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Bio-control ability of *Trichoderma* species against spot blotch disease (wheat) causing pathogen *Bipolaris sorokiniana* under *in vitro* condition

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ABSTRACT

Spot blotch disease of wheat caused by Bipolaris sorokiniana (Shoem.) occurs every year in plain areas of Nepal causing considerable losses of grain yield. Several Trichoderma species possess antagonistic ability against different pathogens. In-vitro studies were conducted to explore the natural antagonistic aptitude of selected Trichoderma species against B. sorokiniana. Dual culture technique was followed to assess the antagonistic ability of five isolates of Trichoderma species against the pathogen. The experiment was conducted following Completely Randomized Design (CRD) with four replications. Radial growth of B. sorokiniana in dual culture with selected isolates of Trichoderma species was measured to compare the antagonistic ability of the selected Trichoderma species. Effects of interaction on viability of mycelium and on sporulation ability of the pathogen were assessed. Prominent antagonistic ability of Trichoderma species was identified from in-vitro dual culture studies. The evaluation of five isolates of Trichoderma species revealed that all the isolates significantly reduced the growth of B. sorokiniana in dual culture. Trichoderma sp. significantly inhibited the growth of B. sorokiniana by entirely covering the colony without completely killing the mycelia; however, the B. sorokiniana's mycelia obtained from overlapped area of the isolates had significantly lower viability than the control. Trichoderma species also significantly reduced the sporulation ability of B. sorokiniana. The tested isolates of Trichoderma spp. had sturdy antagonistic ability against B. sorokiniana under in vitro condition. However, to exploit the isolates for bio-control of spot blotch disease under field conditions, further studies under in-vivo conditions must be needed.

Key Words: *Antagonist, Dual culture, Radial growth, Sporulation and Viability*

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I. Introduction

Spot blotch disease caused by *Cochliobolus sativus* (Ito & Kurib.) Drechsler ex. Dastur [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.] is a serious barrier for optimum yield of wheat in Nepal. The

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disease is most problematic in hot and humid plain regions of Nepal, and has been expanding in mid hill regions having moderate temperature. Spot blotch occurs every year in the warm wheat growing areas with an average yield loss of 15%, but it may reach up to 34% under farmers' field conditions (Sharma *et al.,* 2003; Sharma and Duveiller, 2006; Bhandari, 2013).

The use of host plant resistance and chemical pesticides are the widely used and successful methods of disease managements. However, the persistent and injudicious use of chemicals may create havoc to environment, human health and ecology, and the wide scale use of resistant genotypes may result in sudden breakdown of the resistant ability. Resistant genotypes and foliar sprays with triazole fungicides are effective against the disease (Bhatta *et al.,* 1997; Naitao and Yousan, 1997); nevertheless, unavailability of durable resistant genotypes and high cost of fungicides are major obstacles in the exploitation of these remedies among poor and marginal farmers of South Asia (Saari, 1997; Hobbs *et al.,* 1988). Poor management of the field and lack of effective biological control measures are the other major factors responsible for regular epidemics of the disease.

Search of a disease control measure that could manage the disease without degrading environment and human health has been a burning issue for last many years. Successful researches of last few decades reveal a new frontier of disease management through the use of living organism i.e. biological control. Biological control assumes special significance being an eco-friendly and cost effective strategy which can be used in integration with other strategies for a greater level of protection with sustained yields.

Different strains and races of a micro-organism possessed different levels of antagonistic ability against a pathogen (Ozlem and Gary, 2003; Aggrawal, 2006). Some bio-control agents are effective under both *in -vitro* and field conditions (Kawamata *et al.,* 2004; Adebanjo and Bankole, 2004; Harish *et al.,* 2007; Liggett *et al.,* 1997), whereas many other bio-control agents are ineffective under field conditions (Muthomi *et al.,* 2007; Perello *et al.,* 2002; Duczek and White, 1986). Few micro-organisms have antagonistic ability against the pathogen *B. sorokiniana* under *in vitro* conditions (Bello *et al.,* 2003; Perello *et al.,* 2002), and against various diseases caused by *B. sorokiniana* under field conditions (Zhang and Gary, 1999; Agrawal, 2006). Likewise, *Chaetomium* sp., *Paecilomyces* sp. and some other saprophytes possessed antagonistic ability against spot blotch pathogen *B. sorokiniana* (Perello *et al*., 2001; Perello *et al.,* 2002; Biswas *et al.,* 2003). However, very few studies have been carried out to explore the bio-control agents for spot blotch pathogen in the most problematic areas of south Asia. Therefore, this study was carried out to explore the antagonistic ability of some commercial and local *Trichoderma species* against spot blotch causing pathogen *B. sorokiniana* under *in-vitro* condition at Khumaltar, Lalitpur, Nepal.

II. Materials and Methods

Five different strains of *Trichoderma species* were taken to assess their antagonistic ability against *B. sorokiniana* under *in-vitro* condition at Lalitpur, Nepal.

Isolation and preparation of pure culture: Spot blotch infected and blighted leaves were collected from various wheat fields of Kathmandu valley. Cut pieces of infected and blighted leaf samples were washed, surface sterilized with sodium hypochlorite solution and again finally washed with sterilized distilled water. Sterilized cut pieces of blighted wheat leaf samples were kept in Petri plates containing 3-layered moistened filter papers. The Petri plates were then incubated at 25 ± 1^0 Celsius temperature for seven days. Pure culture of *B. sorkiniana* was prepared following single spore isolation method. Pure cultures of *Trichoderma spp*. were obtained from Plant pathology Division, Khumaltar, NARC and multiplied for experimental purposes.

Dual culture of *B. sorokiniana* and the *Trichoderma spp.*: Dual culture studies as suggested by Morton and Stroube (1955) were carried out under *in vitro* condition to assess the interactions between various *Trichoderma spp.* and *B. sorokiniana*. The study was conducted in Completely Randomized Design (CRD) with four replications. Seven mm diameter mycelial plugs taken from the growing edge of two weeks old pure cultures of *B. sorokiniana* and the *Trichoderma spp.* were inoculated in PDA media in Petri plates. The two plugs were placed with upside down position on

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opposite sides in a Petri dish of 90 mm in diameter at equal distance from each other and from the periphery. The inoculated Petri dishes were incubated at room temperature for 21 days. The effect of interactions between *Trichoderma spp* and *B. sorokiniana* on radial growth of *B. sorokiniana* was measured. The radial growth of *B. sorokiniana* in dual culture with bio-control agents and in sole culture was measured three times at seven days intervals.

Effects of antagonism on sporulation of *B. sorokiniana*: The experiment was conducted in CRD with four replications. The dual culture and control plates were incubated for three weeks at room temperatures. After three weeks, 5 mm diameter plugs of *B. sorokiniana* were cut from the interfaced area of colonies of two fungi. The plugs were also obtained from control plates. Each plug was transferred to separate test tubes containing ten ml sterilized distilled water, and the test tubes were shaken for 10 minutes to make suspension of spores. The suspension was then filtered with a cheese cloth. One ml of the suspension was poured in a nematode counter and total number of spores in one ml of suspension was counted under microscope. The data was analyzed using Genstat software. Normal colony growth data were analyzed directly using ANOVA for CRD. Means were compared using Least Significant Difference and critical difference procedure.

Effects of antagonism on viability *B. sorokiniana* mycelium: The experiment was conducted in CRD with four replications. The dual culture and control plates were incubated for three weeks at room temperatures. Ten mycelial discs of *B. sorokiniana* (5 mm in diameter) from the margin of its colony at the interface zone with the bio-control agents were taken from each dual culture plate after 21 days of incubation. Ten mycelia discs were also taken from each plate of control treatments. The discs were transferred to plane agar plates with four replications, and incubated for 10 days at room temperature. The growth of *B. sorokiniana* was observed under microscope after 10 days of transfer.

III. Results and Discussion

Effects on radial growth of *B. sorokiniana*

Analysis of variance revealed that the treatments were differed significantly (P <0.01) for radial growth of *Bipolaris sorokiniana* in both the years (Table 01). All the tested *Trichoderma species* significantly reduced the radial growth of *B. sorokiniana* under *in vitro* conditions as compare to the control. The antagonistic effect of *Trichoderma* spp. was significant in all the three date of scoring. It shows that antagonistic activities of *Trichoderma* spp. was initiated after a week of dual culture and remain active till third week of dual culture. The result concludes that all the tested bio-control agents have excellent antagonistic ability against *B. sorokiniana* under *in vitro* conditions. The images of *in vitro* interactions between *B. sorokiniana* and the bio-control agents are shown in Figure 01.

Table 01. Effects of *Trichoderma* species on radial growth of *Bipolaris sorokiniana* under dual culture

Bio-control agents	Mean radial growth of *B. sorokiniana* after 7 days of dual culture		Mean radial growth of *B. sorokiniana* after 14 days of dual culture		Mean radial growth of *B. sorokiniana* after 21 days of dual culture	
	2012	2013	2012	2013	2012	2013
Soil guard	9.50	10.25	9.75	10.50	9.75	10.50
Niprot	12.00	10.75	12.50	11.50	12.50	11.50
local isolates (T69)	12.25	6.50	13.00	8.25	13.00	8.25
Trichoderma koningii	8.75	5.25	10.50	7.25	10.50	7.25
Trichoderma sp. (Local isolates)	10.75	10.00	13.50	10.25	13.50	10.25
Control (Only BS)	16.75	18.75	34.25	21.00	34.25	22.25
CV%	*17.8*	*14.2*	*11.80*	*14.3*	*11.80*	*15.00*
LSD	*3.09*	*2.158*	*2.72*	*2.432*	*2.72*	*2.597*
P value	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*	*<0.01*

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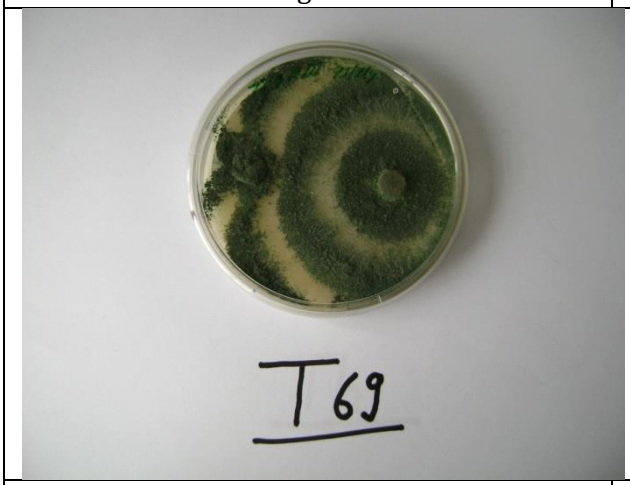
All the *Trichoderma* spp extended directly towards the *B. sorokiniana* colony with a moderate growth rate, and finally overgrew the colony of *B. sorokiniana* within two weeks of dual culture. The growth of *B. sorokiniana* was higher in first week of dual culture as compare to the remaining two consecutive weeks. It reveals that the antagonistic ability of bio-control agents increases as their maturity increases. The radial growth of *B. sorokiniana* was least in dual culture with *Trichoderma koningii* and Local isolates (T69). The results suggest that *Trichoderma koningii* and Local isolates (T69) have robust antagonistic ability against spot blotch fungus *B. sorokiniana.* It also indicates that some of the local Nepalese isolates of *Trichoderma* species are superior to commercial *Trichoderma spp* against *B. sorokiniana*; therefore, the exploration of local bio-control agents against targeted pathogens must be extensively carried out throughout the country

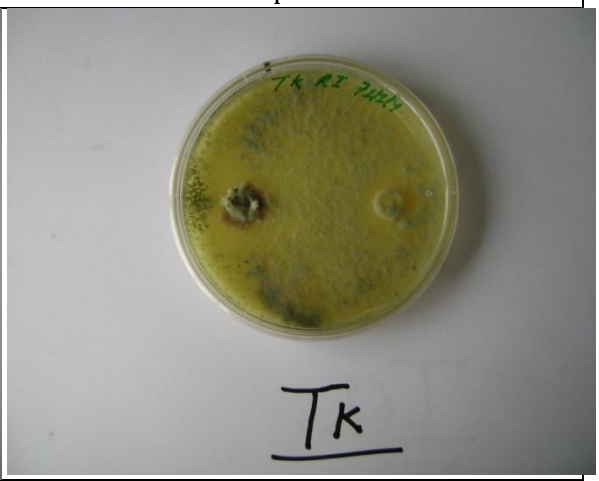
Several species of *Trichoderma* have been tested against various plant pathogens and have been found as a vital source of bio-control measures. The outcomes of several researchers are in accord of our results; however, the mode of action of *Trichoderma koningii,* Local isolates (T69) and other *Trichoderma spp* were not addressed in our study. Plant pathogens controlled by *Trichoderma* species include *Gaeumannomyces graminis tritici, Phythium spp., Botrytis cinerea, Colletotrichum truncatum, Cylindrocladium floridanum, Phytophthora citrophthora, Sclerotinia sclerotiorum* and numerous other fungal pathogens of economically important plants (Kubicek and Harman, 1998). The mechanism of bio-control by *Trichoderma* spp. includes competition for nutrients and sites, mycoparasitism, antibiosis, enzymatic action and hyper parasitism. In addition, a number of recent papers indicate that induced resistance is a widespread and important mechanism governs by *Trichoderma* species to control plant pathogens (Harman *et al.,* 2004). The strong bio-control activities of *Trichoderma* species under *in vitro* and in rhizosphere has been already proved, but the effect of the bio-control agents under field condition on foliage is still suspicious (Kubicek and Harman, 1998).

Soil guard

Niprot



Local isolates (T69)



Trichoderma koningii

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Trichoderma sp. (Local isolates)	Control (Only *Bipolaris sorokiniana*)

Figure 01. Interaction effects of selected *Trichoderma spp* and *Bipolaris sorokiniana* in dual culture under *in-vitro* condition during 2012 at Khumaltar, Kathmandu.

Effects of interaction on sporulation of *B. sorokiniana*

There was significant ($p<0.01$) effects of *Trichoderma* spp. on sporulation of *B. sorokiniana* in dual culture as compared to the control (Table 02). All the tested bio-control agents significantly reduced the sporulation of *B. sorokiniana* under *in vitro* conditions. The results reveal that the antagonistic ability of the tested bio-control agents not only hinders the vegetative growth, but also inhibits the reproductive ability of *B. sorokiniana.* Local isolates *T69* and *T. koningii* significantly suppressed the sporulation of *B. sorokiniana* as compared to the other bio-control agents and the control. It suggests that the bio-control agents who have higher ability to inhibit the vegetative growth of *B. sorokiniana* also have higher ability to inhibit the reproductive growth of the pathogen.

Table 02. Effect of interaction of *Trichoderma spp* and *B. sorokiniana* on sporulation ability of *B. sorokiniana* under dual culture

		Mean numbers of spore /ml (,000)	
S.No.	Pair of fungi in dual culture	2012	2013
1	Soil guard + *B. sorokiniana*	8.30	7.70
2	Niprot + *B. sorokiniana*	9.20	8.20
3	T69 + *B. sorokiniana*	3.50	3.40
4	*T. koningii* + *B. sorokiniana*	2.10	1.90
5	*Trichoderma sp.* (Local) + BS	7.40	6.90
6	Control (Only BS)	12.40	11.70
	CV%	*7.5*	*7.5*
	LSD	*0.80*	*0.74*
	P value	*<0.001*	*<0.001*

Effect of interaction on viability of *B. sorokiniana*

All the tested *Trichoderma* species significantly ($p<0.01$) reduced the viability of *B. sorokiniana*'s mycelia in the dual culture (Table 03). More than 30% samples of mycelia transferred from the margin of *B. sorokiniana* colony at the junction with *T. koningii* and Local isolate T69 had lost their viability at 21 days of dual culture. The ability of the two bio-control agents to decimate the viability of *B. sorokiniana* was higher than the other bio-control agents and the control. It reveals that these two antagonists have higher abilities to demolish the mycelia of *B. sorokiniana*; however, further study is needed to know the mechanisms of decimation, because *Trichoderma* spp. destroys the fungal host by different mechanisms based on pathogens (Gary *et al.,* 2004; Patibanda and Sen, 2007). In myco-parasitism, *Trichoderma Species* produce powerful extra cellular lytic enzymes, which exert necrotrophic action on fungi through lysis of cell walls. Many reviews of bio-control potentials of *Trichoderma* species showed that the most effective mode of action is the lytic activity of fungus on pathogen's cell walls (Chang *et al.,* 1986).

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On the other hand, the percentage of viable mycelia of *B. sorokiniana* transferred from the junction of the *Trichoderma* sp. was substantial (Table 03). The result verifies that the bio-control ability of *Trichoderma* sp. might be also due to the mechanisms other than myco-parasitism. The inhibitions in viability of mycelia along with the inhibition in colony growth signify that the antagonisms of the tested bio-control agents against *B. sorokiniana* are due to myco-parasitism as well as due to the production of some inhibitory substances and competition for space and nutrients. In accord of our outcomes, Harman *et al.* (2004) concluded that the mechanism of bio-control of *Trichoderma* spp. includes competition for nutrients and sites, myco-parasitism, antibiosis, enzymatic action and hyper parasitism.

In addition, *Trichoderma* spp. is known to produce certain volatile and nonvolatile antibiotic metabolites in culture and at sites of interaction with plant pathogens. The metabolites reported to be produced by *Trichoderma* spp. include gliotoxin, gliovirin, viridin, trichodermin, peptide-containing antibiotics, and possibly several other unknown antibiotics. Moreover, several enzymes, including cellobiase, chitinase, endoglucanases, lipase, and protease, which are involved in the mechanism of bio-control activity, are produced by *Trichoderma* spp. (Chang *et al.,* 1986).

Table 03. Effect of *Trichoderma* spp on viability of *B. sorokiniana* under *in-vitro* condition in dual culture

S. No.	Bio-control agents	Combined mean viability of mycelia (%)
1	Soil guard + *B. sorokiniana*	85
2	Niprot + *B. sorokiniana*	80
3	T69 + *B. sorokiniana*	65
4	*T. koningii* + *B. sorokiniana*	60
5	*Trichoderma sp.* (Local) + *B. sorokiniana*	75
6	Control (Only *B. sorokiniana*)	100
	CV%	*3.59*
	CD (0.01)	*5.67*
	P value	*<0.01*

Mean spread of *Trichoderma spp.* in dual culture

The growth rate of *Trichoderma koningii* and Local isolates T69 was highest among the tested isolates, and they covered more than 90% areas of PDA plate with in the first week of dual culture (Table 04). Most of the tested bio-control agents surrounded the pathogen's colony, and finally overgrew the pathogens colony within 14 days of dual culture. The result suggests that the growth rate of different bio-control agents is different based on their natural ability; however, our study did not address the reasons of different growth rate of bio-control agents.

Table 04. Mean spread of various *Trichoderma spp.* under dual culture

Bio-control agents	% Cover in 7 days	% Cover in 14 days	% Cover in 21 days
Soil guard	86	100	100
Niprot	88	100	100
Local isolates (T69)	90	100	100
Trichoderma koningii	90	100	100
Trichoderma sp. (Local isolates)	84	100	100
Control (Only BS)	00	00	00

IV. Conclusion

All the tested *Trichoderma species* (bio-control agents) have sturdy antagonistic ability against spot blotch disease causing fungi *B. sorokiniana* under *in vitro* conditions. All the *Trichoderma species* reduce the growth, sporulation and viability of *B. sorokiniana* under *in vitro* conditions. Among the tested bio-control agents, *Trichoderma koningii* and Local isolates (T69) were more aggressive than the other. The tested bio-control agents had lethal antagonistic ability, so they might possess myco-parasitism against *B. sorokiniana.* In addition, the tested bio-control agents also inhibited the growth

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of *B. sorokiniana*, which might be due to the production of antibiosis or competition for nutrients or space or both. Local Nepalese isolates of *Trichoderma* species had strong bio-control ability against *B. sorokiniana*, so exploration of local isolates of bio-control agents should be given high priority. The study provides basic information on bio-control ability of some commercial and some local *Trichoderma* species against *B. sorokiniana;* however, further test under *in vitro* and *in vivo* conditions should be needed for the enhancement in their efficiency and for the verification of their bio-control ability under field conditions.

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