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Standardize the detection protocol (media recipes and incubation conditions) for easiness, quick and reproducible and results for spot blotch of wheat

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Abstract
Spot blotch in wheat is mainly caused by *Bipolaris sorokiniana* (Sacc.) in India and neighboring South Asian countries and capable of causing losses in yield up to 50% in susceptible varieties as well as results poor grain quality. *Bipolaris sorokiniana* of wheat was investigated with relationship between morphological and pathological variability of isolates on different media namely PDA, Oat agar, Corn agar media and Standard blotter. Isolates were collected from the leaves of field-grown wheat crop at Main Experiment Station, NDUA and T, Kumarganj, Ayodhya. Morphological characters—i.e., colony morphology, growth, color and pathogenic characters – i.e., shape and size, number of septa and color. The single spore cultures were later inoculated on a different media such as PDA, Oat agar, Corn agar media and Standard blotter and incubated at 25±1ºC at 85-95% humidity inside B.O.D. for 7 days. Three types of colony were obtained from the infected wheat leaves. Characteristics of these pure cultures were studied after 7th day of isolation. Colony obtained from wheat leaves were suppressed, dark grey to brownish black in colour with smooth to wavy margin and colony diameter varies from 6.75-9.00 cm. In the initial stage olive, dark brown like mycelial growth occurred on potato dextrose agar medium which later on changes into olive green or gray coloured. After seven days of growth, mycelium becomes dark gray and it was light gray at margins. In petri dishes, fungus develops into clear zones with alternation of dark olive and light brown colour. In 15 day old cultures, mycelium becomes dark greenish grey or black coloured at margins with abundant sporulation. Development of fresh white mycelium on the dark greenish grey mat of mycelium was also observed.

Keywords: Wheat leaves, Spot blotch, Morphological, Pathogenic, *B. sorokiniana*, media recipes, ocular micrometer

Introduction
Spot blotch caused by *Bipolaris sorokiniana* (syn. *Helminthosporium sativum*, teleomorph *Cochliobolus sativus*) is currently regarded as one of the most serious biotic stresses of wheat (*Triticum aestivum*) in India. Globally, total area under wheat production is 215.48 million hectares with production 670.87 million tons and productivity of 31.17 q/ha. India is second largest producer of wheat after China with an area of 29.90 million hectares, production of 94.88 million tons and productivity of 3173.24 kg/ha covering 12 per-cent of world production (FAO Statistics Division 2015-16). It has good nutritional value than other food grains comprising 71.2g carbohydrates, 11.8g proteins, 1.5g fat, 1.2 g crude fiber, 306 mg phosphorus and 41 mg calcium per 100g grains (Rai and Mauria, 1999) [8]. Spot blotch caused by *Bipolaris sorokiniana* (Sacc.) Shoem. (syn. *Helminthosporium sativum*, teleomorph *Cochliobolous sativus*) is an important wheat disease in warmer and humid growing regions of the world such as Eastern India, South East Asia (Joshi *et al*., 2007) [7]. Yield losses were estimated to be 18-22 per cent in India (Saari, 1998) [9]. The control strategy for the diseases caused by *B. sorokiniana* is based on an integrated approach where genetic resistance is a major element, because economic returns have not always resulted in commercial grain production from fungicide inputs (Duveiller and Sharma, 2009) [5]. The pathogen collected from foliar samples of wheat of different agro ecological zones was characterized on the basis of culture/colony colour and texture, conidial morphology and pathogenic nature. They were grouped in 4 classes having black, grayish black, brown and albino (whitish) colony color with profusely sporulated and suppressed type of growth to fluffy and less sporulated type. The conidial average size ranged from 38.3–65.8 µm x 12.3–25 µm with slightly curved, brown to olivaceous brown with 2–13 septa. Some isolates had relatively long and broad slender conidia, while some were uniformly straight and cylindrical and light brown in colour. All the isolates did not show difference in pathogenicity test by producing the symptoms on leaves

but their reaction varied in terms of aggressiveness (Asad *et al.* 2009) [4].

Material and Methods

The experiment was conducted at main experiment station of Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya (U.P.) during crop season *Rabi*, 2017-18.

Collection of disease sample

A number of diseased leaf sample of wheat showing characteristic symptoms of spot blotch were collected from the wheat fields representing Kumarganj Ayodhya district, during *Rabi* season 2017-18and 2018-19. The leaves showing characteristic symptoms of spot blotch were brought to the laboratory, washed with running tap water to remove dust and dirt. The diseased samples were properly dried between two folds of blotting paper and herbarium was made and preserved. To remove excess surface moisture the samples were dried for 24 hrs. in shade and repacked in fresh paper envelopes and maintained at 6 to 8^0C for further study.

Isolation of pathogens

The isolation of pathogens associated with blighted samples was done on potato dextrose agar (PDA), oat agar, corn agar medium and standard blotter. The pathogen was grown on PDA medium. The composition of PDA was as:

Peeled potato	- 200g
Dextrose sugar	- 20g
Agar-agar	- 20g
Distilled Water to make total volume	-1000ml.

Oat agar media

Oat meal	– 60g
Agar-agar	– 20g
Distilled Water to make total volume	-1000ml

Corn agar media

Corn meal	– 60g
Agar-agar	– 20g
Distilled Water to make total volume	-1000ml

The blighted spots were cut into 4-5 mm length pieces along with some healthy green tissue and washed with fresh tap water 2-3 times. These were surface sterilized with 0.1% solution of $HgCl_2$ (Mercuric chloride) for 30-45 seconds to remove contamination followed by 4-5 washing in sterilized water. The bits were later dried using sterilized blotters and plated on the PDA on sterilized Petri dishes (90 mm diameter) @ 1 piece per plate at equal distance. These plates were incubated at 25±1ºC in BOD incubator at 12 h day and night photoperiod cycle to stimulate sporulation in colonies. The colonies developed fully within 10-12 days after inoculation. Pathogens were identified by observing the colony (Alcorn, 1988) under compound stereo binocular microscope initially and later by making slides of spores and observing under compound microscope.

Table 1: To standardize the detection protocol

S. No.	Media Recipes	Incubation Condition					
		Temperature			R.H. (%)	Light	Period
		20ºC± 2	22ºC± 2	25ºC± 2			
1.	Standard Blotter	--	23.5 ºC	--	85-90	12 hr. light /12hr. dark	5-7 days
2.	P.D.A.	--	22.5 ºC	--	85-95	12 hr. light /12hr. dark	5-7 days
3.	Corn meal Agar	--	--	25.5ºC	75-80	12 hr. light /12hr. dark	5-7 days
4.	Oat meal Agar	--	22.5 ºC	--	85-90	12 hr. light /12hr. dark	5-7 days

Maintenance of culture

The cultures obtained using single spores were transferred to PDA slants and after incubation for 10 days these were stored at 40ºC up to 8 weeks in refrigerator after putting Al foil on cotton plugs. The re-culturing was done as per need.

Cultural characters

The circles of 5mm diameter of full-grown colony of each isolate were placed at the center of Petri plates containing PDA. Three replications per isolates were taken. The incubation was done in B.O.D. incubator at 25±1ºC. The Petri plates were observed daily after incubation to record colony characteristics. The colony growth started within 24 h of inoculation. During initial three days only colony growth characters were recorded whereas, colony color and nature of growth were also recorded afterward. Observations were taken up to 7 days and final diametric growth was recorded in each colony.

Morphological characters

The morphological characters of the fungus were observed under *in vitro* conditions. The spores of each isolate were collected from the Petri plates. The conidial characters were observed under the compound microscope after staining with cotton blue dye. The morphological variations for color of spores and its cytoplasm, septation and shape in conidia and conidiophores were observed under microscope by making slides in cotton blue.

Measurement of size of conidia and conidiophores

The length and breadth of conidia and conidiophores were measured with the help of micrometer. The values of ocular micrometer were multiplied with the constant value of stage micrometer to find out the size in µm scale. The record was taken on 10 conidia and conidiophores to get the range of size.

Calibration

For calibration of ocular, it was first placed inside the eye piece of 10 X and stage micrometer was placed on the stage of the microscope. The stage micrometer was brought under focus and ocular divisions were coincided with the divisions of stage micrometer and calculation was made by the following procedure:

Microscope No.: 3180
Eye piece: 10 X
Objective: 10 X
Since 100 divisions of stage micrometer = 1 mm

So 1 division of stage micrometer = 0.01 mm = 10 µm (1 mm = 1000 µm).

In the present case 65 divisions of ocular coincide with 100 divisions of stage micrometer.

1 division of ocular = $\frac{100}{65}$ = 1.538 division of stage micrometer

1 division of stage micrometer = 10 µm

1.538 division of stage micrometer = (10 × 1.538) µm = 15.38 µm = 15.4 µm.

1 division of ocular = 15.4 µm

$$\text{One ocular Division (µm)} = \frac{\text{Number of division on stage micrometer}}{\text{Number of division on ocular micrometer}} \times 10$$

Size (µm) = No. of ocular division occupied by conidia / conidiophores x calibration factor of objective

Results and discussion

Morphological variability

Cultural characters

The data on the cultural characters of conidia and conidiophores of *B. sorokiniana* are presented in Table 2. Three types of conidial colours i.e., olive brown, grayish brown and dark brown was observed. The olive brown colour was observed in 4 media (corn agar, oat agar and standard blotter) whereas other media displayed dark brown or grayish brown colour. The conidiophores showed more variations in terms of colour viz., olive brown, dark brown and grayish brown. The conidiophores of media corn agar, oat agar and standard blotter reflected the olive brown color whereas PDA was of dark brown or grayish brown color.

Septation of conidia and conidiophores

The septa in conidia ranged from 4 –9 (Table 3). Maximum 9 septa were recorded in PDA media while the minimum 4 in corn meal agar, 5 standard blotter and 6 oat meal agar media.

The range of septa in conidiophores of different media varied from 2-8. Minimum 3 septa were observed in oat meal agar whereas, maximum 7 septa were recorded in PDA (Table-4).

Shape of conidia

Variation in the shape of spores was observed under microscope. The shape of conidia were straight in case of PDA, corn meal agar and standard blotter, slightly curved in oat meal agar media (Table 3).

Size of conidia

The length and width of conidia of different media was recorded under microscope, using ocular micrometer. The length of conidia ranged from 50.4- 74.4µm while; width from 11.4-17.8 µm.

The maximum conidia length i.e. 74.4µm, was observed in PDA while the minimum (50.4µm) in standard blotter (Table 3). Likewise, maximum width i.e. 17.8µm was observed in conidia of PDA, followed by corm meal agar (16.2µm) whereas, minimum (11.5µm) in standard blotter.

Table 2: Cultural characteristics of *B. sorokiniana* of wheat *in vitro* on different media

S. No.	Media Recipes	Size in diameter (mm)	Growth	Shape	Colour
1.	Standard Blotter	58.75	Suppressed	R	OB
2.	P.D.A.	89.12	Suppressed	R	DB/GB
3.	Corn Meal Agar	47.25	Suppressed	I	OB
4.	Oat Meal Agar	79.50	Suppressed	R	OB

DB= Dark brown **GB**= Greyish brown **OB**= Olive brown **I**= Irregular **R**= Regular

Size of conidiophores

The average conidiophores length ranged from 126.6-141.2µm. Minimum length (126.6µm) of was exhibited by standard blotter while maximum i.e. 141.2µm in PDA (Table 4).

The conidiophores width was ranged from 5.2-6.6µm. The maximum width i.e. 6.6µm was observed in PDA while; the minimum (5.2µm) was recorded in oat meal agar.

Similar observations were recorded by other workers Kumar *et al.*, (2002) *Bipolaris sorokiniana* is characterized by thick-walled, elliptical conidia (60- 120µm ×12-20µm) with 5-9 cells. In axenic culture, the mycelium is composed of hyphae interwoven as a loose cottony mass and appears as white or light to dark grey depending on the isolates.

Light microscopic study of *Bipolaris sorokiniana* done by Aggarwal *et al.* (2002) shows that the conidia are slightly curved or sometimes straight, fusiform to broadly ellipsoidal, dark olivaceous brown, smooth, thick walled and 3-12 (mostly 6) pseudoseptate.

Table 3: Measurement of Conidia of *B. sorokiniana* of wheat *in vitro* on different media

S. No.	Media Recipes	Size		Length X Width (µm)	No. of septa	Colour	Shape
		Length (µm)	Width (µm)				
1.	Standard Blotter	50.4	11.4	50.4 x 11.4	5	OB	Straight
2.	P.D.A.	74.4	17.8	74.4 x 17.8	9	DB/GB	Straight
3.	Corn Meal Agar	61.8	16.2	61.8 x 16.2	4	OB	Straight
4.	Oat Meal Agar	66.4	14.4	66.4 x 14.4	6	OB	Straight- slightly curved

DB= Dark brown **GB**= Greyish brown **OB**= Olive brown

Table 4: Measurement of Conidiophore of *B. sorokiniana* of wheat *in vitro* on different media

S. No.	Media Recipes	Size		Length X Width (µm)	No. of septa	Colour	Shape
		Length (µm)	Width (µm)				
1.	Standard Blotter	126.6	5.4	126.6 x 5.4	6	OB	Straight- geniculate
2.	P.D.A.	141.2	6.6	141.2 x 6.6	7	DB/GB	Straight geniculate
3.	Corn Meal Agar	135.5	6.2	135.5 x 6.2	5	OB	Straight geniculate
4.	Oat Meal Agar	137.4	5.2	137.4 x 5.2	3	OB	Straight- geniculate

DB= Dark brown **GB**= Greyish brown **OB**= Olive brown

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