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Metabolites in Control of *Rhizoctonia bataticola*

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Agrochemicals used for crop production adversely affect both human and environmental health. The search for bacterial antagonists is a step towards agricultural sustainability. The inhibition of *Rhizoctonia bataticola, causing dry root rot of* chickpea was exhibited by 59 out of 174 rhizobacterial strains. Out of which, 35 strong inhibitors (16 belonging to *Pseudomonas* sp. and 19 representatives of *Bacillus* sp.) were characterized for inhibitory traits. Production of diffusible metabolites by 16 isolates (7 of *Pseudomonas* sp. and 9 of *Bacillus* sp.) reduced the radial proliferation of pathogen suggesting the role of antibiosis in antagonism. Culture filtrate of 28 antagonists @ 50% inhibited growth of test fungus in the range of 11.1-71.25%. Fungal growth inhibition in response to thermo-stable metabolites was observed with 22 rhizobacterial isolates in the range of 11.7-36.6 %. Extracts of antimetabolites in ethyl acetate inhibited the growth of fungus in the range of 28.7-42.5%. Role of volatile antimetabolites in antagonism was evaluated and found positive with 23 antagonistic isolates while all were amylase producers. SEM revealed scanty growth with distorted cell surface morphology of test fungus in response to antagonistic effect.

Key words: Diffusible metabolites, Antibiosis, Amylase.

Chickpea (*Cicer arietinum* L.) is the most important grain legume cultivated globally. In India, it dominates other pulse crops in terms of both area and production. However, the sustainability of chickpea based agriculture in the Indian continent and other countries in Asia is severely threatened by a number of biotic and abiotic factors. Among the several biotic factors, wilt is the major constraint affecting the productivity of chickpea, however recently dry root rot and collar rot are emerging as a major threat to chickpea production[1]. Dry root rot caused by *Rhizoctonia bataticola* Taub (Butler) is becoming severe in most of the chickpea growing regions of India particularly in the central and southern zone because of susceptibility of the crop to drastic climate change[2]. Cultural practices, such as planting date, sowing density and crop rotation proved to be very effective in reducing fungal attacks, but they are insufficient under high disease pressure, especially when weather conditions are particularly conducive to disease development. Among other control measures, such as application of fungicides is exorbitantly expensive, besides, the problems related to environmental pollution, chemical toxicity to humans and animals, fungal resistance and unintended harmful effect on beneficial micro-flora, and is therefore no longer acceptable in modern agriculture which emphasizes the importance of using sustainable technologies for food production.

Realizing the importance of crop and providing focused attention on eco-friendly

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approach to control the pathogen that can be incorporated into integrated disease management strategy of chickpea has popularized the use of naturally occurring biocontrol agents as antagonists. Biological control provides an alternative to the use of synthetic pesticides with the advantages of their perceived level of safety and reduced environmental impact[3]. The determination of their mode of action is of great importance both on scientific grounds and in view of possibly developing one or more of these bacteria as biocontrol agents in the future. The antagonistic properties of biocontrol agents derive from competition for resources, parasitism, systemic induced resistance and antibiosis[4]. Although these mechanisms often work in concert, antibiosis is of great importance in many antagonists as it usually provides the initial mechanism[5], as well as the most rapid means[6], of limiting the growth of a plant pathogen. As such, the present work was framed to screen the soil inhabiting bacterial antagonists and understanding the mechanisms (with special reference to antimetabolites) which they employ to control the dry root rot of chickpea caused by *Rhizoctonia bataticola* under *in vitro* condition.

MATERIALS AND METHODS

Microorganisms Pathogen

Pathogen, *Rhizoctonia bataticola* Taub (Butler) was procured from the Department of Plant Pathology, Punjab Agricultural University. It was maintained on Potato Dextrose Agar slants for further use.

Isolation of rhizobacterial strains

The soil samples were randomly collected from chickpea rhizospheric soil. One gram of soil sample was suspended in 99 ml of sterile water and then serially diluted using sterile water blanks. Pour plating was done on Nutrient agar (NA) for *Bacillus* and on Kings B for *Pseudomonas*, incubated at 30°C for 24 h. The colonies differing in morphology were picked up, sub-cultured and preserved on respective slants at 4 to 5°C.

Screening of rhizobacteria antagonistic to *Rhizoctonia bataticola*

Dual plate assay

Antagonistic activity of the bacterial isolates against *Rhizoctonia bataticola* was evaluated based on dual culture technique. Fifteen ml of PDA was poured into sterile Petri dish. 5mm mycelia plug of *Rhizoctonia bataticola* was inoculated at the centre of plate. Potential antagonistic bacteria were streaked 3 cm apart from the fungal inoculum and plates were incubated at 28p C for 48 h. Radial growth of the test fungus was measured and percentage growth inhibition was calculated.

Assessment of inhibitory traits of rhizobacteria

Production of Non volatile antifungal metabolites

Diffusible antimetabolites production

Production of diffusible antimetabolites by rhizobacteria was assayed by method of Montealegre et al[7]. PDA plates covered with a cellophane membrane were overlaid with nutrient agar and inoculated with 100 µl of the potential bioantagonists. After incubation for 72 hrs at 30ÚC, the membrane along with the bacterial lawn was removed and a fungal bit of pure culture of *R. bataticola* from a well grown lawn of the fungus was placed at the centre of the plate and the growth of the pathogen was measured after 48 hrs.

Culture filtrate antibiosis

For detection of non-volatile antifungal metabolites, selected rhizobacteria were cultured in respective medium incubated at 28° C at 200 rpm for 3 days and culture was centrifuged at 10000 rpm for 20 min. The supernatants were filter sterilized using 0.2 micrometer filter. These supernatants were supplemented in PDA medium (@ 50%). A piece of actively growing *R. bataticola* was placed at the centre of each plate and incubated at 28° C for 2 days. The same volume of sterile distilled water in PDA medium served as control.

Inhibition due to thermostable antimetabolites

Effect of heat stable antifungal compounds on fungal growth was tested by using autoclaved culture filtrate (50%) and supplementation to the sterile potato dextrose agar.Plates were inoculated with test fungus andincubated for 72 h, at 28ºC, and the radial growth of the fungus was measured and compared to that with the control.

Inhibition due to extracted antimetabolites

The antagonism due to antimetabolites produced by bacterial antagonists was evaluated by the method given by Jha and Anjaiah[8]. The antifungal compounds were extracted from the culture supernatants of antagonists grown for 72

hrs in nutrient broth, using ethyl acetate as solvent. The solvent was dried at 40 °C and fractions of these extracts were dissolved in 1ml of 50% methanol and tested for growth inhibition of *R. bataticola* in agar wells made in PDA medium. 50% methanol alone was used as control.

Production of volatile antifungal compounds

The production of volatile antifungal compounds by the isolates was assayed by a sealed plate method as described by Fiddman and Rossal[9]. Bacterial lawn was prepared on Nutrient agar plates. After incubation for 24 h, the lid was replaced by a plate containing an agar block (7mm diameter) of the test fungus grown on PDA. The two plates were sealed together with parafilm. Control sets were prepared in a similar manner, without bacteria in the bottom plate. Such sealed sets of Petri dishes were incubated at 28 °C, and the observations were recorded at intervals of 24 h for 120 h. Percent growth inhibition of the test fungus was calculated.

Production of amylase

Amylase dextrifying assay was carried out by change in blue colour of amylase-iodine complexes. The rhizobacteria were inoculated on plates with minimum salts medium containing (1 % w/v) starch. After an incubation period of 48 h at 30 ºC, plates were flooded with Grams iodine solution and visualized for zones of hydrolysis indicative of amylase production.

Scanning electron microscope (SEM) of the interaction between antagonist and test fungus

Smears for SEM examination were made of the antagonized portions of the test fungi from the sealed Petri dishes. The samples were immersed in 2.5% glutaraldehyde and kept at 4° C for 24 hrs, rinsed thrice with 0.1 M caco buffer. Then, 1% osmium tetraoxide was added and dehydrated by passage through graded aqueous ethylalcohol series (30, 50, 70, 90 and 95%), placed in 100% ethanol at roomtemperature 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.

RESULTS AND DISCUSSION

A total of 174 bacterial strains were isolated from thirty-six chickpea rhizospheric soil samples, 76 isolates from kings B and 98 from nutrient agar.

Screening for bacterial antagonists

All the 174 isolated bacterial strains were screened *in vitro* for their activities against *Rhizoctonia bataticola*. From the total 176 rhizobacterial isolates, 59 had the ability to suppress this pathogen, 19 belonged to *Pseudomonas* sp. and 26 to *Bacillus* sp. Bacterial strains were classified as weak, medium and strong antagonists depending on their ability to inhibit the mycelial proliferation of test fungus under dual culture assay. Thirty-five antagonists inhibited the growth of the fungus in the range of >20.0- 36.25% (categorized as strong antagonists) [Table 1 and 2] and were further evaluated for their antagonistic

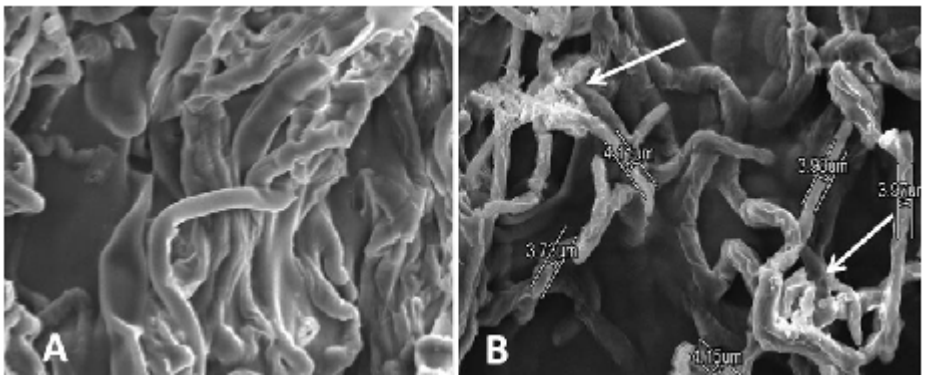

Fig. 1. Scanning electron micrographs of interaction between *Rhizoctonia bataticola* and bacterial antagonists. Scale bar-50 µm. (A) *Rhizoctonia bataticola* hyphae from pure culture (B) Deformed hyphae with sparse fungal growth due to effect of antimetabolites

potential against the pathogen. Maximum antagonism was observed with *Bacillus* isolate B20b (36.75%) followed by *Pseudomonas* strain Ps14c (35%). Rhizobacterial isolates belonging to genera *Bacillus, Pseudomonas, Serratia* and *Arthrobacter* have been reported to be excellent biocontrol agents to soil-borne plant pathogens[10].

Assessment of antagonistic potential of bacterial antagonists

Production of non volatile antifungal metabolites

Diffusible antimetabolites production

Rhizospheric microorganisms have a great potential to produce a number of secondary metabolites that inhibit pathogens. Antibiotics produced by PGPR include phenazine, pyoluteorin, pyrrolnitrin and cyclic lipopeptides all of which are diffusible[11]. In the present study, 45.7% of the rhizobacterial isolates antagonized the growth of *Rhizoctonia bataticola* in the range of 22.25-63.75% under petri dish-based assays. Among these, 7 belonged to *Pseudomonas* sp. [Table 3] while rest 9 was representatives of *Bacillus* sp. [Table 4]. Isolate Ps14c and B20b which exhibited 35% and 36.25% fungal growth inhibition under dual culture assay respectively also contributed to antagonism of fungus due to diffusible metabolites (56.25 and 52.5% respectively). Precultures of antagonists on cellophane membrane produced antifungal compounds that diffused into the medium and inhibited the mycelial proliferation of pathogen. Role of fluorescent *Pseudomonas* and *Bacillus* species in suppression of pathogenic microorganisms by the secretion of extracellular metabolites that are inhibitory at low concentration such as phenazine derivatives is well reported. Even under low concentrations circumstances antibiotic producers are able to control plant diseases due to the involvement of systemic resistance mediated by the antibiotics or due to the interaction of antibiosis with other extra cellular metabolites that may trigger ISR. Sharma and Parihar[12] reported in their investigations, the ability of extracellular antifungal metabolites of *Actinomycetes* against *Rhizopus stolonifer, Aspergillus flavus, F. oxysporum* and *Alternaria* sp.

Table 1. Pseudomonad mediated growth inhibition of *Rhizoctonia bataticola*

Antagonistic isolates	Radial growth (cm)	% growth inhibition
Control	8.0	-
Ps 4b1	5.5 ± 0.11	31.25
Ps7a	5.8 ± 0.28	27.5
Ps13b	5.3 ± 0.17	33.75
Ps14c	6.0 ± 0.57	25.0
Ps14d	5.8 ± 0.11	30.0
Ps15a	5.6 ± 0.08	30.0
Ps16a	5.5 ± 0.23	31.25
Ps16b	5.9 ± 0.11	26.25
Ps18a	5.7 ± 0.08	28.75
Ps19d1	5.5 ± 0.08	31.25
Ps20b	5.7 ± 0.23	28.75
Ps24d	5.4 ± 0.17	32.5
Ps28c	5.7 ± 0.11	28.75
Ps29b	5.8 ± 0.17	27.5
Ps29c	5.5 ± 0.23	31.25
P-I	5.4 ± 0.06	32.5

Table 2. *Bacillus* mediated growth inhibition of *Rhizoctonia bataticola*

Antagonistic isolates	Radial growth (cm)	% growth inhibition
Control	8.0	-
B2a	5.7 ± 0.11	28.75
B5a	5.8 ± 0.14	27.5
B5b	5.4 ± 0.14	32.5
B7a	5.8 ± 0.17	27.5
B8a	5.5 ± 0.23	31.25
B9b	6.2 ± 0.11	22.5
B9c	5.8 ± 0.05	27.5
B9d	5.9 ± 0.17	26.25
B11a	5.7 ± 0.17	28.75
B17b	5.9 ± 0.07	26.75
B18a	5.4 ± 0.11	32.5
B20b	5.1 ± 0.05	36.25
B20d	5.3 ± 0.17	33.75
B21c	5.5 ± 0.17	31.25
B22a	5.6 ± 0.09	30.0
B24b	5.9 ± 0.23	26.25
B-28a	5.8 ± 0.20	27.5
B29c	5.6 ± 0.11	30.0
B-I	5.5 ± 0.11	31.25

Inhibition due to extracted antimetabolites

The synthesis of antibiotics is the mechanism that is most commonly associated with the ability of a PGPR to suppress pathogen development[13]. Antimetabolites extracted from 31.4% of the rhizobacterial antagonists (5 *Pseudomonas* sp. and 6 *Bacillus* sp.) in 50% methanol from culture supernatant inhibited the

Table 3. Effect of different determinants of Pseudomonads on inhibition of *Rhizoctonia bataticola*

Antagonistic isolates	% growth inhibition of *Rhizoctonia bataticola*				
	Diffusible metabolites	Culture filtrate antibiosis	Thermo-stable metabolites	Ethyl acetate extracts	Volatile antimetabolites
Ps 4b1	23.33 ± 0.23	58.8 ± 0.46	22.0 ± 0.31	38.75 ± 0.12	52.94± 0.27
Ps7a	-	11.1 ± 0.05	13.6 ± 0.34	-	31.33± 0.19
Ps13b	-	18.8 ± 0.46	-	40.30 ± 0.28	-
Ps14c	56.25 ± 0.40	29.75 ± 0.36	24.5 ± 0.26	38.75 ± 0.23	73.53± 0.31
Ps14d	27.7± 0.19	30.5 ± 0.28	13.6 ± 0.34	-	31.7 ± 0.40
Ps15a	48.12± 0.26	33.3 ± 0.17	25.0 ± 0.51	-	65.88 ± 0.23
Ps16a	-	14.4 ± 0.23	-	-	33.5 ± 0.28
Ps16b	-	-	-	-	-
Ps18a	32.50± 0.21	-	-	-	30.2 ± 0.12
Ps19d1	22.25± 0.21	15.62 ± 0.35	16.2 ± 0.40	-	73.53 ± 0.19
Ps20b	-	61.1 ± 0.05	25.7 ± 0.17		44.11 ± 0.06
Ps24d	-	23.75 ± 0.36	22.8 ± 0.42	33.30 ± 0.19	37.64 ± 0.21
Ps28c	-	13.3 ± 0.17	-	30.50 ± 0.06	40.20 ± 0.11
Ps29b	-	29.5 ± 0.28	-	-	-
Ps29c	-	15.62 ± 0.47	26.1 ± 0.53	-	-
P-I	63.75± 0.27	16.6 ± 0.34	19.4 ± 0.23	-	72.35 ± 0.20

Table 4. Effect of different determinants of *Bacillus* on inhibition of *Rhizoctonia bataticola*

Antagonistic isolates	% growth inhibition of *Rhizoctonia bataticola*				
	Diffusible metabolites	Culture filtrate antibiosis	Thermo-stable metabolites	Ethyl acetate extracts	Volatile antimetabolites
B2a	32.50 ± 0.12	-	-	-	-
B5a	-	-	11.7 ± 0. 23	35.9 ± 0.22	66.6± 0.51
B5b	56.25 ± 0.42	25.0 ± 0.28	27.8 ± 0.52	-	-
B7a	47.5± 0.32	-	-	-	44.11± 0.32
B8a	-	6.25 ± 0.14	20.3 ± 0.42	-	11.76 ± 0.21
B9b	39.5± 0.28	22.75 ± 0.36	-	-	-
B9c	42.9± 0.22	30.5 ±0.28	14.5 ± 0.23	28.7 ± 0.19	-
B9d	-	63.75 ± 0.36	13.6 ± 0.34	30.4 ±0.42	31.76 ± 0.33
B11a	-	56.25 ± 0.14	16.0 ± 0.51	-	-
B17b	-	51.25 ± 0.20	-	29.3 ± 0.28	50.0 ± 0.14
B18a	-	38.12 ± 0.07	36.6 ± 0.46	-	41.17 ± 0.28
B20b	52.5± 0.36	25.0 ± 0.22	-	-	42.9 ± 0.07
B20d	50.0 ± 0.40	37.5 ± 0.28	26.2 ± 0.40	-	48.6 ± 0.42
B21c		50.0 ± 0.26	25.7 ± 0.17	-	-
B22a	-	71.25 ± 0.14	32.8 ± 0.76	-	38.4 ± 0.21
B24b	-	-	-	-	-
B-28a	45.5 ± 0.17	37.8 ± 0.29	29.1 ± 0.63	42.5 ± 0.32	61.76 ± 0.57
B29c	-	-	-	-	-
B-I	40.5 ± 0.22	37.8 ± 0.19	29.4 ± 0.23	35.6± 0.27	27.64 ± 0.19

growth of the fungus in the range of 29.3-42.5% [Table 3 and 4]. Maximum inhibition was observed with B-28c (42. 5%) followed by Ps-13b (40.3%). The main target of these antibiotics have been reported to be the electron transport chain (phenazines, pyrrolnitrin), metalloenzymes such as copper-containing cytochrome oxidases, membrane integrity (biosurfactants), but their mode of action are still largely unknown[14]. Antimicrobial compounds may act on phytopathogenic fungi by inducing fungistatis, inhibition of germination, lysis of fungal mycelia, or by exerting fungicidal effects[11].

Culture filtrate antibiosis

Three days old culture filtrate @ 50% of the bacterial antagonists inhibited the growth of test fungus, *Rhizoctonia bataticola* in the range of 13.3-71.75% suggesting the role of antibiosis in antagonism. 87% of the pseudomonads were able to inhibit the growth of fungus in perti-plate assay, maximum being exhibited by Ps20b followed by Ps4b1 [Table 3]. Among the *Bacillus* strains, maximum inhibition was exhibited by B9d (71.25%) followed by B 22a (63.75%) [Table 4]. The production of certain antifungal compounds in the absence of pathogen is well known[4] and this may be the reason why different isolates inhibited the growth of the pathogen differently under different techniques. Similar results have been reported where four days old culture filtrate of *Bacillus subtilis* MA-2 completely inhibited the growth of *Alternaria alternate* and *Curvularia andropogonis* at 10% concentration while *Fusarium moniliformae* and *Colletotrichum acutatum* were comparatively less sensitive[15].

Inhibition due to thermostable antimetabolites

The antifungal potential of the culture filtrate was markedly reduced when it was autoclaved, as indicated by the decrease in % growth inhibition of the test fungus.However, 62.8% of bacterial isolates still inhibited the growth of *Rhizoctonia bataticola* in the range of 11.7-32.8% [Table 3 and 4] signifying the role of some heat labile metabolites in antagonism. However, reduction in % growth inhibition due to autoclaved filtrate may be attributed to the fact that isolate's active metabolites such as hydrolytic enzymes produced during exponential phase (as suggested by higher growth inhibition under dual culture technique) may have got denatured or detoxified.

Production of amylase

Biocontrol agents are known to be good producers of a number of hydrolytic enzymes including amylase. In the present investigation, all the isolates were found to be producers of amylase as indicated by the change in blue colour of the amylase-iodine complex to yellow. The amylase has been reported to have only a discrete effect on phytopathogens cell wall as there is no report of the presence of starch in fungal cell wall. However, glycogen which is closely related polysachharide is present in cell walls of some fungi. It is unlikely that this enzyme plays a key role in the early phase of antagonistic association[16]. Alternatively, as starch is widely distributed in nature, these enzymes could assist easy survival of microorganisms by mediating hydrolysis of starch or any other readily available related ±-glucan elsewhere[17].

Production of volatile antifungal compounds

Another potential weapon that microorganisms have developed against phytopathogens is production of antifungal volatile compounds. Volatile compounds such as alkanes, alkenes, alcohols, aldehydes, ammonia, esters, ketones, sulfides, and terpenoids known to be produced by a number of rhizobacteria are reported to play an important role in biocontrol[18]. 65.7% of the rhizobacterial antagonists produced antifungal volatile compounds as evident from the reduction in growth of *Rhizoctonia bataticola* under sealed plate technique. The production of volatile antifungal compounds by pseudomonads has been reported[19]. However, in the cited studies, the effect of inhibitory volatile metabolite(s) received less importance than the inhibitory diffusiblemetabolite(s). In the present investigation, it was demonstrated that the volatile metabolite(s) produced by pseudomonads had a predominant inhibitory role in the antagonism of the test fungi, *Rhizoctonia bataticola* and the diffusible metabolite(s) played only a supplementary role inthe antagonism. Maximum inhibition in fungal growth was observed with isolate Ps19d1 and Ps14c (both 73.53%) [Table 3] after 48 h of incubation. Among the *Bacillus* isolates, 11 out of 19 inhibited fungal growth due to volatiles production [Table 4]. Rhizobacterial isolates comprising *Serratia plymuthica, Serratia odorifera, Pseudomonas fluorescens, and*

Pseudomonas trivialis synthesize and emit complex blends of volatiles that inhibit growth of many phytopathogenic and non phytopathogenic fungi[19, 20].

Scanning electron microscope (SEM) of the interaction between antagonistand test fungus

The microscopic examination by SEM revealed scanty growth with distorted cell surface morphology in response to the bacterial effects as indicated in figure 1. This illustrates the antagonistic behavior of rhizobacterial isolates against *Rhizoctonia bataticola*. A clear distortion of fungal mycelium and induction of hyphal surface flaking was observed due to lysis and bursting of cytoplasmic material revealing the role of antagonism in this interaction.

Screening is a critical step in the development of biocontrol agents. The success of all subsequent stages depends on the ability of a screening procedure to identify an appropriate candidate. This study and the results are particularly useful for identifying likely candidates for biocontrol and for making educated guesses concerning the mechanisms by which they reduce pathogen damage.

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