

SELECTION OF *IN VITRO* TECHNIQUE FOR PATHOGENICITY AND SCREENING OF WHEAT CULTIVARS AGAINST *BIPOLARIS SOROKINIANA*

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Abstract

Bipolaris sorokiniana (*Cochliobolus sativus*) is a seed and soil borne pathogen causing leaf blight or spot blotch of wheat worldwide. In Pakistan the pathogen was identified as predominant leaf spot causing fungus in wheat growing areas during 2004 – 2006. A lab based technique has been standardized to study the pathogenic nature of the pathogen, its variability/ aggressiveness and preliminary screening of cultivars/ germplasm before going to explore the sources of resistance under natural conditions. The purpose of this study was to adopt a time and cost effective methodology which can be referred as *In-vitro* technique. Five methods were used to select the best one and later that method was standardized. This may be used for pathogenicity test, to check the Pathogen variability/ aggressiveness of the pathogen and preliminary screening of varieties/ germplasm against the pathogen. These methods include pot and test tube experiments by application of inoculum and seed in soil simultaneously, by dipping roots in spore suspension before planting in soil, by foliage spray with spore suspension, seed coating with spore suspension before planting in test tube with cotton swab method. Out of these five procedures the test tube with cotton swab method has been found the most appropriate and effective for the study of *B. sorokiniana*.

Introduction

Bipolaris sorokiniana (*Cochliobolus sativus*) is a world wide economically important leaf blight / foliar spot causing pathogen of the wheat. It also causes root rot and black point on grains (Wiese, 1998), most frequently associated with poor germination and abnormal seedlings of wheat (Ammara *et al.*, 2001). The fungus is one of the constraints for crops in warmer growing areas and causing significant yield losses (Aftabuddin *et al.*, 1991). In north eastern and north western plains of India the yield losses ranged from 27 - 56.6% during 1998-99, incited due to the leaf blight caused by *B. sorokiniana* (Satvinder *et al.*, 2002; Singh *et al.*, 2002). Pathogen has also been reported to be the one of principal fungus involved in the seedling blight and root rot of wheat in Pakistan (Bhatti *et al.*, 1986; Hafiz, 1976; Kishwar *et al.*, 1992).

Different methods are being employed to study the *In vitro* disease development by different workers in the research world. These methods include pot experiments by using wheat seedlings and with different ways of inoculation (Lamari & Bernier, 1989), plantlets regeneration of embryonic callus through *In-vitro* method (Chang *et al.*, 1997) and test tube method by using inverted cone of filter paper (Kishwar *et al.*, 1992). To study the seed and soil borne pathogen different methods have been tried to check the pathogenic nature of the pathogen, its variability and aggressiveness and preliminary screening of cultivars/ germplasm before going for identification of source of resistance under natural conditions. The purpose of the current study was to standardize a procedure

by using the *B. sorokiniana* as a model organism having a seed and soil borne nature. The method can be used to see the response of the host to the pathogen, variation of the pathogen and its interaction to the host and varietal behaviour against the pathogen.

Materials and Methods

Five different methods were used for pathogenicity test to select the method which may be standardized for pathogenicity, pathogen variability/ aggressiveness analysis and screening of varieties against the seed and soil borne pathogen. During the current study, (*Bipolaris sorokiniana*) the most frequently isolated pathogen of leaf spot was used. The methodologies are:

Seed with inoculum disk method: Wheat seeds disinfected with 1% Clorox were sown in the autoclaved soil media (1:1:1 soil, sand and peat moss) in the plastic pots. Three seeds / pot were sown with a 5 mm disk of 7 days old fungal culture (approximately containing 3.2×10^4 spores/ disk). Disk was placed adjacent to seeds. The pots were incubated at 22 - 25°C in growth room by placing on the racks randomly. The data was recorded after 30 days by noting the spots on leaves on 0-5 scale (Anon., 1996) and confirmed the Koch's postulate.

Root Dip method: Wheat seeds were sown in autoclaved sand and watered daily. After 6-8 days of the sowing, the raised seedlings were removed from the sand very carefully so that roots remain intact. These seedlings were dipped in the spore suspension of *B. sorokiniana* (3.2×10^4 spores /ml) and were planted in pots, already filled with autoclaved soil containing silt loam, sand and peat moss (1:1:1). The pots were placed in growth room at 22 - 25°C and the symptoms observed after four weeks on 0-5 scale on foliage (Anon., 1996) and confirmed the Koch's postulate.

Spore suspension spray method: Pure culture of *B. sorokiniana* was obtained from single spore culture maintained on PDA. The mycelium of the full grown culture was scraped with a bent metal rod. Then conidial suspension was prepared by flooding the plates with sterilized distilled water and the spore concentration was determined with a haemocytometer (Lamari & Bernier, 1989). A drop of Tween-20 was added per 100ml of suspension before application. Five wheat seeds of Wafaq-2001 were sown in plastic pots containing sterilized soil (as prepared in above methods) and placed in a growth room. At seedling stage (at 2-3 leaf stage after planting for 17-18 days) plants were sprayed with a spore suspension containing 3.2×10^4 spore/ ml. Approximately 15 ml of inoculum was sprayed per plant with a sprayer. The plants were covered with plastic bag for attaining high humidity for 30 hours and then plants were placed in growth room at 22 - 25°C and the disease severity observed as above and confirmed the Koch's postulate.

Seed Coating method: Wheat seeds were surface sterilized with 1% Clorox and was rinsed thrice with sterilized distilled water. Few drops of Tween-20 were sprayed on these seeds and then were put on the actively grown culture of *B. sorokiniana*. The seeds were mixed in the mature culture with the help of rod so that maximum spores may stick with the seeds. Later these seeds were sown in the pots as in the above methods and placed in growth chamber. Disease severity was observed on 0-5 scale upon the appearance of spots on the leaves and confirmed the Koch's postulate.

Test Tube Cotton Swab Method: Test tubes (20 cm x 3 cm) were prepared by filling 1/4th of cotton in the bottom of the tube. Sterilized distilled water (20 ml) was added in each tube and lids were covered with aluminum foil and were autoclaved. Wheat seeds (three seeds/tube) were surface disinfected with 1% Clorox and placed on the moist cotton swab in the test tube. One disk of 5 mm of fungal isolate (3.2×10^4 spores/ disk) was placed adjacent to the seeds (Giri *et al.*, 2001). After inoculation tubes were again covered with aluminum foil and were placed in growth room at 25°C.

Standardization of method: Method was standardized by the application of two treatments.

i. Inoculum with seeds: Described as above (Giri *et al.*, 2001).

ii. Inoculum after initiation of roots: The seeds were first allowed to germinate on cotton swab. When the roots appeared and seedlings became 5 days old, the inoculum disc was placed on the roots. After 30 days when symptoms appeared, the data was recorded in same fashion as above. The data was recorded on a revised (0-5) scale (Anon., 1996). The revised scale was 0 = no symptoms, 1 = 1-5% spots on leaves, 2 = 6-20% spots on leaves, 3 = 21-40% spots on leaves, 4 = 41-60% spots on leaves, 5 = 61% and above spots on leaves. Later the data was subjected to analysis of variance and means interaction by Computer Soft Ware Package M-Stat-C.

Results and Discussion

Among the five *In-vitro* methods, four were conducted in pots and one in test tubes. Out of five methods cotton swab test tube method (Fig. 1) was found the best one for pathogenicity test and subsequently determined the most efficient method to be used for pathogenicity/screening/aggressiveness analysis. The results revealed that there was no significant effect of replications. However, there was a highly significant effect of isolate, method and isolate x method interaction (Table 1). The mean values of different methods revealed that M1 and M5 showed highly significant results as compared to one another and the rest of the methods. However there was no significant difference between M2, M3 and M4 (Table 2). The interactive means of the methods M1, M3 and M5 showed highly significant difference from one another while M2 and M3 had slightly significant difference. However M1 and M4 had no significant difference. The results showed that M5 had highly significant difference compared to other methods (Table 3).

After statistical analysis of the data it was found that the test tube moist cotton swab method proved to be the most effective and efficient method for pathogenicity test and to check the aggressiveness of the pathogen. The method is also more convenient for preliminary screening of the varieties/ germplasm before going to field. In our studies this method was specially compared with the method used by Lamari & Bernier (1989) which was later adopted by Iram & Ahmad (2005), in which the plants at seedling stage planted in pots needed to be sprayed with spore suspension with a particular concentration and for the maintenance of humidity the pots with plants need to be covered with plastic bags for 30 hours, whereas the test tube method proved to be more efficient as in this method there is no need to maintain humidity. The method was taking the long time to develop the symptoms and the symptoms developed were not as good as compared to the symptoms developed in test tube method (Fig. 2). Another advantage of this method was that the infection on the roots (if required) can be measured in the same experiment. So the seed and soil borne pathogens can be easily tested by this method.



Fig. 1. Cotton Swab Test Tube Method



Fig. 2. Symptom on wheat leaves during pathogenicity test by cotton swab test tube method.

Table 1. Analysis of variance of different methods applied for testing the most efficient one for pathogenicity test of *Bipolaris sorokiniana*.

Sources of variation	Degree of freedom	Sum, of squares	Mean square	
			Leaf	Root
Replication	4	1.00	0.250 NS	0.200 NS
Isolates	1	5.780	5.780**	3.920**
Methods	4	65.400	16.350**	13.400**
Isolate x method	4	2.520	0.630**	0.720**
Error	36	7.800	0.217	0.078

**Mean squares of isolates, methods and isolate x methods interaction are highly significant at probability level 1.

Table 2. Mean value of different methods applied for pathogenicity of *Bipolaris sorokiniana*.

S. No.	Methods	Mean values	
		Leaf	Root
1.	M1	1.900B	2.200B
2.	M2	1.300C	2.400B
3.	M3	0.900C	0.00C
4.	M4	2.200C	2.4..B
5.	M5	4.200a	3.00b
LSD 5%		0.4225	0.2533

Column means followed by a common letter are not statistically different at 5% by LSD test

*M1 = Seed + inoculum disk in the soil together.

*M2 = Root dip of seedlings in spore suspension.

*M3 = Spray of spores suspension on foliage at seedling stage.

*M4 = Seed + conidia/ spores (coated on seed).

*M5 = Test tube moist cotton swab method.

Table 3. Mean value of interaction of different methods applied for testing the most efficient one.

S. No.	Methods	Leaf (0-5 scale)	Root (0-3 scale)
1.	M1	2.600c	2.600
2.	M2	1.400de	2.800ab
3.	M3	1.000e	0.000
4.	M4	2.600a	3.000a
5.	M5	4.600a	3.000a
LSD value		0.5975	0.3580

Column means followed by a common letter are not statistically different at 5 %

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