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EEFICIENCY OF TWO SPORE COUNTING METHODS AND VALIDATION OF SSR MARKERS ASSOCIATED WITH SPOT BLOTCH RESISTANCE

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***Key words**: Bipolaris sorokiniana, spore load, QTLs, spot blotch and wheat*

Abstract–An experiment was conducted to determine the efficiency of two spores counting methods from wheat seeds. Seeds 40 genotypes collected from the Wheat Association mapping population from the Varanasi for the recovery of *Bipolaris sorokiniana* spores. The correlation(r) between these two methods was (0.8). Results indicated that Nematode Plate count Method (NPM) was superior to the Washing Test method (WT). The mean recovery of spore in the nematode count plate was significantly higher than the washing test method. In Nematode Plate Method, the spore count distributed with a mean of 126.47/50 seed while in the Washing Test, the spore count mean was 64.15. Recovery of different of black and mixed population was recorded from the seed. White population could not be detected from samples. In the present study, validation and utility of two SSR markers associated with spot blotch resistance in wheat (*Triticum aestivum* L.) was undertaken. Two primers were used for assessment of genetic variability among 40 wheat genotypes. The phenotypic data in the form of spore load of individual genotypes was compared with the genotypic data. The selected SSR primers showed a high level of polymorphism. *Xgwm148* produced 170 bp band in resistant cultivar, while *Xgwm111* amplified 150 bp band in resistant cultivar. SSR marker *Xgwm148* linked with *QTL, QSb.bhu-2B* and *Xgwm111* is linked with *QSb.bhu-7D*.

INTRODUCTION

Bipolaris sorokiniana (Sacc.) Shoem. *(Helminthosporium sativum),* telomorph *(Cochliobolus sativum),* the causal agent of spot blotch disease of wheat one of the major biotic stresses in the warm humid tropic encompassing many countries of the world such as India, Bangladesh, Nepal, Brazil, Argentina and Peru.(Kumar *et al*., 2002, Mathre, 1997; Duczek and Jones and Flort, 1994). The pathogen is ubiquitous in nature but highly seed borne and transmitted by wheat seed (Rashid and Neergaard, 1996; Fakir *et al*., 1997). While carrying and disseminating the pathogen seed itself be victimized, resulting the black point and shrivelled. Thus the pathogen lowers the market as well as planting value of the seeds which ultimately effect the cultivation of wheat (Rashid *et al*., 2003). Seed infection of *B. sorokiniana* causes germination failure with the coleoptiles and coleorhizae infection indicate the increasing trend of deteriorating planting value of the seed with the increasing rate of transmission of pathogen from seed to the germinating seedlings (Sultana and Rashid, 2012). Different morphological groups in *B. sorokinina* have been reported (Chand *et al*., 2004; Pandey *et al*., 2007) and associated with melanin content and survival of pathogen. Maritte *et al*., 1998 reported the selection of aggressive strain on resistant wheat genotypes. Transmission of the pathogen through the seeds is reported. However, variation in the wheat genotypes for spore load is not known. At the same time different morphological group associated with the seed is also lacking. The efficiency of seed counting method against established method for other pathogen is not compared. This experiment was taken to find out the answer of above questions.

MATERIAL AND METHODS

Collection of seeds of wheat genotypes

Two hundred ninty four genotypes grown for wheat association mapping were grown at 3 locations i.e.

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BHU, Varanasi, UBKV, Coochbehar and DWR, Karnal) in the year 2011-2012, Seed of all the genotypes were collected separately by hand threshing from all the locations. The seed of each genotype was packed in individual envelop with proper labeling and brought to Banaras Hindu University for the spore analysis. Out of 294 wheat genotypes, 40 genotypes were selected for the comparison between two methods of spore count. Based on the spore load data obtained by the Nematode plate count method, 40 genotypes were selected on the basis of mean number of spores. The 40 genotypes were divided in to 4 groups based on spore load of 50 seeds i.e. group 1 < 60 spore 2=.61-120, 3=121 -180, 4=> 180.

Quantification of spore load by using Nematode Counting Disc

Fifty seeds from each genotype was selected randomly from each replication and kept in a 10 ml test tube. These seed were submerged in 5 mL distilled water with Tween 80 50µl. (1ml/100 mL of water). Tubes were kept on the bench for 5 minutes and hand shaken end to end for 100 times to dislodge the spores. The same time and procedure was followed for all the samples. The spore suspension was taken in the nematode counting disc. The entire spores were counted under the microscope with the magnification of 10×5.

Quantification of spore load by centrifugation method (Washing test)

The washing test is a seed health testing method, which is used solely to test for externally seed borne pathogens, which is present loosely on the seed surface. The washing test is a qualitative test for which no standard working sample has been approved so far by the International Seed Testing Association (ISTA). In this method, 50 seeds were taken from each genotype random from each replication and kept in a 10 mL test tube. The seeds were submerged in 5 mL of distilled water and 50 µl. sterile Tween 20 was added to the each tube. The tubes were kept on a bench for 5 minutes. The tubes were shaken for 10 minutes on a mechanical shaker to dislodge the spores from the seed. The suspension was transferred to a 10ml tube and centrifuged at a speed of 3000 rpm for 10 minutes. Supernatant was discarded from the tube. The pellete contain the spores. One ml of pellet was separated in to 2 mL micro centrifuge tube. From this suspension, 200 µl of suspension was drawn with the help of a micro pipette and spotted on clean sterilised glass slide. This glass slide was observed under microscope (10×) and spore load was quantified. The same centrifugation speed and time were followed for all the samples. For every genotype samples were drawn from 3 replications, for each replication 5 slides were examined.

Monitoring of pathogen population form the seed

Four seeds from each genotype were randomly selected from each replication. Four seeds were inoculated in each plate containing Potato Dextrose Agar Medium. Inoculated plates were incubated at 25 C for 5 days. Appearance of *B. sorokiniana* colony was recorded with their morphological group (Chand *et al.* 2004).

Measurement of characters of wheat genotypes

Leaf angle

Leaf angle was measured two week after ear emergence (Joshi and Chand, 2004) in all the genotypes using a protractor, dividing the genotypes in four groups; erect (flag leaf non-drooping and making an angle of 60 to 90% with respect to the horizontal plane); semi-drooping (less than half the length of the flag leaf, from tip to base, was drooping); and drooping (more than half the length of flag leaf was drooping).

Days to heading

Days to heading (DH) was recorded when spikes of approximately 50% of the plants in a plot were fully emerged. Two replications were taken from the two plots.

Leaftip necrosis

Leaftip necrosis of flag leaf was recorded after the emergence of spike. If tip necrosis is present, it is *Ltn+* or otherwise, it is *Ltn-* (Joshi *et al.* 2002). Eight plants were taken from each plot and recorded the data for leaftip necrosis. The data was represented in percentage.

Molecular probing of the genotype with spot blotch resistant markers

Young leaves were collected from 15 days seedlings and immediately stored in 80 ^{0}C till further processing. The DNA was extracted following CTAB extraction method (Doyle and Doyle, 1984) with few modifications as described below. Two mg of leaf sample was placed in 1.2 ml collection micro tube. Two tungsten carbide beads (3 mm) were

dispensed with the help of tissue lyser bead dispenser in each micro tube and closed with micro tube caps. All microtubes were placed in the tissue lyser adapter set 2× 96 and pre cooled at -80 ° C for two hours. Pre cooled adapter sets were placed on to tissue lyser arms tightly. Leaf samples were disrupted and homogenized by operating tissue lyser for 30 sec at 30 Hz. After homogenization, 1ml pre warmed CTAB buffer (1M Tris base, 0.5M EDTA, 5M NaCl, CTAB 10%, β-mercaptoethonol, distilled water) was added to each sample and mixed vigorously. The homogenate was transferred in to fresh 2ml micro-centrifuge tube and incubated at 65°C in water bath for 1 hour with gentle shaking after each 10 minutes. After incubation, samples were taken out and kept at room temperature for few minutes to cool down.

Equal volume of Phenol : Chloroform : Isoamylalchohol solution (P: C: I, 25:24:1) was added in each micro centrifuge tubes. Samples were mixed gently by inverting the microfuge tubes for a period of 10 minutes at shaker (Bangalore Genei, Bangaluru). After shaking, samples were centrifuged at 13000rpm at room temperature. Supernatant was taken out and transferred to new microcentrifuge tube (2ml) without disturbing the middle layer. Five micro litre of RNAase A solution was added in each sample and incubated at 37°C for 45 min. Equal volume of chloroform: isoamylalchohol (C:I, 24:1) was added and mixed gently for 10 minutes at shaker and centrifuged at 13000 rpm at room temperature. Supernatant was taken out and transferred to new micro centrifuge tube (1.5 mL) and 2/3 rd volume of Isopropanol (chilled) was added. Tube was mixed gently by inverting the tubes. Samples were placed in -20°C for 30 minutes and centrifuged at 13000 rpm for 10 minutes at 4°C. Solution was discarded and 200 micro litre Ethanol (70%) was added and centrifuged at 13000 rpm for 10 minutes for washing the DNA pellets. The solution was discarded and tubes were inverted for overnight on blotting paper for drying the DNA pellets. DNA pellets were dissolved in 50 micro litre distilled water and stored at -20°C.

Polymerase chain reaction was performed to selectively amplify in vitro a specific segment of the total genomic DNA to a billion fold (Mullis *et al.,* 1986). The most essential requirement of PCR is the availability of pair of short (typically 20-25 nucleotides) primers having sequence complementary to either end of the target DNA segment (called template DNA) to be synthesized in large amount. The PCR conditions standardized. The amplified DNA fragments generated through SSR primers were resolved through electrophoresis in 2.5% agarose gel prepared in TAE (242 g Tris-base; 57.1 ml glacial acetic acid and 100 ml 0.5M EDTA (Ph 8.0) bring final volume to 1000 ml) buffer. Ethedium bromide solution at a final concentration of 0.03 ng/ml was added to the agarose solution. For electrophoresis, 15 ml of the PCR product was mixed with 2 ml of 6x loading dye (0.25% bromophenol blue in 30% glycerol) and loaded in the slot of the agarose gel. In order to determine the molecular size of the amplified products, each gel was also loaded with 1mg DNA of a 100 bp DNA size marker (Fermentas, USA). Gel electrophoresis was performed at a constant voltage of 65V for about 3.5 hours. Finally, the gels were visualised under a UV light source in a gel documentation system (Gel Doc TM XR+, BIO-RAD, USA) and the images of amplification products were captured and stored in a computer for further analysis and future use.

Statistical data Analysis

Data of two methods was compared by PROC Paired t test of SAS.

RESULTS

Comparison of two spore counting methods

The mean spore loads of 40 selected wheat genotypes by the two methods were compared by 'Paired t-test' and the results were presented in the Table 1. The mean spore loads of two methods are presented in the Table 1. The results of the "paired t-test" clearly indicated that Nematode plate count method was superior to the washing test method. The mean recovery of spore in the Nematode plate count method was always higher (126.46) than the Washing test method (62.31).

Association of different morphological group of the pathogen

Total 130 colonies were examined from seed samples of different wheat cultivars. Out of these 95 were black and 20 mixed group. White group could not recovered from the wheat seed. Among the black group 65 showed suppressed colony growth and 45 profuse fluffy growth, Association of group was not genotypic specific both the black and mixed colonies type were recovered from the seed of most

Table 1. Mean number of spores calculated by two spore counting methods for lines.

S. No.	Genotype	Mean spore load/50 seed	
		Nematode plate count method	Washing test method
1	WAMI-75	47	42.5
2	WAMI-203	47.33	27.5
3	WAMI-236	49.66	38.75
4	WAMI-85	50.33	31.25
5	WAMI-162	51.66	26.25
6	WAMI-148	52	25
7	WAMI-166	52.66	31.25
8	WAMI-86	55.66	30
9	WAMI-283	57.66	31.25
10	WAMI-136	58.66	36.25
11	WAMI-270	104	42.5
12	WAMI-218	104.66	38.75
13	WAMI-111	105	40
14	WAMI-229	105.66	48.75
15	WAMI-28	106	31.25
16	WAMI-291	106.33	52.5
17	WAMI-21	107	38.75
18	WAMI-244	107.33	35
19	WAMI-156	107.66	41.25
20	WAMI-68	108.33	43.75
21	WAMI-113	144.33	55
22	WAMI-88	145.33	51.25
23	WAMI-44	146.66	41.25
24	WAMI-97	147	78.75
25	WAMI-71	148.33	41.25
26	WAMI-186	153.66	61.25
27	WAMI-206	154.33	41.25
28	WAMI-187	155.66	65
29	WAMI-165	156	70
30	WAMI-90	158	42.5
31	WAMI-205	185.33	120
32	WAMI-138	188	128.75
33	WAMI-147	189	113.75
34	WAMI-24	189.66	71.25
35	WAMI-227	195.66	148.75
36	WAMI-15	198.33	136.25
37	WAMI-48	199.66	136.25
38	WAMI-258	200.66	156.25
39	WAMI-232	205.33	62.5
40	WAMI-96	213	138.75
	Mean	126.46	62.31
	t- value	12.32*	

*Significant at 0.0001.

of the genotypes (Table 3).

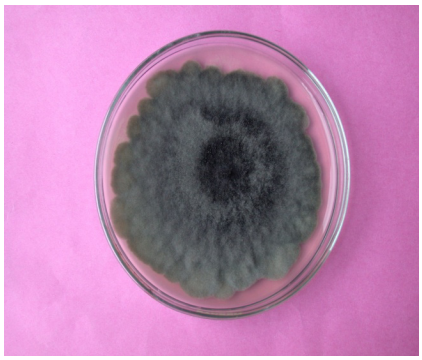
Genetic diversity of selected wheat genotypes

Leaftip necrosis

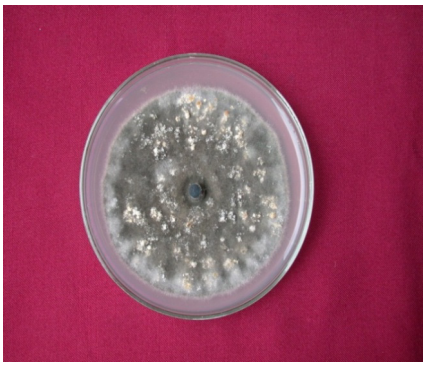
The no leaftip necrosis was observed in genotypes WAM-165, WAM-48, WAM-258, WAM-113, WAM-28, WAM-283 and WAM-148. The highest percentage (100%) was recorded in the genotypes WAM- 205, WAM- 138, WAM-147 and WAM-162. The overall mean of leaftip necrosis (*Ltn*) was 44.37 % (Table 4).

Leaf angle

The leaf angle ranged from 1 to 3 with a mean of



(1)



(2)

Figure. 1.
(1) Black isolate with suppressed growth
(2) Mixed isolate with profuse growth

Table 2. Differences between Nematode Plate count and Washing Test method.

Method	Sample size (N)	Mean	Standard deviation	SE mean	Mean time taken for counting /1 sample
Nematode plate count method	40	126.47	54.36	8.59	15 min
Washing Test method.	40	62.31	39.23	6.20	35 min
Differences		64.15	32.93	5.21	

1.37(Table 4). Leaves of most of the genotypes were semi drooping.

Days to heading

It varied from 78 days to the 86.5 days and with the overall mean of 82.57 days. The genotypes *i.e.* WAM-147 and WAM-187 had minimum 78 days for heading where as the cultivars WAM-203, WAM-229, WAM-291, WAM-88 and WAM-138 had 86.50 days to heading.

Correlation between quantitative characters

The estimates of linear correlation coefficients among 5 quantitative traits, viz., spore load of Nematode Plate Method, spore load of Washing method, Leaftip necrosis, Leaf angle and Days to Heading are presented in the Table 5. The spore load of Nematode Plate Method was positively and significantly correlated with Washing Test method (r = 0.80, p≤0.01). However, negative but significant associations were found between Days to Heading (DH) and Nematode count Method (r= -0.27, p≤0.05) and Days to Heading to Washing Test method (WT) (r= -0.22, p≤0.05).

Association molecular makers with genotypes carrying low spore load

Amplification of resistance markers were recorded in the wheat genotypes. However, it was not associated strictly associated with the genotype carried low spore load. Although it was absent in the susceptible genotype Sonalika carrying maximum spore load. However the association of maker was found in 60% cases (Fig. 2).

DISCUSSION

The seed spore estimated of the 40 selected genotypes from the Varanasi region by the two spore counting methods *viz.*, Nematode Plate Method (NPM) and Washing Test Method (WTM). It has revealed a significant correlation (r=0.8) between these two methods. Nematode Plate Method was superior to the Washing Test method. In the present study, the mean recovery of spore in the Nematode Plate Method was always higher than the Washing Test method. The comparison of the two methods by the group wise also gave the

Table 3. Morphological characters of isolates

Group	Colony morphology		Population	
	Colour	Growth behaviour	Number	Percentage
1.	Black	Suppressed growth	65	50
		Profuse growth	45	35
2.	Mixed type	Profuse growth	20	15

Table 4. Mean values of the 40 wheat lines for the five traits.

Parameters	Min.	Max.	Mean	± SE
Nematode plate	47.0	213.00	126.46	8.59
Washing test method	25.0	156.25	62.31	6.20
Leaftip necrosis	0.0	100.00	44.37	5.00
Leaf angle*	1.0	3.00	1.37	0.11
Days to heading	78.0	86.50	82.57	0.36

*Leaf angle: three types of leaf angles were found, Semi-drooping(sd) = 1; Erect(e) = 2 and Drooping(d) = 3.

Table 5. Simple correlation coefficients among five traits in 40 wheat lines.

Trait	NPM	WT	LTN	LA
WT	0.80**			
Leaf Tip Necrosis	0.08	0.11		
Leaf Angle	-0.06	-0.14	-0.02	
Days to headig	-0.27*	-0.22*	0.08	-0.01

Abbreviation of traits provided in Table 1.
* Significant at p<0.05
** Significant at p<0.01

Table 6. Performance of the 40 selected wheat lines for different traits and SSR markers associated with spot blotch resistant QTLs.

Line no.	Entry no.	Mean spore count		Leaf tip necrosis (%)	Leaf angle	Days to 50% Heading	*Qsb.bhu-7D* *Xgwm-111* (140 bp)	*Qsb.bhu-2B* *Xgwm-148* (180 bp)
		Nematode plate method	Washing Test					
9	WAMI-75	47	42.5	75	sd	84.5	-	-
28	WAMI-203	47.33	27.5	50	sd	86.5	+	+
35	WAMI-236	49.66	38.75	25	sd	82	+	-
10	WAMI-85	50.33	31.25	12.5	sd	82	+	-
23	WAMI-162	51.66	26.25	100	sd	84	-	+
21	WAMI-148	52	25	0	e	86	-	+
25	WAMI-166	52.66	31.25	75	sd	82	+	+
11	WAMI-86	55.66	30	50	sd	84	+	-
39	WAMI-283	57.66	31.25	0	e	81.5	-	-
18	WAMI-136	58.66	36.25	37.5	sd	82	-	-
38	WAMI-270	104	42.5	75	d	84	-	-
31	WAMI-218	104.66	38.75	50	d	81.5	-	+
16	WAMI-111	105	40	25	sd	82	+	-
33	WAMI-229	105.66	48.75	50	sd	86.5	+	-
4	WAMI-28	106	31.25	0	sd	82	-	-
40	WAMI-291	106.33	52.5	12.5	sd	86.5	-	+
2	WAMI-21	107	38.75	50	sd	82	-	+
36	WAMI-244	107.33	35	62.5	d	82.5	+	+
22	WAMI-156	107.66	41.25	50	e	80	+	+
7	WAMI-68	108.33	43.75	87.5	e	82	-	+
17	WAMI-113	144.33	55	0	sd	82.5	+	+
12	WAMI-88	145.33	51.25	75	sd	86.5	-	-
5	WAMI-44	146.66	41.25	25	sd	82	+	-
15	WAMI-97	147	78.75	37.5	sd	79.5	+	-
8	WAMI-71	148.33	41.25	50	e	80	+	-
26	WAMI-186	153.66	61.25	37.5	e	84.5	+	+
30	WAMI-206	154.33	41.25	50	e	84.5	+	+
27	WAMI-187	155.66	65	12.5	sd	78	+	+
24	WAMI-165	156	70	0	sd	80	+	+
13	WAMI-90	158	42.5	12.5	sd	82	+	+
29	WAMI-205	185.33	120	100	sd	80	+	+
19	WAMI-138	188	128.75	100	sd	86.5	+	+
20	WAMI-147	189	113.75	100	sd	78	-	+
3	WAMI-24	189.66	71.25	50	sd	82	+	+
32	WAMI-227	195.66	148.75	50	sd	80	+	-
1	WAMI-15	198.33	136.25	62.5	sd	81.5	-	-
6	WAMI-48	199.66	136.25	0	d	82	+	+
37	WAMI-258	200.66	156.25	0	sd	84	-	+
34	WAMI-232	205.33	62.5	75	sd	84	+	-
14	WAMI-96	213	138.75	50	sd	82	+	+

SSR marker *Xgwm-111* associated with *Qsb.bhu-7D* amplified a band of approx. 140 bp (+) in resistant lines and 150 bp (-) in susceptible lines.

SSR marker *Xgwm-148* associated with *Qsb.bhu-2B* amplified a band of approx. 180 bp (+) in resistant lines and 170 bp (-) in susceptible lines.

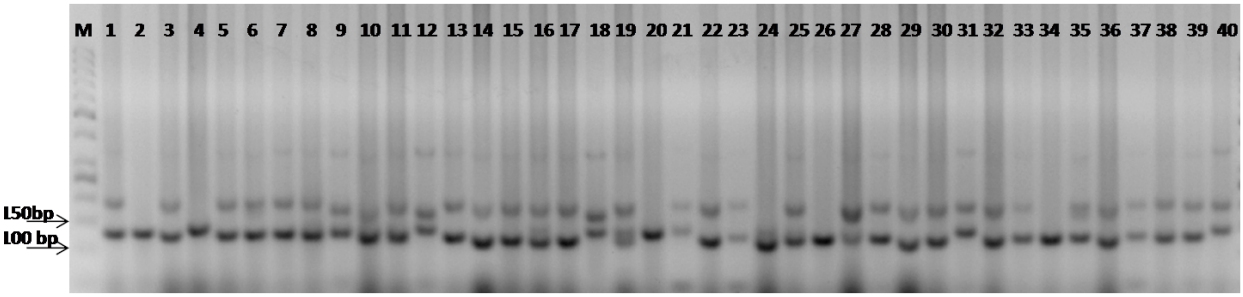


Fig. 2. SSR marker *Xgwm111* associated with *Qsb.bhu-7D* amplified a band of approx. 140 bp in resistant lines and 150 bp in susceptible lines. M= DNA ladder of 50 bp. 1-40 are selected wheat genotypes.

significant results. The time taken for counting number of spore by the Nematode Plate Count method was nearly 15 minutes for the individual sample. In Washing Test method, for each sample it took nearly 35 minutes (soaking-5 min, shaking in mechanical shaker-10 min, centrifugation-10 min, counting-10 min) for individual sample. The Washing Test, also need equipments like shaker, centrifuge etc. and it is a complex process. While Nematode Plate Count Method, one can perform the quantification of the spore easily, precisely and efficiently. At the same time it is less expensive as it requires only Nematode counting plates and a simple microscope.

The higher efficiency of nematode plate count method is due to count of spore in the from the 5 ml spore suspension spread over the counting plate. The mean conidium size of *Bipolaris sorokiniana* varied 60- 70 um easily visible in the microscope in the combination of 10 X 5 of eye piece and objective. Sultana and Rashid, 2012 reported the importance of seed transmission of *Bipolaris sorokiniana* and their effect in the failure of germination of seed. However, none of workers suggested the method for the quantification of spore for various decisions related to seed health, certification and treatment. Present study also revealed that there is variation in the wheat genotypes for the seed infection of course it is also influenced by the many other factors also. This information is important to wheat breeders of warm and humid climate where spot blotch is problem to select the only those genotype who are good in agronomic performance with low seed inoculum. Molecular markers for the spot blotch resistance gave variable results. Markers developed for spot blotch was based on leaf infection and it has given good result in case of susceptible check Sonalika however not able to produce the same result with the genotype carrying similar spore load.

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