mg^{++} of 1.5 mM; 10X buffer; 0.2 mM each of the four dNTPs and 30 ng template DNA, 25 pmol each of the primers. Thirty-five amplification cycles were performed in an automated DNA thermocycler with the following parameters: denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, elongation at 72°C for 1 min and final extension for10 min. Primer sequences were as under:

Oligo 1 Forward sequence - 5'-AGAGTTTGATCC TGGCTCAG-3'

Oligo 2 Reverse sequence - 5'-TACGGCTACCTT GTTACGACTT-3'

Five microliters of PCR amplifications was run on 1.2% (wt/vol) agarose gel electrophoresis in Tris Borate EDTA buffer (pH 8.3) (1.5 h at 40 V), stained with ethidium bromide, and photographed on a UV transilluminator.

III. EFFECTS ON FUNGAL GROWTH

(a) Non-volatile allelochemicals

(i). Dual culture method: The mycelial growth inhibition of fungus was determined in broth based dual culture technique. One ml of 24 h old bacterial culture and a disc of test fungus (5 mm) from a well-grown fungal colony on PDA plates was inoculated into 50 ml broth of potato dextrose media in 250 ml conical flasks at 25°C. Broth inoculated only with fungus served as control. The differences in dry weights between the fungus and the bacterium or the control cultures were recorded by passing 5 days grown dual cultures through pre-weighed filter paper (Whatmann No.1). The filter papers were dried for 24 h at 70°C and weighed. The percent reduction in weight of test fungus was calculated as under:

$$\% \text{ reduction in weight} = (w1-w2)/ w1 \times 100$$

Where, w1 : Weight of test fungus in control flasks and w2 : Weight with the bacterial antagonists.

(ii). Culture filtrate antibiosis*:* Antagonistic rhizobacteria were cultured in 100 ml of nutrient broth, incubated at 28° C at 200 rpm for 3 days and culture was centrifuged at 10000 rpm for 20 min. The cell free filtrate was filter sterilized using 0.2 micrometer filter. These supernatants were supplemented in PDA medium (@ 50%). A piece of actively growing *F. oxysporum* was placed at the centre of each plate and incubated at 28° C for 5 d. The same volume of sterile distilled water in PDA medium served as control. Plates were incubated at 22˚C for 72 h and the growth of pathogen was measured.

(iii) Thermostable allelochemicals*:* The autoclaved culture filtrates (50%) of rhizobacteria were mixed with sterile potato dextrose agar, inoculated with the test fungus and the plates were incubated for 5 d at 28ºC. The radial growth of the fungus was measured and compared to the control.

(iv) Diffusible allelochemicals*:* The effect of diffusible allelochemicals produced by rhizobacteria in liquid medium on fungal growth was assayed by method of Montealegre *et al* (19). PDA plates covered with a cellophane membrane (pore size 33 mm) were overlaid with nutrient agar and inoculated with 100 µl culture filtrate. After incubation for 48 h at 25˚C, the membrane along with the bacterial growth was removed and a 10 mm disc of a pure culture of *F. oxysporum* was placed at the centre of the plate and the growth of the pathogen was measured after 72 h.

(iv) Ethyl acetate extracts of non-volatile allelochemicals*:* The antifungal compounds from the cell free filtrate of *Bacillus* sp., grown for 72 h in nutrient broth, were extracted using ethyl acetate as solvent in 1:1 ratio**.** Ethyl acetate was allowed to evaporate at 40˚C in a vacuum evaporator and the concentrated filtrate was dissolved in 1ml of 50% methanol and tested for growth inhibition on *F. oxysporum* in agar wells made in PDA medium. 50% methanol alone served as control (11).

(b) Volatile allelochemicals: The release of volatile antifungal compounds by the rhizobacteria was determined using the sealed plate method of Fiddman and Rossal (8). From 72 h old cultures, 200 µl was plated on nutrient agar and after incubation at 37**°**C for 24 h, a second petri dish containing PDA inoculated with a 6-mm plug of the test fungus was placed over the bacterial culture. The two plates were sealed with parafilm and incubated at 25**°**C. As a control, a petri plate containing only agar medium without bacteria was placed over the PDA medium inoculated with the fungal pathogen. The radial growth of test fungus was measured at 24 h intervals for a period of 5 days.

(c) Effects of allelochemicals on spore germination*:* Forty µl of the spore suspension of pathogen (1×10^5) was suspended in 40 µl of sterilized culture fluid antagonistic bacteria on a sterile glass slide placed in sterile petri dish lined with wet filter paper to maintain moisture and were incubated at 28 ºC. Slides were examined using light microscope at 6, 8 and 12 h to observe the pattern of spore germination.

IV. Scanning electron microscopy (SEM) of the interaction between *Bacillus* sp. and test fungus*:* The antagonistic bacteria and pathogen were grown under dual plate assay and a portion of agar (8 mm dia) from the zone of inhibition containing some portion of hyphae of the pathogen was taken for SEM examination. The samples were immersed in

2.5% glutaraldehyde and kept at 4° C for 24 h, rinsed thrice with 0.1 M caco buffer. Then, 1% osmium tetraoxide was added and dehydrated by passage through graded aqueous ethyl alcohol series (30, 50, 70, 90 and 95%), placed in 100% ethanol at room temperature for few minutes. It was then dried with a critical point dryer unit mounted on aluminum stubs with silver glue and coated with gold-palladium using anion sputtering unit. The samples were then examined under scanning electron microscope, SEM unit at EMN lab, Punjab Agricultural University, Ludhiana.

V. Detection of allelochemicals

(i). β- 1,4 glucanase: For its detection, plates containing minimum salts medium **(**Na_2HPO_4 **:** 33.9g/l, KH_2PO_4 : 15g/l, NaCl: 2.5g/l, NH_4Cl: 5g/l, pH: 7.0) with (1 % w/v) carboxy methyl cellulose (CMC) were spot inoculated with the *Bacillus* sp. isolates at the centre. After incubating for 48 h at 30 ºC, the agar medium was flooded with an aqueous solution of Congo red for 15 min, washed and visualized for zones of hydrolysis (18) for detecting β- 1,4 glucanase.

(ii). Chitinase: For its detection, the rhizobacteria were inoculated on plates with minimum salts medium containing (1 % w/v) colloidal chitin and incubated for 48 h at 30ºC and formation of clearance zones of hydrolysis indicated chitinase production.

(iii). Protease: For its detection, the rhizobacteria were inoculated on plates with minimum salts medium containing (1 % w/v) casein for 48 h at 30 ºC. Plates were examined for zones of hydrolysis suggestive of protease production.

(iv). Siderophore: It was detected on agar plates containing the chrome azurol S (22). Formation of Orange halos around the colonies on blue medium indicated siderophore production. Catechol-type siderophores production by rhizobacterial antagonists were assayed using the method of Arnow (1).

(v). Salicylic acid (SA): Its production by the rhizobacteria was examined by growing the bacterial strains in standard succinate medium **(**K_2HPO_4: 6.0 g/l, KH_2PO_4: 3.0 g/l, $MgSO_4$: 0.2 g/l, $NH_4SO_4$1.0 g/l, Succinic acid: 4.0 g/l, pH: 7.0**)** at 28 ˚C for 48 h and the cultures centrifuged at 8000 rpm for 5 min. Four ml cell free culture filtrate was acidified with 1 N HCl to pH 2.0 and SA was extracted twice with equal volume of $CHCl_3$. Four ml of water and 5 µl of 2M $FeCl_2$ were added to the pooled $CHCl_3$ phases. The absorbance of the purple iron- SA complex in the aqueous phase was read at 527 nm. Sodium salicylate was used as reference standard (17).

(vi). HCN: Its production was detected by incubating the petri plates containing 10% Trypticase soya agar (TSA) supplemented with 0.44 % of glycine, inoculated with the bacteria and covered with the lid containing filter paper wetted with 0.5% picric acid in 2 % sodium carbonate (3). The plates were incubated at 28 °C for 3 to 5 d. Change in colour of the filter paper from yellow to orange-brown indicated cyanide production.

(vii). Ammonia: For its detection, overnight grown cultures were inoculated in 10 ml peptone water and incubated for 48-72 h at 30 °C. Nessler's reagent (0.5 ml) was added in each test tube and the development of brown to yellow color was indicative of ammonia production (4).

RESULTS AND DISCUSSION

The rich microbial diversity in plant rhizosphere possesses a resource which could replace the synthetic agrochemicals to control phytopathogens. Because of their sporulating ability, members of the genus *Bacillus* survive under extremes of environment and can be exploited as biocontrol agents. Several *Bacillus* species are known to be very efficient producers of antimetabolites/allelochemicals and are used in plant disease control (21).

A total of 98 rhizobacterial isolates were isolated from thirty-six chickpea rhizospheric soil samples and were subjected to antagonism tests against *F. oxysporum;* of these, 28 isolates were found to inhibit *F. oxysporum* (Figure 1) and among these 16 were strong inhibitors (Figure 2). These were further examined for morphological, biochemical, molecular characters and identified as *Bacillus* sp**.**

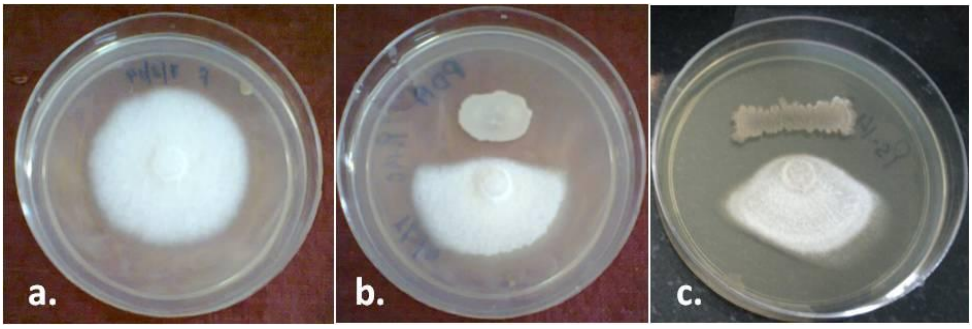

Figure 1. Antagonistic activity of *Bacillus* sp. isolates against *F. oxysporum*

a. Control
b. Isolate B-I
c. Isolate B20d

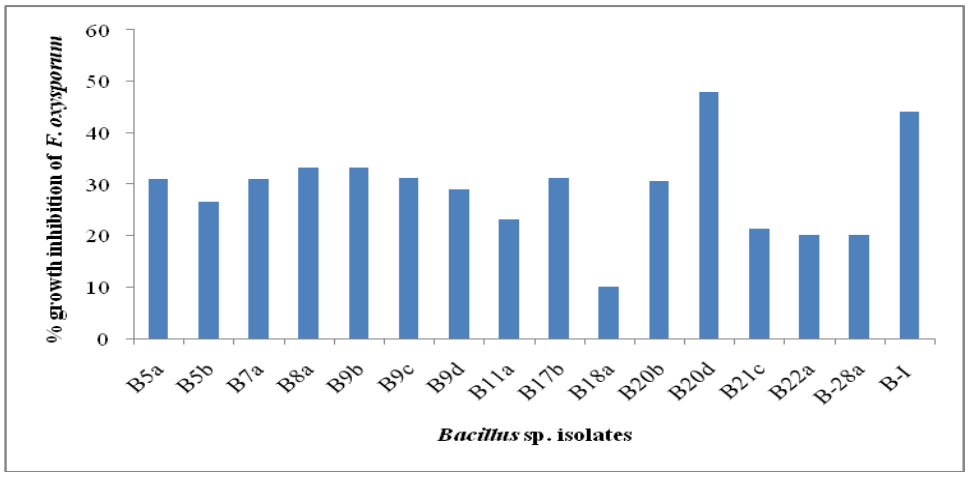

Figure 2. Inhibitory effect of *Bacillus* sp. isolates on *F. oxysporum*

Strain identification

All the isolates were Gram positive, sporulating rods and positive for catalase, nitrate reduction, Voges Proskauer, starch hydrolysis and negative for methyl red. However, molecular characterization using *Bacillus* genus specific primers showed amplification only in 12 out of 16 isolates and yielded a product of about 1.5 Kb (Figure 3) confirming their identity as *Bacillus* sp. *Bacillus* sp. are found to be promising agents for disease management due to their abundance and persistence in soil (11).

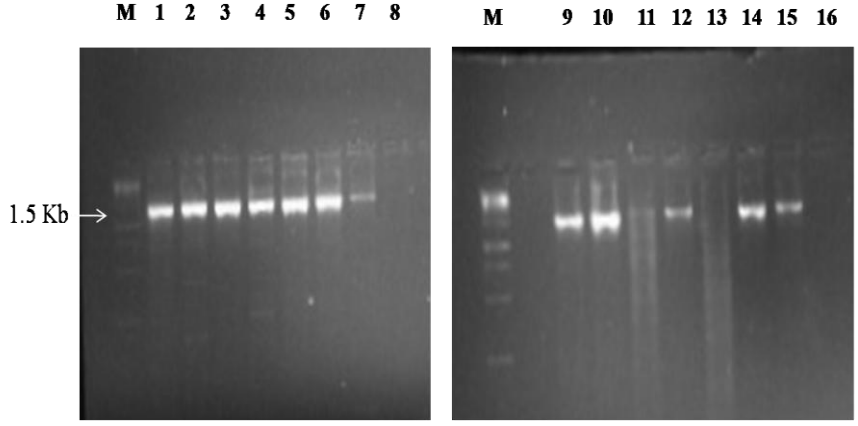

Figure 3. Gel electrophoretic analysis of PCR products amplified using *Bacillus* genus specific primers
M: 1 Kb DNA ladder, 1. B-5b; 2. B-9d; 3.B-20d; 4.B-17b; 5.B-I; 6.B-7a; 7.B-21c; 8.B-20b; 9.B-9b; 10.B-18a; 11.B-28a; 12.B-5a; 13.B-22a; 14.B-9c; 15.B-8a; 16.B-11a

Bacilli are reported to produce a number of volatile, non-volatile and thermostable metabolites which are part of their inhibitory mechanism against the pathogens (11). Some of these metabolites are produced in presence or absence of pathogen, while others may be produced in varying amount depending on medium used (liquid or solid). The allelopathic effect of the isolated *Bacillus* sp. was assessed with regards to specific conditions governing the differential production of antifungal compounds from selected bacteria.

II EFFECTS OF NON-VOLATILE ALLELOCHEMICALS

(i). *Dual culture method:* In confrontation assays in liquid media, the fungal proliferation was inhibited by *Bacillus* isolates as indicated by decrease in dry weights of the fungal cultures grown with antagonists than control and inhibition in fungal dry weight ranged from 11.6-62.97% (Table 1), with isolate B20d being the best followed by B5b, B-20b and B-I. *Bacillus* sp. have been reported for their inhibitory activity against plant pathogens and allelopathy/antibiosis is probably the mechanism that limits pathogen invasion in host plant tissues. Ashwini and Shrividya (2) reported complete inhibition of *Colletotrichum gleosporioides* OGC1 when co-inoculated with *Bacillus* isolate (BC2) under broth based dual culture technique than test fungus alone, suggesting that metabolites produced by bacteria were inhibitory to pathogens**.**

(ii). *Culture filtrate antibiosis:* Three days old culture filtrates of all test isolates inhibited the growth of *F. oxysporum* (13.6-61.7%), with maximum inhibition by B-17b followed by B28a, B-I, B22a and B20d (Table 1). The production of certain antifungal compounds in the absence of pathogen is well known (15). Mishra *et al*. (17) reported that four days old culture filtrate of *Bacillus subtilis* MA-2 completely inhibited the growth of *Alternaria alternata* and *Curvularia andropogonis* at 10%, while *F. moniliformae* and *Colletotrichum acutatum* were comparatively less sensitive.

Table 1. Inhibitory effects of *Bacillus* sp. on growth of *F oxysporum*

Isolates	*F. oxysporum* growth inhibition (%)					
	Dual culture technique	Culture filtrate antibiosis	Autoclaved Culture filtrate	Diffusible allelo-chemicals	Ethyl acetate extracts	Biocidal volatiles
B5a	11.6 ± 0.34	29.4 ± 0.57	20.0 ± 0.28	-	33.6 ± 0.65	14.0 ± 0.57
B5b	60.1 ±1.79	38.2 ± 0.69	13.6 ± 0.34	-	-	-
B7a	43.7 ± 1.15	13.6 ± 0.34	-	-	-	24.5 ± 0.86
B8a	24.2 ± 0.57	26.4 ± 0.51	14.5 ± 0.23	13.3 ±0.17	-	20.1 ± 0.57
B9b	19.6 ± 0.34	35.3 ± 0.17	13.6 ± 0.34	10.0 ± 0.40	-	13.3 ± 0.75
B9c	20.1 ± 0.63	26.4 ± 0.46	16.0 ± 0.51	10.0 ± 0.23	26.9 ± 0.57	-
B9d	17.4 ± 0.23	32.3 ± 0.28	-	16.6 ± 0.65	30.4 ± 0.28	-
B11a	-	35.3 ± 0.26	36.6 ± 0.46	-	-	10.4 ± 0.23
B17b	25.3 ± 0.75	61.7 ± 0.40	-	46.6 ± 0.65	30.4 ± 0.23	8.0 ± 0.51
B18a	17.6 ±0.34	32.5 ± 0.28	26.2 ± 0.40	-	-	14.4 ± 0.34
B20b	53.9 ± 0.49	35.3 ± 0.57	25.7 ± 0.17	16.6 ± 0.23	-	-
B20d	62.9 ± 0.51	41.3 ± 0.83	42.8 ± 0.76	-	-	6.4 ± 0.23
B21c	20.1 ± 0.63	17.6 ± 0.34	-	-	-	-
B22a	-	44.1 ± 0.23	-	10.0 ± 0.57	-	9.6 ± 0.34
B-28a	48.9 ± 0.51	50.0 ± 0.57	29.1 ± 0.63	10.0 ± 0.17	40.8 ± 0.46	5.6 ± 0.23
B-I	52.4 ± 0.23	45.4 ± 0.23	29.4 ± 0.23	16.6 ± 0.41	39.1 ± 0.63	23.1 ± 0.34

Values represent mean of three replicates

(iii). *Thermostable anti-metabolites:* The antifungal potential of the autoclaved culture filtrates was markedly reduced, as indicated by the % growth inhibition of the test fungus **(**Table 1). This suggests that some of the metabolites produced are heat labile. It is possible that the active metabolites such as hydrolytic enzymes produced may have got denatured or detoxified, however, 68.7% of *Bacillus* isolates still inhibited the

growth of *F. oxysporum* in the range of 13.6-45.4% due to presence of thermostable antimetabolites. Fungal growth inhibition by isolate B20d by both membrane-sterilized and autoclaved culture filtrate was at par, suggesting the production of thermostable metabolites.

(iv). ***Production of diffusible anti-metabolites:*** Disease suppression in plants is attributed to production of diffusible non-volatile allelochemicals produced in low concentrations by bacteria (12). In the present study, growth inhibition due to diffusible antifungal metabolites varied between 10-46.6%. Maximum inhibition was observed with B17b (46.6%) after 5 days of incubation (Table 1) followed by B-20b and B-I. Although isolate B17b showed only 25.3% fungal biomass inhibition, it exhibited maximum fungal growth inhibition with culture filtrate antibiosis and diffusible metabolites. Martinez *et al*. (15) in their studies against *H. solani* have reported similar observations. The present study suggests that production of inhibitory substances may also be influenced by the type of medium used. Séveno *et al*. (23) reported that production of the antibiotic phenazine, by *Pseudomonas aureofaciens* was six times higher in solid culture than with liquid culture. However, this is in contrast to our finding that higher fungal growth inhibition by isolate B-17b was recorded due to culture filtrate antibiosis than diffusible metabolites.

(v). ***Effects of non-volatile allelochemical extracts:*** Ethyl acetate fractions of the culture filtrate of *Bacillus* sp. showed inhibition zones ranging from 25.5-40.8% (Table 1), suggesting the role of bioactive metabolites in antagonism. Inhibitory effects of metabolites of native *Pseudomonas* and *Bacillus* isolates against growth of fungal plant pathogens had been well documented (11).

II. EFFECTS OF VOLATILE ALLELOCHEMICALS

Production of volatile antifungal compounds by rhizobacteria is reported to alter the physiological activities of pathogenic fungi, inhibit sclerotial activity and limit ascospore production thereby managing disease incidence (7). A reduction in the radial growth of the test fungus *F. oxysporum* under sealed plate technique was used after 120 h of incubation due to volatile antifungal compounds and it varied between 6.4-24.5% (Table 1). Trivedi and Pandey (27) also reported that volatile antifungal compounds produced by *Pseudomonas corrugata* inhibited the growth of *A. alterneta* and *F. oxysporum* in sealed petri dishes. Fernando *et al.* (7) demonstrated the production of volatiles by bacteria in the soil and their capacity to prevent sclerotial germination. This would help in the management of overwintering structures (chlamydospores and sclerotia) in soil which act as primary inoculum production and establishment of the disease in the crop.

III. EFFECTS OF AELLELOCHEMICALS ON SPORE GERMINATION

Although cell free culture filtrate of all the antagonistic *Bacilli* inhibited mycelial proliferation of test fungi, fungistatic effect against spores of *F. oxysporum* was observed with 10 isolates which significantly inhibited conidial germination in the range of 20.0-95.7% with maximum by B20b followed by B20d (Table 2). This suggests that metabolites other than those responsible for mycelial growth inhibition could be implicated in conidial germination inhibition**.** Germ tube length was also affected as

evident in Figure 4. In a similar report by Mishra *et al*. (17), 3 days old culture filtrate of *Bacillus subtilis* at 60% concentration inhibited the spore germination of *Alternaria alternata*, *Curvularia andropogonis*, *Fusarium moniliforme* and *Colletotrichum acutatum* by 72.5%, 82%, 67.6% and 80.7% respectively over control.

Table 2. Effects of allelochemicals produced by *Bacillus* sp. isolates on spore germination

Isolates	Effects on spore germination of *F. oxysporum*	
	Spore germination inhibition (%)	Germ tube length
B5a	-	-
B5b	28.5 ± 0.28	+
B7a	60.0 ± 1.88	+
B8a	20.0 ± 1.15	+
B9b	-	-
B9c	20.0 ± 1.73	-
B9d	-	-
B11a	30.0 ± 0.57	+
B17b	-	-
B18a	-	-
B20b	95.7 ± 0.40	+
B20d	92.5 ± 0.28	+
B21c	60.0 ± 1.73	+
B22a	-	+
B-28a	55.0 ± 1.15	+
B-I	37.9 ± 0.51	+
Control	0.0	-

(-) sign indicates no spore germination, (+) effect on germ tube length

Figure 4. Effects of bacterial filtrate metabolites on spore germination of *F. oxysporum*
(a) Control (b) With culture filtrate.

*Arrows show spore germination in control 6 h after incubation

IV. SCANNING ELECTRON MICROSCOPY (SEM) OF INTERACTIONS BETWEEN *BACILLUS* SP. AND TEST FUNGUS

The microscopic examination by SEM revealed sectors of intumescent hyphae, apparent inhibition of fungal growth and hyphal distortion in response to the allelopathic effects of bacteria (Figure 5). This is associated with a decrease in fungal growth and higher levels of non-viable cells due to hyphal transformations and death (11). Intumescent hyphae could also be the result of chitin deposition in the fungal cell wall due to stress caused by the effect of antagonistic bacteria. The extensive accumulation of chitin is part of complex defense mechanism used by fungi to prevent the penetration of pathogens and fungitoxic molecules. Nonetheless, it has been reported that *Bacillus* sp. are able to overcome such barriers and cause severe fungal cell injuries (6).

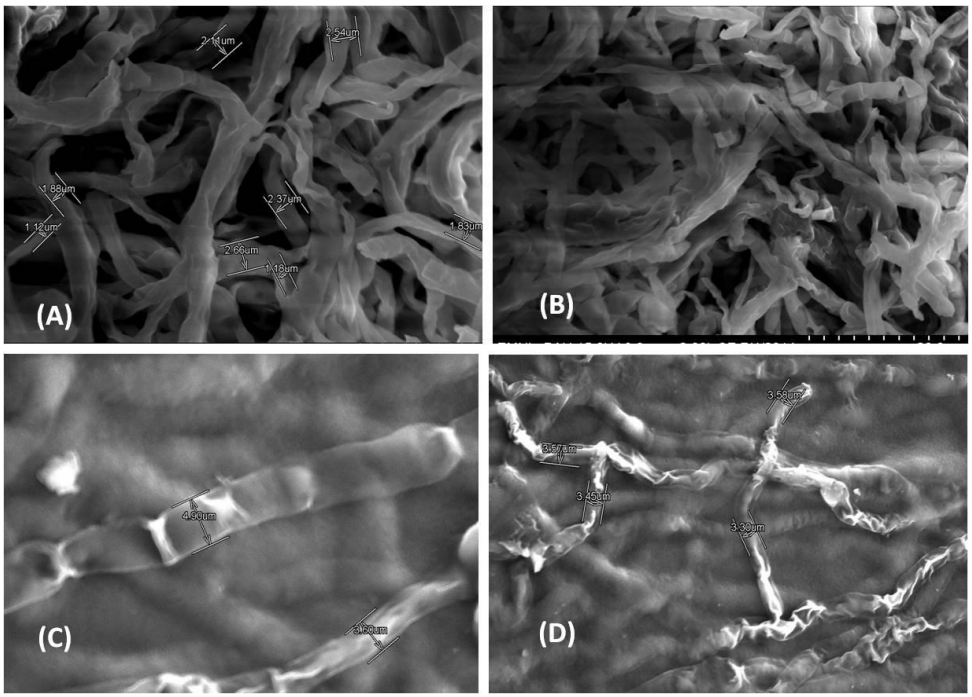

Figure 5. Scanning electron micrographs of interaction between *F. oxysporum* and *Bacillus* sp.
(A) Scale bar-10 µm (B) scale bar-40 µm: *F oxysporum* cells from pure culture; (C) Scale bar-10 µm (D) scale bar-40 µm: Swollen and deformed hyphae with sparse fungal growth as a result of allelopathy

V. DETECTION OF ALLELOCHEMICALS

(i). *Extracellular hydrolytic enzymes*: Several bacteria produce enzymes able to hydrolyze the chitin, proteins, cellulose and hemicellulose, thus contributing to direct

suppression of plant pathogens. There are few examples of biocontrol agents (BCA)'s able to produce enzymes, effective against certain plant pathogens. However, such activities are indicative of the need to obtain carbon nutrition (20). So, microorganisms showing a preference for colonizing and lysing plant pathogens are considered effective biocontrol agents**.**

Nine of the *Bacillus* isolates (including B20d and B-I) produced β,1-4 glucanases, fifteen were positive for protease and three (B5b, B17b and B-I) produced chitinase as indicated by the production of a halo (Table 3). It is known that different types and concentrations of hydrolytic enzymes produced by pseudomonads play an active role in antagonism against different root pathogens such as *Phytophthora capsici* and *Rhizoctonia solani* (10). Isolate B-I, which inhibited the growth of test fungus by 44.0%, produced β, 1-4 glucanase, protease and chitinase signifying the possible role of these lytic enzymes in antagonism. The findings of our study substantiate the antagonistic potential of some chickpea rhizobacteria against *F. oxysporum* due to production of different metabolites.

Table 3. Allelochemicals produced by *Bacillus* sp. isolates

Bacillus Isolates	Salicylic acid (µg/ml)	Siderophore		Lytic enzymes		
		Siderophore index	Catechol-type siderophore (µg/ml)	Chitinase	β 1,4 glucanase	Protease
B5a	28.5 ± 0.28	-	-	-	-	+
B5b	17.9 ± 0.82	-	-	+	+	+
B7a	26.6 ± 0.34	-	-	-	+	+
B8a	50.6 ± 0.34	-	-	-	-	+
B9b	12.8 ± 0.34	1.2 ± 0.05	-	-	+	+
B9c	1.8 ± 0.17	1.3 ± 0.30	15.3 ± 0 .17	-	-	+
B9d	-	-	-	-	-	+
B11a	32.2 ± 0.20	-	-	-	+	+
B17b	1.3 ± 0.11	1.2 ±0.04	42.4 ± 0.23	+	-	+
B18a	1.3 ± 0.17	-	-	-	+	+
B20b	13.2 ± 0.11	1.8 ± 0.05	51.9 ± 0.23	-	+	+
B20d	28.5 ± 0.28	1.9 ± 0.11	75.4 ± 0.23	-	+	-
B21c	15.2 ± 0.28	-	-	-	-	+
B22a	19.5 ± 0.28	-	-	-	-	+
B-28a	65.5 ± 0.34	-	-	-	+	+
B-I	23.0 ± 0.23	2.11 ± 0.06	91.6 ± 0.34	+	+	+

Values represent mean of three replicates

(ii). ***Siderophore and salicylic acid as allelochemicals:*** The production of siderophores (24) or salicylic acid (19) by *P. putida* has been implicated as one of the modes of action of antagonistic activity. The production of siderophores under iron deficiency by the biocontrol agents in quantities sufficient to limit Fe^{3+} availability to the pathogen, may lead to induction of host resistance against the pathogen. Out of the16 isolates, 6

produced siderophore and highest siderophore index was recorded with *Bacillus* isolate B-I (2.1) followed by B20d (1.9) Catechol-type siderophore production was observed with 5 isolates which showed similar trend as on CAS plate, maximum being observed with B-I (91.6 µg/ml) followed by B20d (75.4 µg/ml (Table 3**).** *Bacillus* isolates B20d and B-I, categorized as strong siderophore producers, also produced extracellular hydrolytic activities and inhibited the growth of test pathogen under dual culture technique and culture filtrate antibiosis. This suggests a plethora of mechanisms involved in antagonism, each having synergistic effect against the pathogen.

Salicylic acid production was seen in 15 isolates, with maximum in B28a (65.5 µg/ml) > B8a (50.6 µg/ml) > B20d (28.5µg/ml) (Table 3). Salicylic acid is plant phenolic hormone- simulating endogenous regulator to combat the biotic and abiotic stress (26).

(iii). *Biocidal volatiles as allelochemicals:* Five *Bacillus* sp. isolates produced HCN, with B-I being the strongest producer; 93.4% of the isolates produced ammonia including B20d and B-I. Fernando *et al* (7) reported the antifungal nature of organic volatiles like HCN and inorganic volatiles such as ammonia.

CONCLUSIONS

For an effective antagonistic agent to be used for seed treatment against *F. oxysporum*, selection is of utmost importance. Determining the various mechanisms involved in disease inhibition provide a great awareness for their application and possibly commercializing these bacteria as a part of biocontrol strategy. This study suggests the role of different allelochemicals produced by *Bacillus* isolates as a major component in the control of fusarium wilt of chickpea. Further research to fine tune combinations of different metabolites, plant-microbe-pathogen interaction will ultimately lead to better disease control**.**

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in the publication.

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