JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 2071-2078

Role of Defense Enzymes in Biocontrol of Spot Blotch and Leaf Rust of Wheat (*Triticum* sp. L.) by *Chaetomium globosum*

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(Received: 07 September 2015; accepted: 03 November 2015)

Spot blotch caused by *Bipolaris sorokiniana* (Sacc.) Shoem. (syn. *Helminthosporium sativum*, teleomorph *Cochliobolous sativus*) and leaf (brown) rust caused by (*Puccinia triticina* Eriks) are serious diseases of wheat inflicting heavy yield losses. A highly antagonistic *Chaetomium globosum* strain, Cg-2 that inhibited the mycelial growth of *B. sorokiniana* was selected for assessing spot blotch and leaf rust control by pot culture experiments in the greenhouse. Pre-inoculation foliar spray of Cg-2 challenge-inoculated with *B. sorokiniana* and *P. triticina* resulted in significantly lower incidence relative to the pathogen control. On challenge inoculation with *B. sorokiniana*, the lower disease incidence coincided with increased plant vigor index (4007), relative to the pathogen control. Analysis of leaf samples also indicated increased induction of peroxidase (PO) by 2 and 3.8-fold, polyphenol oxidase (PPO) by 2 and 4-fold, phenylalanine ammonia lyase (PAL) by 5 and 6-fold and catalase by 1.8 and 2.4-fold, more than those of the pathogen controls, *B. sorokiniana* and *P. triticina*, respectively. In quantitative PCR assays, Cg-2 with challenge-inoculation, showed maximum expression of PO at 5 (5.6 fold) and 7 days (8.5 fold) of challenge inoculation with *B. sorokiniana* and *P. triticina*, respectively. Also, the maximum expression of PAL was noticed at 7 days of challenge inoculation with *P. triticina*. It is presumed that the induced peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and catalase may be involved in the reduction of spot blotch and leaf rust development in wheat. Reduced disease severity coupled with enhanced enzyme production elicited by *Chaetomium globosum* in glasshouse experiments by foliar spray indicate that its mode of action for spot blotch and leaf rust suppression in wheat is through induced resistance.

Keywords: Induced resistance, wheat, spot blotch, leaf rust, *Chaetomium globosum,* defense enzymes.

Induced resistance is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli[1]. Disease reduction by induction of resistance has been accounted for a large number of defence enzymes, including peroxidase (PO) and polyphenol oxidase (PPO) that catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) that is involved in phytoalexins and phenolics bio-synthesis. Chitin and glucan oligomers released during degradation of fungal cell wall act as elicitors that stimulate various defence mechanisms in the plants[2]. The induced protection by selected strains of plant growth promoting rhizobacteria (PGPR)[3-5] and *Trichoderma*[6] have been well documented. Besides PGPR and *Trichoderma*, species of *Chaetomium* belonging to Ascomycetes have considerable potential for the biocontrol of plant diseases[7-10]. These species normally exist in soil or organic compost[11] and some are being good cellulase and laccase producers, are important for the biotechnological industry[12,13]. Biocontrol

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mechanisms of *Chaetomium* spp. are generally attributed to the secretion of a variety of degrading enzymes[14] and antibiotics[15-17]. They are also known for stimulating plant growth, and inducing resistance of plant host[7]. Among *Chateomium* spp., *C. globosum* represent a potentially attractive alternative disease management approach since this is known for growth promotion and disease reduction in crops, has been reported to be an effective biocontrol agent against several soilborne and foliar diseases[18, 7, 10].

Wheat (*Triticum* spp.) is an important cereal crop, whose cultivation is often hampered by the foliar diseases, spot blotch caused by *Bipolaris sorokiniana* (Sacc.) Shoem. (syn. *Helminthosporiu msativum*, teleomorph *Cochliobolous sativus*) and leaf (brown) rust caused by (*Puccinia triticina* Eriks). The yield loss was accounted to be 6.3 to 50.6% for spot blotch[19], whereas, it was 10-65% for the latter[20]. Chemical control measures create imbalances in the microbial community, which may be unfavorable to the activity of the beneficial organisms and may also lead to the development of resistant strains of pathogen. Developing resistant varieties can be difficult in the absence of dominant genes and development of new races of the pathogen overcoming host resistance. *Chaetomium globosum* (Kunze) Fr. therefore, was identified as a potential biocontrol agent against the diseases[21-22, 8]. In biocontrol of plant diseases, though induced resistance through induction of host defence enzymes is well documented for rhizobacteria and *Trichoderma*[3-6], little is known in case of *Chaetomium* spp.

The present study therefore assessed the efficacy of an *in vitro* selected *C. globosum* isolate, Cg-2 that exhibits maximum antagonism against *B. sorokiniana* for spot blotch and leaf rust reduction in wheat under greenhouse conditions and the role of defense enzymes in suppression of these diseases.

MATERIALS AND METHODS

Fungal pathogens and inoculum preparation

A highly virulent isolate of *Bipolaris sorokiniana*, BS 75[23] (GenBank Accession No. HM 195259) was used as the test pathogen. For inoculum preparation, the pathogenic fungus was cultured on potato dextrose agar (PDA) for 7–10 days in Petri plates. The spore suspension was prepared by pouring 20 mL of sterile distilled water into each Petri plate and then filtering the suspension through a fine nylon mesh to remove larger mycelial parts. The suspension was then quantified using a haemocytometer and the spore concentration adjusted to 4 x 10^4 spores/mL and mixed with 0.05 % Tween 80 for inoculation.

Puccinia triticina pathotype, 77-5 was used as the test pathogen for leaf (brown) rust. The inoculum was obtained from the regional station of Indian Institute of Wheat and Barley Research (IIWBR), Flowerdale, Shimla. The pathogen was maintained as single spore derived culture by inoculating 10 mg of uredospores ($6x10^5$) suspended in petroleum spirit and mineral oil (70: 30) on the brown rust susceptible genotype, 'Agra Local'.

Evaluation of *C. globosum* isolates for antagonism against the spot blotch pathogen

Isolates designated as Cg-1 to Cg-9 that are maintained in the laboratory were evaluated for their antifungal activities against the highly virulent strain of *B. sorokiniana* (Bs75) by dual culture assays on PDA. Both C. *globosum* isolates and the fungal pathogen were co-inoculated on the same day. Plates inoculated with the pathogen alone served as control. The plates were incubated at 28±2 ºC for 7 days and the linear growth of the pathogen was measured when the control plates showed full growth. The colony diameter of *B. sorokiniana* in dual culture with each isolate of *C. globosum* was measured and growth inhibition was expressed as a percentage of the control. The treatments and controls were replicated three times in the antifungal assays.

Greenhouse evaluation of selected antagonists for spot blot and leaf rust management

The isolate, Cg-2 identified to be highly antagonistic against the spot blotch pathogen was cultured on PDA for 15 days in Petri plates and the spore suspension was prepared as described earlier for the spot blotch pathogen and its population was adjusted to 3X 10^6 spores/mL. The experiments on wheat (cv Sonalika) was carried out over a 30days period (from the day of sowing) in completely randomized design (CRD) in a polyhouse with 3 replicates of 30 plants per treatment. The seeds were surface sterilized with

1% sodium hypochlorite for 2 min and sown in individual 10-cm-diameter pots containing 400g steam sterilized (121 °C, 30 min for two consecutive days) soil @10 seeds per pot. Ten days old seedlings were pre-inoculated with Cg-2 as a foliar application (10^6cfu/mL). The seedlings were subsequently inoculated after two days with of *B. sorokiniana* inoculum containing 4x 10^4spores/mL. Untreated plants and plants treated with pathogen alone served as control. The cultivars developed infection from 6-10 days of inoculation when the humidity and temperature were 90-95% and 20–30°C, respectively. A similar set of experiment challenge-inoculated with *P. triticina* was conducted on Agra Local.

Tissue collection, enzyme extraction and biochemical assays

For each treatment, the leaf samples (6 g) per replication were collected after 1 through 9 d of pathogen inoculation at 48-h intervals. The samples were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized tissues were stored at -80 ºC. One gram of powdered sample was extracted with 2mL of 100 mM sodium phosphate buffer (pH 7.0) at 4 ºC. The homogenate was centrifuged for 20 min at 10,000 rpm at 4 ºC, and the protein content of the supernatant was determined by the Bicinchoninic acid kit. The supernatant was used as a crude enzyme source to assay the activities of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase[24]. One unit of PO is defined as the amount of enzyme catalyzing the oxidation of 1 5ØßM of guaiacol in 1 min. PAL activity is defined as the quantity of enzyme that catalyzes the formation of 1µg of cinnamic acid per hour under the usual assay conditions. One unit of PPO activity is defined as that amount of enzyme which catalyzes the transformation of 1micro mole substrate/min under the conditions of the assay. One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute. Enzyme activity was expressed as nkat/mg protein

Quantitative RT-PCR assays

Total RNA was isolated from the tissues with Tri reagent (Amresco) according to the manufacturer's protocol. For RT-PCR, 2mg of RNA was treated with RNase free DNase (Promega) for 30 min at 37°C followed by DNase stop solution for 10 min at 65°C for DNase inactivation. The treated RNA was used for reverse transcription with oligo(dT)17V primer following the protocol (Promega, M170A). Two microlitres of an eight fold dilution of this cDNA was used as template for amplification by qPCR (SYBRmix, Thermo Scientific). The *B. sorokiniana* actin (AY748990) was used as 'housekeeping' gene to balance the amount of cDNA. For real-time RT PCR reactions performed in an MJ miniBiorad thermal cycler, 20 ng of cDNA were used as template. The 20 µl of reaction volume included 10 µl of 2XAbsolute SYBR mix (Thermo scientific) and 100 nM final concentrations of specific primers for the gene of interest. Reactions were performed in triplicate. The transcript abundance was calculated following the procedure of Liu and Saint[25].

RESULTS AND DISCUSSION

Plants are bestowed with various defence genes that need appropriate stimuli or signals to be activated. Induction of plant defence mechanisms by prior application of a biological inducer is a novel technique of plant protection. Despite major advances in our understanding of defence responses of plants, little information is available on *Chateomium globosum* mediating induced enzymes. It is possible that the defence mechanisms induced by the biocontrol agent may result in reduction in symptoms of each pathogen inoculated. In the present study, identification of a potential isolate for biocontrol experiments involved dual culture assays as a screening strategy against the spot blotch pathogen. The antifungal activity assays for the *Chaetomium* spp. against *Bipolaris sorokiniana* revealed that the strains in general significantly inhibited the mycelial growth of the pathogen with 40.0-77.2 percent mean inhibition (Table 1). One isolate, Cg-2 exhibited significantly higher mean mycelial growth inhibition of the pathogen (77.2%) over the control (Table 1).

In evaluating the biocontrol efficacy of Cg-2, application through seed treatment of the suspension culture with or without challenge inoculation with *B. sorokiniana* significantly reduced the disease incidences besides promoting wheat growth over the pathogenic either control despite challenge inoculation with the pathogen.

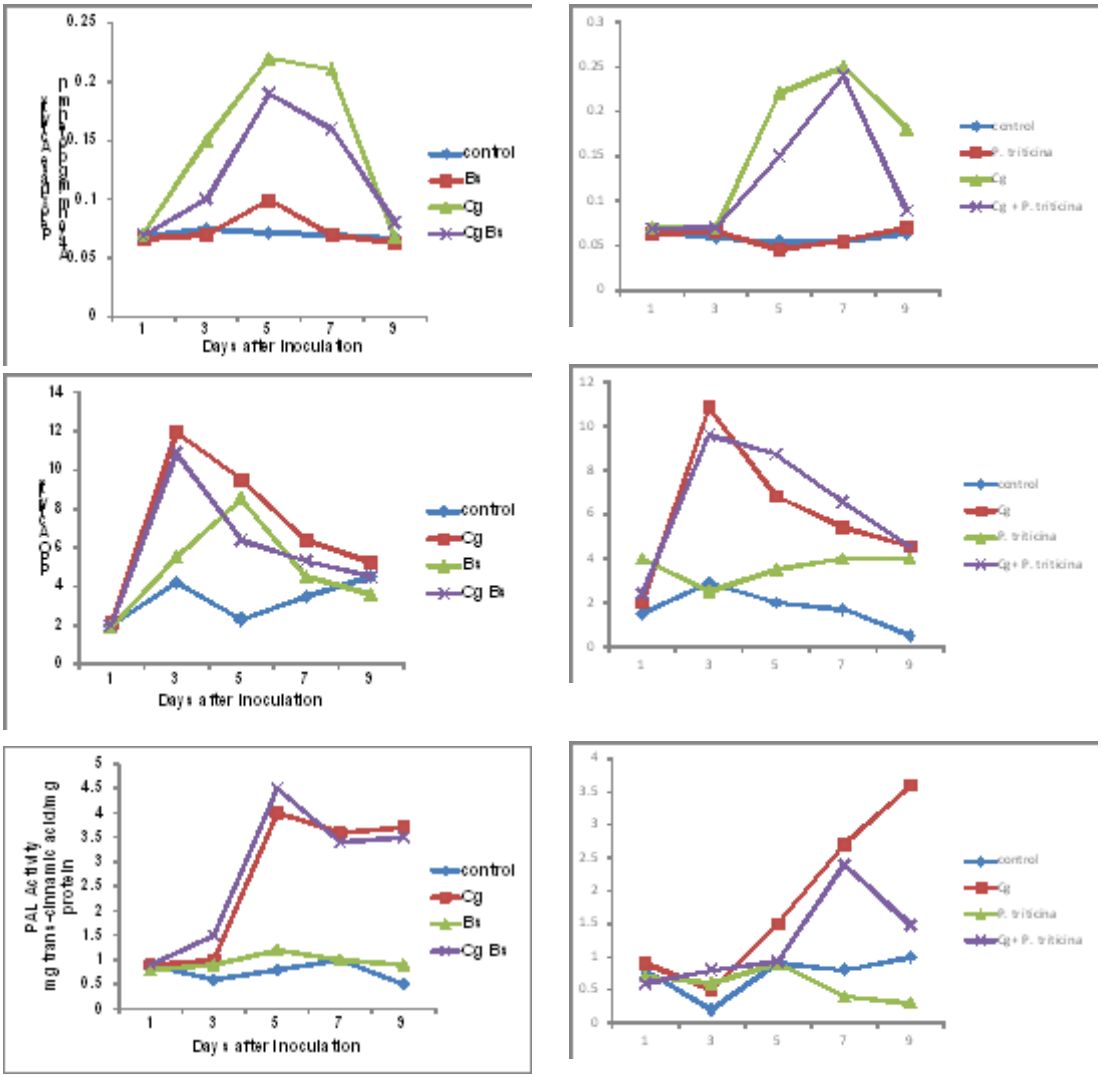

Fig. 1. Induction of defense enzymes a) peroxidase b) polyphenol oxidase and c) phenyl alanine ammonia lyase in "Agra Local" wheat treated with Cg-2 and challenge inoculated with either of the pathogens, *Bipolaris sorokiniana* and *Puccinia triticina*

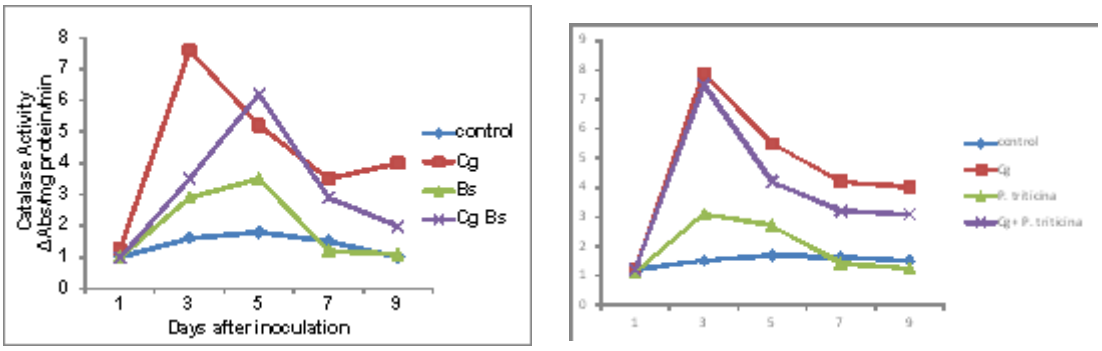

Fig. 2. Induction of catalase in "Agra Local" wheat treated with Cg-2 and challenge inoculated with the pathogens a) *B. sorokiniana* and b) *P. triticina*

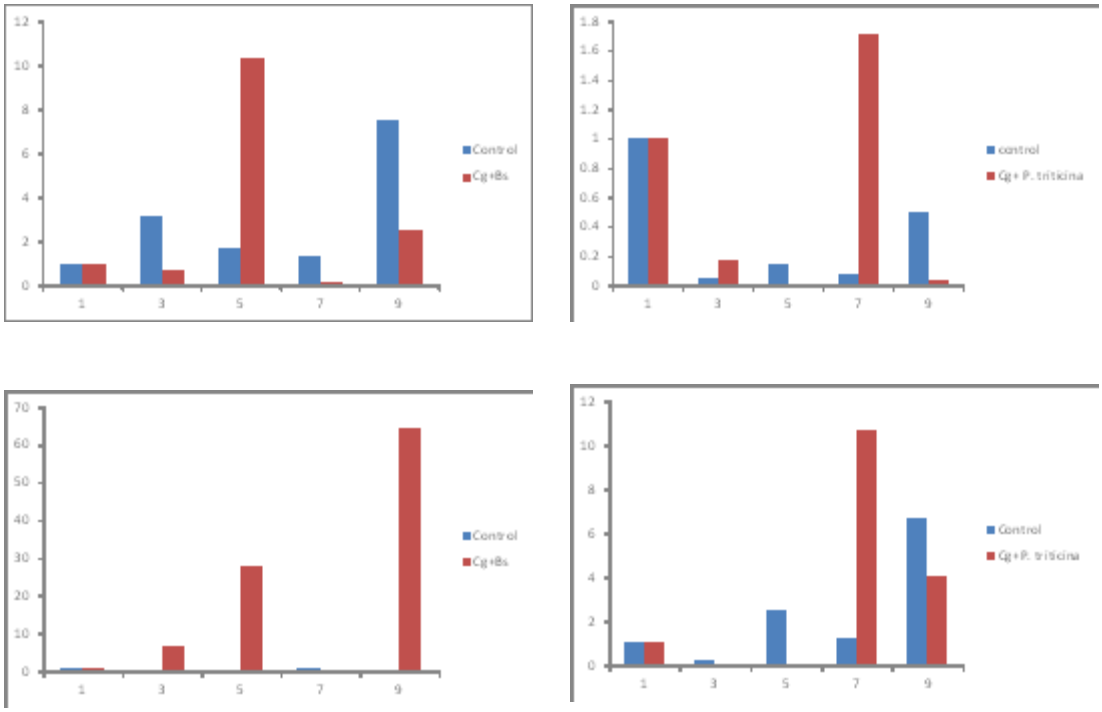

Fig. 3. Quantitative expression of the defense enzymes, PO (a) and PAL (b) in wheat treated with Cg-2

This indicated the ability of the strain to exert biocontrol systemically. Though the Cg-2 treatment resulted in maximum (cent per cent) germination and vigour index (6050) of wheat plants, the challenge inoculated plants also exhibited significantly higher germination and vigour index than those of the pathogen control. The latter resulted in 59.2% and 48.30 % increase in the shoot and root lengths relative to BS control, respectively following challenge inoculation (Table 2).

The efficacy of biocontrol agents can be improved by studies on the mechanism of action of the biological agents. The capacity of biocontrol agents to stimulate plant defence activities is a widely recognized mechanism[3-6]. However, little information is available on *Chaetomium* mediating induced enzymes. The present study revealed enhanced activities of the defense related enzymes PPO, PO, PAL and CAT in wheat plants treated with Cg-2 and challenged with *B. sorokiniana* and *P. triticina*. Time course analyses indicated that the biocontrol agent, with or without challenge inoculation with either pathogen, stimulated maximum enzyme production than that of either control at 3 through 7 days for the defense enzymes (Fig. 1 and 2). However, the biocontrol agent stimulated enzyme production was significantly higher than those with challenge inoculations and those of the pathogen control. At maximum induction, the treatment induced 2.4 and 4.2, 2.5 and 4, 3 and 6.5, 2.5 and 2.6-fold increase in PO, PPO, PAL and catalase, respectively than those of the pathogens *B. sorokinana* and *P. triticina*, respectively (Fig. 1 and 2). Activation of defence reactions in *Chaetomium* only treated plants in this study suggests that even a beneficial microbe may be perceived by the plant as a potential threat, and that such perception involves production of resistance-eliciting compounds that act mechanistically similar to elicitors produced by plantpathogenic fungi and bacteria[3]. The challenge inoculated plants though stimulated relatively lesser amount of enzymes, the amount of induction was significantly higher than those of the pathogenic control and the untreated control.

Among the challenge inoculated biocontrol treatments, though no difference for maximum induction (3 days) of PPO (Fig. 1b) and catalase (Fig. 2) was observed for the fungal pathogens, an earlier induction (5 days) of PO (Fig. 1a) and PAL (Fig. 1c) was observed for *B. Sorokiniana* than that for *P. triticina* (7 days). The biocontrol strain on challenge inoculation stimulated PO by 2 and 3.8-fold, PPO by 2 and 4-fold, PAL by 5 and 6-fold and catalase by 1.8 and 2.4-fold, more than those of the pathogen controls, *B. sorokiniana* and *P. triticina*, respectively. The maximum induction of the defense enzymes by the challenge inoculated plants was further confirmed by gene expression studies employing quantitative PCR assays. Similar to enzyme assays, maximum expression of PO at 5 (5.6 fold) and 7 days (8.5 fold) of challenge inoculation with *B. sorokiniana* and *P. triticina*, respectively was observed (Fig. 3a). Also, the maximum expression of PAL was noticed at 7 days of challenge inoculation with *P. triticina*. In contrary to the enzyme assays, the maximum expression of PAL was noticed at 9 days when challenged with *P. triticina* which was greater than that of the untreated control (Fig. 3b). Since the lower disease incidence and greater yield realized in the biocontrol treatment compared with pathogen control in the greenhouse were associated with an increase in known defence gene products, such as PO, PPO, and PAL, we speculate that these are involved in the mechanism of disease suppression by the biocontrol agent Cg-2. Stimulation of plant defence activities resulting in reduced disease incidences have been reported earlier[3].

Table 1. Screening of *Chaetomium globosum* isolates for antagonism against *B. sorokiniana* inciting spot blotch of wheat in dual culture assays

Isolate	Accession No	Place of collection	Radial growth (cm) 7 days after inoculation		
			Pathogen	Antagonist	Percent inhibition over control
Cg1	ITCC 1627	New Delhi	3.2^{b}	5.5^{a}	59.4
Cg2	WSCG 3	New Delhi	1.8^{e}	5.3^{a}	77.2
Cg3	ITCC 2401	Nainital	2.9^{bc}	4.7^{abc}	63.2
Cg4	ITCC 2034	New Delhi	2.2^{de}	5.1^{ab}	72.15
Cg5	WSCG 1	IARI Farm	2.5^{cd}	4.8^{abc}	68.35
Cg6	WSCG 2	IARI Farm	3.4^{b}	5.0^{ab}	56.96
Cg7	WSCG 4	Dhaulakuan, H.P	4.7^{a}	3.5^{d}	43.03
Cg8	WSCG 5	Samastipur, Bihar	4.5^{a}	3.7^{cd}	40.03
Cg9	WSCG 6	Jammu, J & K	4.1^{a}	4.0^{cd}	48.10
Control	-	-	7.9^{a}	-	-
CD (P=0.05)	-	-	0.52	1.06	-

Values superscripted with same letter are not significantly different at (P=0.05) according to Duncan Multiple Range Test (DMRT)

Table 2. Greenhouse evaluation of *Chaetomium globosum* Cg-2 against spot blotch of wheat (*Bipolaris sorokiniana*)

Treatments	% germination (30 DAS)**	Shoot length (cm)*	Root length (cm) *	Vigour index*
C. globosum	100	40.5	20.0	6050
C. globosum + *B. sorokiniana*	70	39.8	17.5	4007
Pathogenic control (*B. sorokiniana*)	10	25.0	11.8	36

** DAS: days after sowing; *Average of three replications

CONCLUSIONS

Based on the results of our study, we conclude that Cg-2 may represent an important biocontrol agent to control spot blotch and leaf rust diseases. Hence, the strain could be ideally exploited for field trials to achieve the goal of practical applications of biocontrol, which are currently under progress.

ACKNOWLEDGMENTS

The authors are thankful to the Head, Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi for support and encouragement during the course of this investigation. The financial support received from Indian Council of Agricultural Research, New Delhi in the form of National Fellow Project Grant (F. No. 27-2/2005) is gratefully acknowledged.

REFERENCES

1. Van Loon, L. C., Bakker, P. A. H. M., Pieterse, C. M. J. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.*, 1998; **36**: 453–83.
2. Frindlender, M., Inbar, J., Chet, I. Biological control of soil borne plant pathogens by a -1, 3-glucanase producing *Pseudomonas cepacia*. *Soil. Biol. Biochem*. 1993; **25**: 1211-1221.
3. Shanmugam,,V., Kanoujia, N. Biological management of vascular wilt of tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* by plant growth-promoting rhizobacterial mixture. *Biol. Control*. 2011; **57**: 85-93.
4. Shanmugam , V., Atri, K., Gupta, S., Kanoujia, N., Naruka, D. S. Selection and differentiation of *Bacillus* spp. antagonistic to *Fusarium oxysporum*f.sp. *lycopersici* and *Alternaria solani* infecting tomato. *Folia Microbiol.* 2011a; **56**: 170–177.
5. Shanmugam,, V., Kanoujia, N., Singh, M., Singh, S., Prasad, R. Biocontrol of vascular wilt and corm rot of gladiolus caused by *Fusarium oxysporum* f.sp. *gladioli* using plant growth promoting rhizobacterial mixture. *Crop Prot.* 2011b; **30**: 807-813.
6. Shanmugam ,V., Chugh, P., Sharma, P. Cold-tolerant *Trichoderma* species for the management of Fusarium wilt of tomato plants. *Annals* of *Microbiol.* 2015; **65**:543–551.
7. Soytong, K., Kanokmedhakul, S., Kukongviriyapa, V., Isobe, M. Application of *Chaetomium* species (Ketomium®) as a new broad spectrum biological fungicide for plant disease control: A review article. *Fungal Divers.* 2001 **7**:1-15.
8. Biswas, S. K., Srivastava, K. D., Aggarwal, R., Dureja, P., Singh, D. V. Antagonism of *Chaetomium globosum* to *Drechslera sorokiniana*, the spot blotch pathogen of wheat. *Indian Phytopath.* 2000; **53**:436-440.
9. Biswas, S. K., Srivastava, K. D., Aggarwal, R., Praveen, S., Singh, D. V. Biochemical changes in wheat induced by *Chaetomium globosum* against spot blotch pathogen. *Indian Phytopath.* 2003; **56**(4): 324-379.
10. Aggarwal, R. *Chaetomium globosum*: a potential biocontrol agent and its mechanism of action. *Indian Phytopath*. 2015; **68**(1): 8-24.
11. Seonju, L., Richard, T. H. Phylogenetic relationships of *Chaetomium* and similar genera based on ribosomal DNA sequences. *Mycologia* 1999; **91**(3): 434-442.
12. Ankudimova, N.V., Baraznenok, V. A., Becker, E. G., Okunev, O. N. Cellulase complex from *Chaetomium* cellulolyticum: isolation and properties of major components. *Biochem.*. 1999; **64**: 1068- 1073.
13. Zhihua, L., Qian, Y. Gene cloning and sequence analysis of dienelactone hydrolase from *Chaetomium globosum*. *J. Northeast. Forestry. Uni.* 2005; **33**(4):48-51.
14. Inglis, G. D., Kawchuk, L. M. Comparative degradation of oomycete, ascomycete, and basidiomycete cell walls by mycoparasitic and biocontrol fungi. *Can. J. Microbiol.* 2002; **48**(1): 60-70.
15. Aggarwal, R., Tiwari, A. K., Srivastava, K. D., Singh, D. V. Role of antibiosis in the biological control of spot blotch (*Cochliobolus sativus*) of wheat by *Chaetomium globosum*. *Mycopathologia* 2004; **157**(4): 369- 377.
16. Ahammed , S. K., Aggarwal, R., Sharma, S., Gupta, S., Bashyal, B. M. Production, partial purification and characterization of extra-cellular beta-1,3-glucanase from *Chaetomium globosum* and its antifungal activity against *Bipolaris sorokiniana* causing spot blotch of wheat. *J. Mycol. Plant Pathol* 2012; **42**:146-152.
17. Kanokmedhakul, S., Kanokmedhakul, K., Nasomjai, P., Loungsysouphanh, S., Soytong, K., Sobe, M., Kongsaeree, K., Prabpai, S., Suksamran, A. Antifungal azaphilones from the fungus, *Chaetomium cupreum* CC 3003. *J. Natural Products* 2006; **69**:891-895.
18. Di Pietro, A., Gut-Rella, M., Pachlatko, J. P.,

Schwin, F. J. Role of antibiotics produced by *Chaetomium globosum* in biocontrol of *Pythium ultimum*, a causal agent of damping off. *Phytopathol* 1992; **82**: 131-135.

19. Singh, D. P., Sharma, A. K., Tewari, A. N., Singh, K. P., Singh, A. K., Singh, R. N., Singh, S.P., Kalappanawar, I. K., Dodan, D. S. and Singh, V. K. Assessment of losses due to leaf blight in popular varieties of wheat under different sowing conditions and agro climatic zones in India. *Indian J. Agric. Sci.* 2004; **74**(2): 110-113.
20. Prabhu, K. V., Gupta, S. K., Charpe, A.., Kaul, S., Cherukuri, D. P., Dhaliwal, H. S., Vikal, Y., Chunneja, P, Haq, Q. M. R Molecular marker detects redundancy and miss-identity in genetic stocks with alien leaf rust resistance genes *Lr32* and *Lr28* in bread wheat. *J. Plant Biochem. & Biotech* 2003; **12**: 123-129.
21. Mandal, S, Srivastava, K. D., Aggarwal, R., Singh, D. V. Mycoparasitic action of some fungi on spot blotch pathogen (*Drechslera sorokiniana*) of wheat. *Indian Phytopath.* 1999; **52**: 39-43.
22. Park, J. H., Gyung, J., Choi, K. S., Jang, H., Kyoung, L. Kim, H. T., Kwang, Y. C., Kim, J. C.Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum. FEMS Microbiol. Lett.* 2005; **252**(2): 309–313.
23. Aggarwal, R., Singh, V. B., Shukla, R., Gurjar, M. S., Gupta, S., Sharma, T. R. URP-based DNA Fingerprinting of *Bipolaris sorokiniana* isolates causing spot blotch of wheat. *J. Phytopathol.* 2010; **158**: 210–216.
24. Purwar, S., Gupta, S. M., Kumar, A. Enzymes of phenyl propanoid metabolism involved in strengthening the structural barrier for providing genotype and stage dependent resistance to Karnal bunt in wheat. *Am. J. Pl. Sci.*, 2012; **3;** 261-267.
25. Weihong, L., Saint, D. A. Validation of a quantitative method for real time PCR kinetics. *Biochem.Bioph. Res. Co.* 2002; **294**: 347-353.