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RESEARCH ARTICLE

DUAL CULTURE APPROACH FOR THE EVALUATION OF ANTAGONISTIC ACTIVITY OF NATIVE TRICHODERMA ISOLATES AGAINST BIPOLARIS SOROKINIANA

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Abstract

Spot blotch disease caused by *Bipolaris sorokiniana (Sacc.)* shoem. is one of the most destructive diseases of wheat (*Triticum aestivum L.)* in plain areas of Nepal. A laboratory experiment was conducted to evaluate the antagonistic activity of seven test *Trichoderma* isolates obtained from the soil sample by employing dual culture method against spot blotch pathogen of wheat *Bipolaris sorokiniana* at Plant Pathology Division, Nepal Agriculture Research Council, Khumaltar, Nepal. The experiment was laid out in a Completely Randomized Design (CRD). The result revealed that all *Trichoderma* isolates showed control over the pathogen. Isolate T517 stands out to be the most effective one giving 67.81% mycelial growth inhibition but isolate T528 was the least effective. Native *Trichoderma* can have significant biocontrol potential and may provide an effective solution against plant fungal diseases.

Introduction:-

Spot blotch disease caused by *Bipolaris sorokiniana* (Sacc.) shoem. is one of the prime concerns for wheat (*Triticum aestivum L.*) production in plain areas of Nepal. The fungus is transmitted by seed (Sultana and Rashid, 2012) and multiplies asexually (Malaker et al., 2007). Grain is the common substrate for the pathogen. It targets the necrotic tissue of the wheat plant and survives in the infected stubble and survives as a saprophyte on dead tissues (Manandhar et al., 2016). Excessive fertilization, particularly nitrogen fertilization, promotes disease progression by encouraging vegetative growth, which increases transportation and accelerates plant water stress. High temperatures and relative humidity favor disease outbreak, especially in South Asia's "intensive irrigated wheat–rice production systems," where rice favors the rapid multiplication of the foliar blight pathogen (*Bipolaris sorokiniana*) and rice stubble serves as a substrate for the fungi after rice harvest (Kumar et al., 2002).

In 1996, a study was conducted in Nepal to investigate and identify the national issue of 'poor germination in wheat' which confirmed *Bipolaris sorokiniana* as a major responsible factor (Shrestha et al., 1997). *Bipolaris sorokiniana* induced spot blotch disease causes severe yield losses to wheat crops in South East Asia (Saari, 1998). Yield loss range from 23 – 40% depending on the genotypes and other environmental factors (Bhandari and Tripathi, 2005; Sharma and Duveiller, 2006) in warm regions of Nepal. This disease has been increased in areas having the moderate temperature too and will be a problem in future for wheat growers of Kathmandu valley and similar mid-hills regions of Nepal (Bhandari, 2017). Therefore, there is an urgent need for constant improvement of crop yield to prevent this disease. Progress in rust resistance breeding through the deliberate use of vertical genes in

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various epidemiological zones around the country has helped to prevent rust outbreaks, but the same variables have also fostered the spread of diseases like spot blotch (Savary et al., 1997).

Chemical usage in agricultural farming has resulted in various threats to the environment and human health in recent years. Biological agents may be essential components in the control of *Bipolaris sorokiniana* if effective and reliable formulations were widely accessible and used with chemical fungicides. *Trichoderma spp.* has been used as a biocontrol agent for several years and has recently gained popularity. *Trichoderma* species have been extensively researched for their antagonistic activity against various plant diseases. However, there is little or limited information on the effectiveness of *Trichoderma* species against *Bipolaris sorokiniana*-caused wheat spot blotch disease. It's important to note that not all *Trichoderma* strains are effective in controlling plant diseases. The ecological adaptation of various *Trichoderma* species varies. Exotic *Trichoderma* species may not function for native diseases owing to issues like climate adaptation and soil colonization, thus it is critical to identify *Trichoderma* with biocontrol capability, which may be done by in vitro testing subsequently followed by pot and field trials.

Materials And Methods:-

To create the test pathogen, cells from a previous year's pure culture were transferred to Petri-dishes containing fresh growth medium (Potato Dextrose Agar) and incubated at 25^{o}C for 6 days (*Bipolaris sorokiniana*). The pure culture was isolated from infected leaves of wheat crop showing characteristic blight symptoms in the research field of Plant Pathology Division (PPD) under Nepal Agriculture Research Council (NARC).

Twenty-four soil samples were gathered from various geographical locations of Nepal, including the Hilly and Terai regions. Fresh and decaying soil samples were collected from old and new heaps in clean polythene bags cultivated with crops such as potato, banana, areca nut, lentil, gerbera, garlic, mustard, cardamom, and wheat from various parts of the country, including Jhapa, Chitwan, Kavre, Dang, Banepa, Kapurkot, Itahari, Dharan, Morang, Dhading, Gorkha, Biratnagar, Sindhupalchowk, Kathmandu, Baglung, Parbat and Pokhara. Soil samples were taken primarily from a zone that had only been exposed to organic fertilizer with no pesticides, fungicides, or other chemical agents. It is because biocontrol agents are limited in an agricultural area where chemical fertilizers are used excessively, resulting in the inhibition of biocontrol agents already present in the soil. The soil samples used in this study for Trichoderma isolation were named and registered in PPD as 251, 367, 386, 396, 392, 404, 406, 420, 434, 436,437,425,496, 498,501, 503, 506, 508, 509, 510,516, 517, 528, and 530.

Trichoderma colony was isolated employing the serial dilution method from 24 soil samples available in PPD, NARC. For the serial dilution, conical flasks were labeled with the numbers representing soil samples and test tubes were labeled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} up to 10^{-5} for each number of soil samples. In each test tube, 9 ml of distilled water was poured and in each conical flask, 45 ml of distilled water was poured. Test tubes and conical flasks were plugged with non-absorbent cotton and were sterilized in an autoclave.

After cooling, the initial solution was prepared in conical flasks by adding 5 gm of representative soil sample. Contents were mixed in a shaker for 30 minutes to obtain uniform distribution of the soil sample. From the solution in conical flasks, 1000 µl of suspension was transferred to the tube labeled as 10-1 having 9 ml sterilized water with a freshly sterilized pipette. Again same procedure was repeated, till the original sample was diluted to 10-5. Each time freshly sterilized pipette was used. After the serial dilution, with the help of a sterilized pipette, 100 µl of suspension from the test tube (10-1, 10-3, and 10-5) was placed in the center of TSM. In an aseptic condition, the suspension in TSM was spread using a sterile glass rod. Plates were labeled with the dilution and soil number, inverted, and incubated in an incubator at 30^{o}C for a week. Incubated plates were observed every day for the growth of *Trichoderma spp.*

To isolate the test *Trichoderma* from several colonies of microorganisms, preliminary screening for *Trichoderma* was carried out by observing both macroscopic and microscopic features of the colonies. Confirmation about the identity of the *Trichoderma* colony was done and approved by Plant Pathology Division, NARC, Khumaltar.

Among 24 soil samples tested, *Trichoderma* isolates were obtained from 7 soil samples. Antagonistic activity of seven test *Trichoderma* isolates was evaluated in-vitro for their biocontrol efficacy against *Bipolaris sorokiniana* by

dual culture method. There were three replications for each treatment with a control test and the experiment was arranged in a complete randomized design (CRD).

For the dual culture method, 20 ml of prepared PDA was poured into each Petri plate and allowed to solidify. After solidification of PDA media, 5 mm diameter mycelial disc from the margin of a three-day-old culture of *Trichoderma* isolates and seven days old test pathogen (*Bipolaris sorokiniana*) were placed on the opposite ends of the plate at equal distance from the periphery on the same day. In the control plate, the test pathogen was kept without an antagonist. The experiment was performed in complete aseptic condition and both the control plates and test plates were incubated at 25 °C for six days. The colony diameter of radial growth of targeted fungal pathogens was measured on the 2nd, 3rd, 4th day, 5th day, and 6th day after inoculation at two locations from the center of the test placed with the help of vernier caliper, and average diameter were calculated. Finally, percent growth inhibition of the pathogen was calculated by using the following formula of Vincent (1947).

$$I\ \% = \frac{C-T}{C} \times 100$$

Where,
I= inhibition percentage
C= Colony diameter in control and T=Colony diameter in treatment

The data collection started from 48 hours after dual culture i.e. 26th February to 4th of March.The data were recorded by measuring the growth of the test pathogen after each 24 hours by using Vernier caliper in mm. The data obtained from the experiment were analyzed using the software Gen Stat for the analysis of variance (ANOVA) to test the significance of treatments effect on mycelial growth of Bipolaris sorokiniana. Means of significant treatments at 5% level of significance were compared following Duncan's Multiple Range Test (DMRT) and Microsoft Excels.

Results And Discussion:-

Isolation of *Trichoderma* isolates:

Out of 24 soil samples tested, *Trichoderma* isolates were obtained from 7 soil samples. *Trichoderma* colonies were present in 1:10 dilution in case of all soil samples. *Trichoderma* colonies were obtained from (29%) of the total 24 soil samples using serial dilution method. Other microorganisms excluding *Trichoderma* were present in (71%) of the remaining soil samples as shown in Figure 1.

Table 1:- Number of *Trichoderma* colonies obtained from soil samples.

SN.	Soil samples	Location	Date of serial dilution	Date of isolation	Dilution	No. of colonies obtained
1.	501	Charkose Jhadi, Dharan	Jan 24, 2021	Feb 1, 2021	10^{-1}	3
2.	503	Biratnagar, Morang	Jan 24, 2021	Feb 1, 2021	10^{-1}	4
3.	506	Biratnagar,Morang	Jan 24, 2021	Feb 1, 2021	10^{-1}	2
4.	516	Dhobighat, Kathmandu	Jan 26, 2021	Feb 3, 2021	10^{-1}	2
5.	517	Kirtipur, Kathmandu	Jan 26, 2021	Feb 3, 2021	10^{-1}	2
6.	528	Baglung, Pokhara	Jan 26, 2021	Feb 3, 2021	10^{-1}	4
7.	530	Kusma, Parbat	Jan 26, 2021	Feb 3, 2021	10^{-1}	2

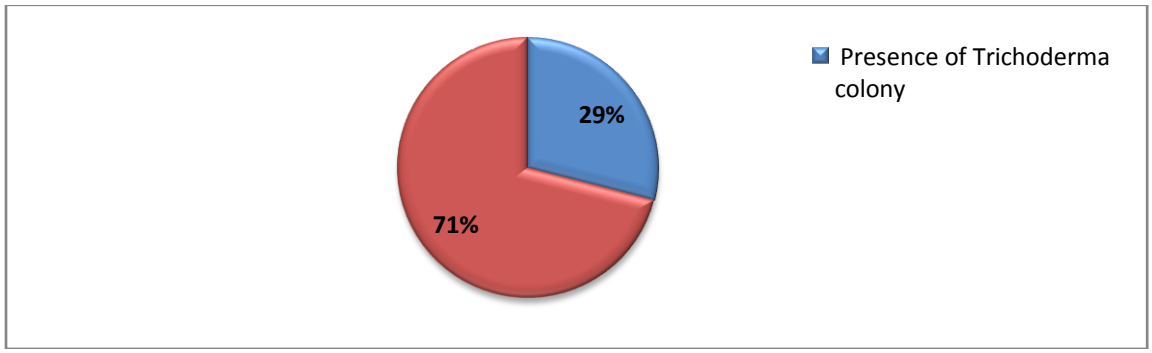


Figure 1:- Presence of *Trichoderma* colonies in percentage.

Effect of *Trichoderma* isolates on the radial growth of mycelium of *Bipolaris sorokiniana* by dual culture method

In the table below, *Trichoderma* isolates are named after the soil sample from which they are obtained. Data were transformed to standard unit (cm) to avoid statistical complications during analysis. Dual culture test showed that all isolates of *Trichoderma* tested inhibited the growth of *Bipolaris sorokiniana.*

Table 2:- Mean radial growth of mycelium of *Bipolaris sorokiniana* in presence of *Trichoderma* isolates.

	Mean radial mycelial growth (cm)				
Treatment	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
T501	1.16 [c]	1.83 [b]	2.21 [b]	2.28 [b]	2.35 [bc]
T503	1.36 [b]	1.67[c]	1.78 [e]	1.89 [d]	1.96 [d]
T506	1.16 [c]	1.68 [c]	1.78 [e]	1.83 [d]	1.88 [d]
T516	1.20 [c]	1.94 [b]	2.05 [c]	2.34 [d]	2.42 [b]
T517	1.13 [c]	1.71 [c]	1.76 [e]	1.80 [b]	1.86 [d]
T528	1.23 [c]	1.87 [b]	1.92 [d]	2.01 [c]	2.12 [cd]
T530	1.19 [c]	1.50 [d]	1.80 [de]	1.87 [d]	2.01[d]
Control	1.55 [a]	2.10 [a]	3.03 [a]	4.18 [a]	5.80 [a]
Grand Mean	1.25	1.79	2.05	2.27	2.55
LSD	0.1199	0.1070	0.1182	0.1138	0.2379
CV	5.50%	3.50%	3.30%	2.90%	5.40%
SEm(±)	0.04	0.0357	0.0394	0.0380	0.0793
P value	<0.001	<0.001	<0.001	<0.001	<0.001

CV:Coefficient of variation, LSD: Least significant difference, Means followed by the same letter in a column are not significantly different by Duncan's Multiple Range Test at 5% level of significance, SEm (±) indicates standard error of the mean, cm is centimeters

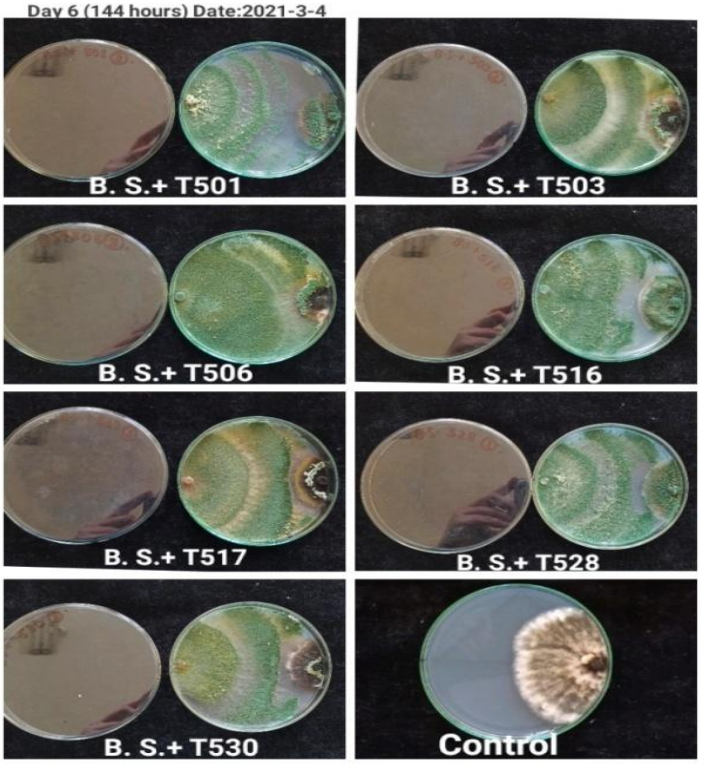
Means with the different letters are significantly different at 5% level of significance using LSD. In (Table 2), different letters in a column signifies that the treatment means are significantly different with each other at P-value <0.001.

All *Trichoderma* isolates showed control over the pathogen, *Bipolaris sorokiniana* in varying degrees of difference with control treatment. Coefficient of variation (CV) are 5.5%, 3.5%, 3.3%, 2.9% and 5.4% on 2nd, 3rd, 4th,5th and 6th day respectively. The highest radial mycelial growth was observed in control i.e. 1.55, 2.10, 3.03, 4.18 and 5.18 cm on 2nd, 3rd, 4th, 5th and 6th day respectively which was statistically different with other treatments.

On Day 3, isolates T501, T516, T528 and T503, T506, T517 showed no significant difference whereas T530 was highly significant as compared to control.

On the last day (Day 6) of data collection i.e. 144 hours after inoculation, the significant isolates are T517, T506, T503, T530, T528 and T501, T528 and T501, T516. T516 made the highest radial growth which was significantly higher as compared to other treatments followed by T501. Isolate T517 (2.423 cm) made very slow growth of the pathogen as compared to the control. Furthermore the effect of T517, T506, T503, T530, and T528 on the growth of *Bipolaris sorokiniana* is not statistically different. The results clearly demonstrated that the radial growth of the pathogen was highly influenced by the isolate T517 in all days of data collection as compared to the control treatment.

Radial mycelial growth observed on Day 3 of dual culture



Radial mycelial growth observed on Day 6 of dual culture

Table 3:- Effect of *Trichoderma* isolates on the inhibition percentage of *B. sorokiniana* by dual culture method.

Treatment	Mycelial growth inhibition (%)				
	Day 2	**Day3**	**Day4**	**Day5**	**Day6**
T501	25.16	12.86	27.13	45.38	59.48
T503	12.26	20.48	41.31	54.78	66.09
T506	25.16	20.00	41.31	56.22	67.53
T516	22.58	7.62	32.41	44.02	58.22
T517	27.10	18.24	41.97	56.94	67.81
T528	20.65	10.81	36.70	51.91	63.33
T530	23.23	28.57	40.65	55.26	65.34
Control	0	0.00	0.00	0.00	0.00

Table 3 shows that the maximum mycelial growth inhibition percentage is 27.10% by isolate T517, 28.57% by T530, 41.97 % by T517, 56.94 % by T517 and 67.81 % by T517 whereas the minimum mycelial growth inhibition percentage is 12.26% by T503, 7.62% by T516, 27.13% by T501, 44.02% by T516 and 58.22% by T516 on 2nd, 3rd, 4th, 5th and 6th day respectively. The mycelial growth inhibition percentage ranged between 58.22 to 67.81 % on Day 6 of dual culture. For the control of *Bipolaris sorokiniana*, T517 showed greatest inhibition percent (67.81%) of mycelia growth at 6th day of dual culture followed by T506 (67.53%) and T503(66.09%) as compared to other isolates while inhibitory effect of T516 was the lowest i.e 58.22% . Among the isolates, T517 in almost all days of data collection was found to be significantly superior showing highest mycelial growth inhibition throughout the whole experiment.

Biocontrol agent reduce the growth of fungal pathogen through one or more mechanism consisting of mycoparasitism, a competition for space and nutrients, antibiosis involving enzymes and secondary metabolites and elicitation of plant defense system (Thangavelu and Mustaffa, 2012). The growth of *Trichoderma* isolates were faster than the test pathogen *Bipolaris sorikiniana*. All the T*richoderma* isolates extended directly towards the *B. sorokiniana* colony with a moderate growth rate, and finally overgrew the colony of *B. sorokiniana.*

The antagonistic ability of bio-control agents increases as their maturity increases. It reached the pathogen mycelium within 144 hours and overgrew it, inhibiting further growth of pathogen. It could be due to the mechanism as described by Weindling in 1941. The presence and growth of *Trichoderma* isolates in dual culture controlled the growth of pathogen after *Trichoderma* entered the hyphae of the pathogen and produced extracellular proteolytic, glucanolytic and chitinase enzymes, which were responsible for release of bioactive molecules namely proteins and cell wall fragments and lysis of pathogen cell (Weindling, 1941). These released molecules and cell wall fragments were responsible for elicitation of induced systemic or localized resistance. *Trichoderma* grown over the pathogenic fungal hyphae coils around them and degrades the cell walls (Reddy et al., 2014). This action of parasitism restricts the development and activity of pathogenic fungi (Asad et al., 2014). The secondary metabolites produced by *Trichoderma* species such as volatiles and antibiotics were responsible for antibiosis (Thangavelu and Mustaffa, 2012). Further *Trichoderma* isolates also helped in removing the mycotoxin produced by the fungal pathogen.

Conclusion:-

All the tested *Trichoderm*a isolates (bio-control agents) have sturdy antagonistic ability. Among the tested *Trichoderma* isolates, Local isolate (T517) followed by (T506) were the most aggressive as compared to other treatments against the pathogen. Local Nepalese isolates of *Trichoderma* species have strong bio-control ability against *B. sorokiniana*, so exploration of local isolates of bio-control agents should be given high priority and exploit them in commercial formulations rather than to import biopesticides from other countries. The study provides basic information on bio-control ability of local *Trichoderma* species against *B. sorokiniana*.

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Conflict Of Interest
Author declares no conflict of interest regarding the publication of manuscript.

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