

Screening for disease resistance in barley cultivars against *Bipolaris sorokiniana* using callus culture method

Ramesh Chand¹, Devyani Sen², K D Prasad¹, A K Singh², B M Bashyal¹, L C Prasad² & A K Joshi²

¹Department of Mycology and Plant Pathology, ²Department of Genetics and Plant Breeding,
Institute of Agricultural Sciences, Banaras Hindu University Varanasi 221 005, India

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Screening for resistant barley genotypes in response to fungal toxin of *Bipolaris sorokiniana* was assessed on standing barley plants as well as in selected callus lines of the same. For the standing lines tested, those manifesting chlorosis in response to toxin infiltration showed a significantly slower disease progress as compared to the necrotic lines. Also, necrosis in the callus tissues of the susceptible cultivar in MS medium supplemented with different concentrations of the crude toxin was significantly higher than in the callus tissues of the chlorotic lines studied. Similar host response to the toxin in *in vitro* and field situations open up the possibility of screening barley cultivars for resistance to spot blotch using callus culture as against classical methods of screening in order to increase accuracy and save time and space.

Keywords: *Bipolaris sorokiniana*, Callus culture, Disease screening, Toxin

Bipolaris sorokiniana (Sacc.) Shoem, [perfect stage *Cochliobolus sativus* (Ito and Kurib.) Drechsler ex Dastur] which causes spot blotch disease is a serious pathogen in wheat and barley. The fungus is capable of producing non-host-specific toxin of sesquiterpenoid nature¹ in liquid minimal media that is pathogenic on barley as well as other Poaceae hosts^{2,3}. Although, most of the toxins reported from *B. sorokiniana* are host non-specific, it has been used to differentiate between susceptible and resistant cultivars under laboratory conditions⁴. In plants, pathotoxins play a critical role in development of disease and also help determine the host range and parasitic ability of the pathogen. Screening for resistance by conventional method involves generations of stringent selection after hybridization⁵. As against this, *in vitro* techniques present an immediate and inexpensive method to assist plant breeders⁶. Several host-pathogen interactions have been successfully assessed using the callus-fungal method, which involves *in vitro* inoculation of callus cultures with the crude toxin⁷⁻¹⁰. Results obtained under *in vitro* conditions have been found to be similar to those obtained from field data¹¹. Keeping this principle in view, the present study has been undertaken to determine the possible use of *Bipolaris*

sorokiniana crude toxin as a tool for assessing resistance of barley lines by way of *in vitro* selection and correlating the results with observed *in vivo* resistance.

Materials and Methods

Partial purification of *B. sorokiniana* toxin—The pure culture of isolate (BCUBIPOL) was prepared by the single spore isolation method¹². Pure culture BCUBIPOL was multiplied in liquid minimal medium¹³ for extraction of crude toxin, and incubated for 8 days at 25^o±2^o C. The mycelium was then filtered with a double layer cheese cloth, the partially purified toxin extracted¹⁴ and stored at 8^oC till further use. Pathogenicity tests to confirm the disease producing ability of the toxin were conducted on penultimate leaves of 40 days old susceptible barley cultivar Jyoti by infiltrating 100 µl of the crude toxin (diluted 1000 times in double distilled water) with the help of a hypodermic syringe.

Screening of barley germplasm for spot blotch under natural and artificial conditions—Barley cultivars (1685) obtained from the Directorate of Wheat Research, Karnal India were screened under natural conditions for spot blotch susceptibility in the winter season of 2005-2006. Each genotype was sown at the Agricultural Farm, Banaras Hindu University, in rows of 2 meter length, 25 cm apart under irrigated conditions with the recommended basal dose of NPK fertilizers (20:40:40). Based on the screening results,

58 genotypes exhibiting chlorosis were identified. These chlorotic lines were further verified thrice by artificial infiltration of toxin for their disease severity during the winter of 2006-2007. All the plants were inoculated with a 10^4 /ml spore suspension of *B. sorokiniana* 52 days from sowing and the disease severity percent was calculated¹⁵. Area Under Disease Progress Curve (AUDPC) was measured to assess disease resistance using the formula¹⁶:

$$\text{AUDPC} = \sum_{i=1}^a \left[\frac{(Y_i + Y_{i+1})}{2} \times (t_{i+1} - t_i) \right]$$

where, Y_i = disease level at time t_i and,

$t_{i+1} - t_i$ = time (days) between two disease scores

The experiments were laid out in randomized block design with three replications.

Callus culture—Based on AUDPC results, upon verification, young seeds from standing plants of the barley cultivars BCU 3412, BCU4394, BCU3418 and the susceptible cultivar Jyoti were removed from their spikes, and surface sterilized. The caryopses of the seeds were then aseptically cut open and the young embryos (1.0-1.5 mm in length) removed and placed on culture tubes containing MS medium¹⁷ supplemented with 2,4-D adjusted to a concentration of 2 mg/l. The calli were sub cultured every 3-4 weeks by transferring them into fresh medium with similar composition. The partially purified crude toxin was adjusted to final concentrations of 1, 5, and 10 µl per ml of growth medium in separate flasks and re-distributed equally into 8 culture tubes per treatment. Callus sub cultured from 3-4 week old callus of the 4 barley genotypes (Jyoti, BCU-3412, BCU-4394, BCU-3418) were placed on these culture tubes

amended with different dosage of the crude toxin and incubated at 25°C for 11 days. Changes in callus colour due to effect of toxin were recorded by visual scoring as white, when it was similar to that of the healthy callus and brown when necrotic areas were observed in the callus tissues.

A tentative nine point scale (1-9) was developed for scoring the differential browning of the callus. The interpretation of the scale is as follows:

1=White healthy callus, 3=Scattered brown necrotic lesions, 5=Easily observed brown necrotic patches, 7=Brown necrotic patches very common and, 9=Dark brown necrotic tissue

These different groups of calli were then photographed for recording the change in their colour spectrum with a Nikon Digital Camera (995 pixels) and the photographs were assessed for the spectral value (Red, Green and Blue) of the callus using Adobe Photoshop. Spectral values (RGB) of individual callus grown at different concentrations of toxin for the four genotypes of barley were noted randomly from five different areas of the callus tissue. Lower the RGB value of the callus, greater was the effect of necrosis in medium supplemented with different concentrations of the crude toxin.

Results

Out of 1685 germplasm lines screened, 1627 lines produced typical necrotic symptoms four days from occurrence of the disease while 58 lines manifested only chlorotic symptoms. The disease progress in the chlorotic lines was slower and differed from the AUDPC results of the lines manifesting necrosis. These lines obtained were further verified for their expression of the chlorosis and were found to be true to type (Table 1). Based on appearance of symptoms

Table 1—AUDPC in chlorotic barley lines screened for natural disease and by artificial inoculation

Accession No.	2005-2006 Disease progress under natural conditions	2006-2007 Disease progress under artificial inoculation
BCU2949, BCU3511, BCU3549, BCU3610, BCU3617, BCU3618, BCU3800, BCU3806, BCU3820, BCU3839, BCU3912, BCU4410, BCU4447, BCU4588, BCU4612, IC405267, IC437851	200-300	386
BCU3412, BCU3418, BCU3501, BCU3507, BCU3527, BCU3552, BCU3554, BCU3564, BCU3566, BCU3568, BCU3571, BCU3573, BCU3574, BCU3575, BCU3576, BCU3577, BCU3579, BCU3585,	301-400	485
BCU3657, BCU3669, BCU3691, BCU3700, BCU3919, BCU3927, BCU3931, BCU3940, BCU3941, BCU2780, BCU4394, BCU4456, BCU4466, BCU4468, BCU4687		
BCU2740, BCU3532, BCU4513, BCU4674, BCU4675, BCU4678, IC82794	401-600	741
BCU3555, BCU4329	601-700	995
JYOTI	1285	1800

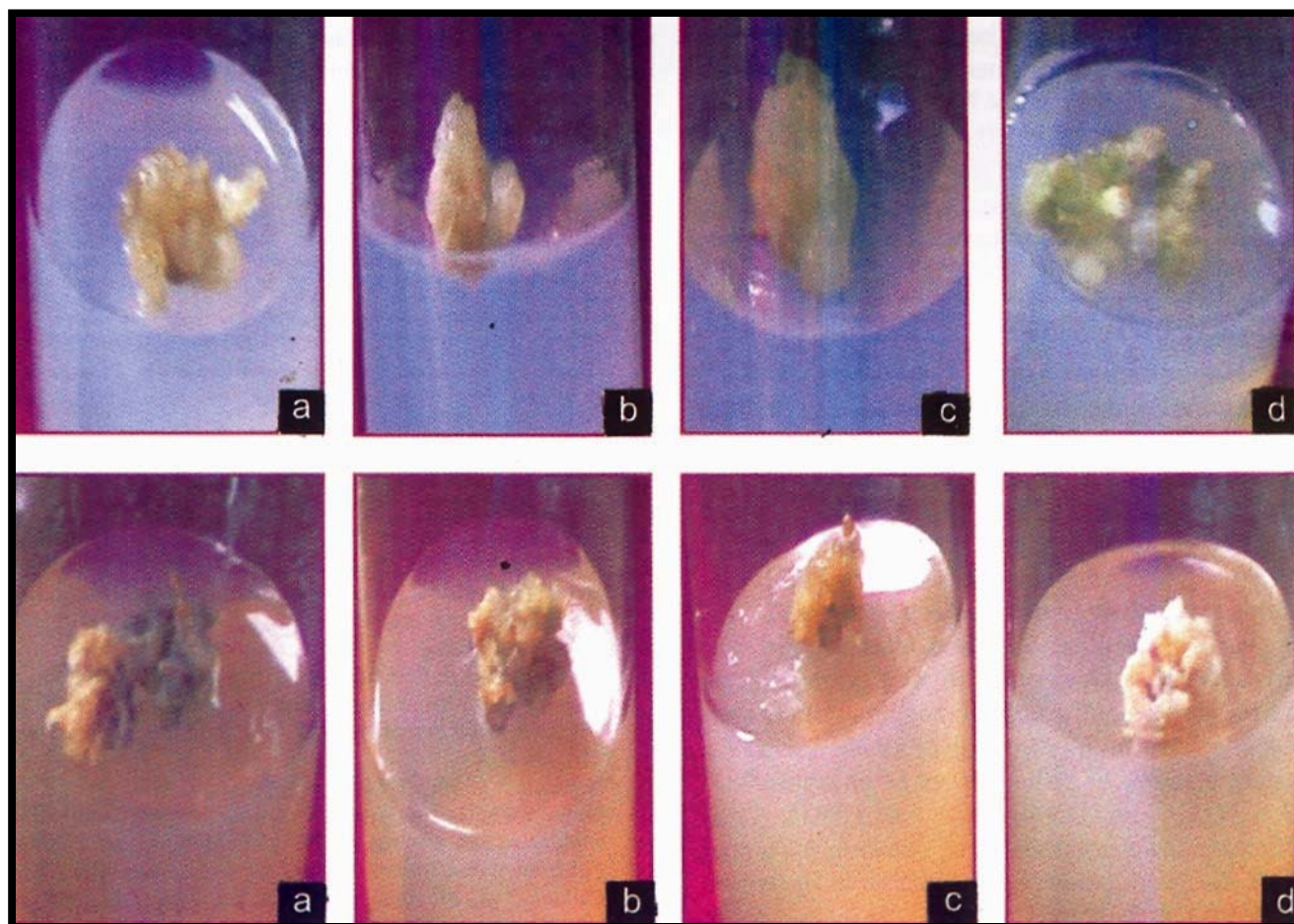


Fig. 1—Response of chlorotic lines BCU-3412, BCU-3418, BCU-4394 and the susceptible check Jyoti to toxin of *B. sorokiniana* [Upper panel: Untreated barley calli of different genotypes studied; Lower panel: Barley calli treated with 10 µl/ml of *B. sorokiniana* toxin. a = Jyoti; b = BCU 3412; c = BCU 3418; d =BCU4394]

in treated as well as control barley lines, cultivars BCU 3412, BCU4394, BCU3418 and Jyoti were selected for callus regeneration.

Studies on effect of toxin revealed that while severely damaged necrotic patches in the callus tissues of the check cultivar Jyoti were observable within 72 hr when placed in growth medium containing 10µl of crude toxin/ml medium, the chlorotic lines treated with the same level of toxin and for similar length of time turned partially brown in colour (Fig. 1).

It was observed that while the control treatment (C0) for all the four genotypes comprising of white healthy callus tissues deflected maximum amount of light and registered the highest RGB values, RGB values of the same callus lines when placed in growth medium supplemented with different levels of the toxin (Table 2), was significantly lower. Significant

Table 2—RGB values of effect of toxin concentration on callus colour (G × C)

Toxin concentration (µl/ml)	Genotypes			
	JYOTI	BCU3412	BCU4394	BCU3418
Control	102.533 [1]	96.578 [1]	95.067 [1]	96.733 [1]
1	88.133 [3]	93.8000[1]	96.800 [1]	89.044 [3]
5	78.889 [7]	83.822 [3]	84.556 [3]	83.956 [3]
10	59.044 [9]	64.289 [7]	77.489 [5]	65.867 [7]
SE	±0.2309			
L.S.D (0.05)	0.6399			
L.S.D (0.01)	0.2			
1=White healthy callus, 3=Scattered brown necrotic lesions, 5=Easily observed brown necrotic patches, 7=Brown necrotic patches easily observable, 9=Dark brown necrotic tissue				
Figures in brackets indicate estimated score of necrosis				

differences for RGB values were also obtained with a change in concentration of the crude toxin indicating differential callus response to different concentrations of the crude toxin. Results for susceptibility to the

crude toxin also varied significantly between the chlorotic lines which had been assigned a similar AUDPC trend during assessment of disease progress in field conditions, with BCU4394 showing maximum tolerance in presence of the 10 µl toxin/ml followed by BCU3418 and BCU3412 respectively. Interaction studies also revealed that the same genotype reacted differently to different concentrations of the crude toxin.

Discussion

The host reaction to *B. sorokiniana* partially purified toxin in the infiltrated standing barley lines was indicative of genotype specificity of the *B. sorokiniana* toxin in barley. This result is in accordance with the findings of Lee *et al.*¹⁸, which stated that a diethyl ether soluble toxin extract produced by *Cochliobolus sativus* responsible for causing foliar disease in barley was host specific.

In vitro screening revealed that while the regenerated callus tissues of the susceptible cultivar Jyoti exhibited a significantly high amount of necrosis in response to the *B. sorokiniana* crude toxin, the browning reaction in callus tissues of chlorotic lines BCU 3412, BCU4394 and BCU3418 was significantly lower. A possible explanation for higher necrosis in Jyoti could be attributed to increased endogenous ethylene production in response to *B. sorokiniana* toxin as had been observed in expression of a susceptible reaction in *Poa pratensis*¹⁹. As against this, a lower degree of damage in the chlorotic lines was possibly due to neutralization of the *B. sorokiniana* pathotoxin which is one of the many defense mechanisms employed by resistant genotypes²⁰.

For all the four lines tested in *in vitro* conditions, susceptibility to the toxin increased with an increase in the toxin concentration. The degree of damage to the callus tissues was directly related to the dosage²¹. This was due to increased intensity of metabolic processes such as increased heat emission to neutralize the fungus metabolites²². Additionally, the response of the individual genotypes could be adjudged better in *in vitro* conditions.

Thus, the response of the standing barley cultivars as well as that of callus cultures of the same *B. sorokiniana* which attacks its host directly with the toxin although along similar lines can be more accurately assessed in *in vitro* conditions. These results therefore open up the potential of screening

resistant lines of barley *in vitro* employing much less space to provide for an immediate method of generating valuable information.

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References

- 1 Storbel G A, Phytotoxins, *Ann. Rev Biochem*, 51(1982) 309.
- 2 Xiao J Z, Tsuda M, Doke N & Nishimura S, Phytotoxins produced by germinating spores of *Bipolaris oryzae*, *Phytopathology*, 81 (1991) 58.
- 3 Wolf S J & Earle E D, Effect of *Helminthosporium carbonum* race-1 toxin on host and non-host cereal protoplast, *Plant Sci*, 76 (1991) 127.
- 4 Singh D P, Maraite H, Duveillier E, Diego M & Renard R, Comparison of host resistance to *Bipolaris sorokiniana*, the causal agent of leaf blotches and toxin in wheat, *Proceeding of the fourth international wheat tan spot and spot blotch workshop* (2002) 74.
- 5 Joshi A K, Singh R H & Rai B, Effect of three generation of back crossing on maturity duration, grain yield and its attributes in intervarietal crosses of wheat, *Malays Appl Bio.*, 126 (1997) 7.
- 6 Hadrami A E, Idrissi-Tourane A E, Hassni M E, Daayf F & Hadrami I E, Toxin-based *in vitro* selection and its potential application to date palm for resistance to the bayoud *Fusarium* wilt, *Comptes Rendus Biologies*, 328 (2005) 732.
- 7 Carlson P S, Methionine sulfoximine-resistant mutants of tobacco, *Science*, 180 (1973) 1366.
- 8 Helgeson J P & Haberlach G T, Disease resistance studies with tissue cultures, In: Ingram DS, Helgeson JP, (eds.), *Tissue Culture Methods for Plant Pathologists*. Oxford: Blackwell Scientific Publications, (1983) 179.
- 9 Daub M E, Tissue culture and the selection of resistance to pathogens, *Annu Rev Phytopathol*, 24 (1986) 159.
- 10 Gayatri MC, Roopa darshini V & Kavyashree R, Selection of turmeric callus for tolerant to culture filtrate of *Pythium graminicolum* and regeneration of plants, *Plant Cell Tiss Org Cult*, 8 (2005) 333.
- 11 Ragazzi A, Moricca S & Dellavalle I, Growth of axenic cultures of *Cronartium flaccidum* on callus tissue from *Pinus nigra* var. *laricio* and *Pinus sylvestris*, *Eur J F Pathol*, 25 (1995) 31.
- 12 Chaurasia S, Chand R & Joshi A K, Relative dominance of *Alternaria tritici* and *Bipolaris sorokiniana* (Sacc.) Shoemaker, in different growth stage of wheat (*T. aestivum* L.), *J Plant Dis Prot*, 107 (2000) 176.
- 13 Leach J, Lang B R & Yoder D C, Methods for selection of mutants and *in vitro* culture of *Cochliobolus heterostrophus*, *J Microbiol*, 128 (1982) 1719.
- 14 De Mayo P, Spencer E Y & White R W, Helminthosporal, the toxin from *Helminthosporium sativum*. I. Isolation and characterization, *Can J Chem*, 39 (1967) 1608.
- 15 Joshi A K, Ortiz-Ferrara G, Crossa J, Singh G, Alvarado G, Bhatta M R, Duveillier E, Sharma R C, Pandit D B, Siddique

- A B, Das S Y, Sharma R N & Chand R, Associations of environments in South Asia based on spot blotch disease of wheat caused by *Cochliobolus sativus*, *Crop Sci*, 47 (2007) 1.
- 16 Zadoks J C, Chang T T & Konzak C F, A decimal code for the growth stages of cereals. *Weed Res*, 14 (1974) 415.
- 17 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, 15 (1962) 473.
- 18 Lee S S, Vick V A & Stack R W, Biological activities of the diethyl ether soluble toxin produced by *Helminthosporium sativum*, *Korean J Mycol*, 19 (1991) 128.
- 19 Hodges C F & Campbell D A, Endogenous ethane and ethylene of *Poa pratensis* leaf blades and leaf chlorosis in response to biologically active products of *B. sorokiniana*, *Eur J Plant Pathol*, 105 (1999) 825.
- 20 Lamb C J, Lawton M A, Dron M & Dixon R A, Signals and transduction mechanisms for activation of plant defences against microbial attack, *Cell*, 56 (1989) 215.
- 21 Ludwig, R B, Toxin production by *Helminthosporium sativum* and its significance in disease development, *Can J Bot*, 35 (1957) 291.
- 22 Trillas M I & Azconbieto J, Short- and long-term effects of *Fusarium oxysporum* elicitors on respiration of carnation callus, *Plant Physiol Biochem*, 33 (1995) 47.