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Six potential plant growth promoting rhizobacteria – *Bacillus methylotropicus* (NAIMCC-B-01492), *B. symbiont* (NAIMCC-B-01489), *B. altitudinus* (NAIMCC-B-01484), *B .megaterium* (NCBI JX312687), *B. pumilus* (NAIMCC-B-01483) and *P. polymyxa* (NAIMCC-B-01491) showing *in vitro* antagonistic activity against *Bipolaris sorokiniana* were screened for their effects on growth of sorghum plants and induction of resistance against *B. sorokiniana*. Among these rhizobacteria, *B. methylotropicus* and *B. megaterium* induced maximum growth and accumulation of phenols, and enhanced activities of defense enzymes. Root colonization with dominant abruscular mycorrhizal fungi followed by soil application with *Trichoderma asperellum* in combination with talc based formulation of *B. methylotropicus* reduced disease incidence markedly in sorghum plants which was evident by enhanced accumulation of phenols and activities of defense enzymes in comparison with single treatment with bioinoculants (AMF,PGPF or PGPR). Time course accumulation of chitinase, â 1-3 glucanase, peroxidase and phenylalanine ammonia lyase were also evaluated. Pathogen infestation in leaf tissues in all treatments following challenge inoculation was evaluated by indirect immunofluorescence assay using polyclonal antibody raised against *B. sorokiniana* and compared with untreated healthy leaf. Accumulation of phenolic compounds in treated and inoculated plants in comparison with untreated healthy plants was analysed by HPLC.

Key words: Sorghum, *Bipolaris* sorokiniana, AMF, PGPF, PGPR, spot blotch, defense enzymes

INTRODUCTION

Sorghum [*Sorghum bicolor* (L) Moench] is the fifth most important cereal crop in the world; and its wide range of other applications are now being explored with worldwide interest in renewable resources (Dahlberg *et al,*2011). Spot blotch has been a serious problem in north-eastern region as well as in north-western parts of India. *Bipolaris sorokiniana* (Sacc.) Shoemaker (syn. *Helminthosporium sativum* teleomorph: *Cochliobolus sativus*), a hemibiotrophic phytopathogenic fungus is a well known cause of Spot blotch disease in Barley (*Hordeum vulgare* L.) and Wheat (*Triticum aestivum* L.). *B. sorokiniana* usually induces symptoms on the leaf, sheath and stem. Yield losses due to Spot blotch vary from 16 to 33% in Barley. *B. sorokiniana* is widely distributed in the areas where cereals are grown and forms a continuous genetic pool of isolates varying in virulence and aggressiveness to various cereals and grasses. The infection process on the leaves usually occurs through natural wounding, stomata or with the use of an appresorium-like structure through the cell wall. The presence of other hosts plays an important role in disease epidemic. The primary inoculum of *B. sorokiniana* comes from several sources such as weed hosts, soil, crop debris

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which enhances the disease level (Bashyal *et al,*2011). Recently, Chakraborty *et al*, (2016) have reported the serological and molecular characterization of *B. sorokiniana* causing spot blotch disease of wheat.The present investigation was undertaken to evaluate the potential of different bioinoculatns in growth improvement, Spot blotch suppression and activation of defense mechanisms in sorghum

MATERIALS AND METHODS

Plant material

Seeds of sorghum [*Sorghum bicolor* (L) Moench] obtained from local market was selected. Seeds were surface sterilized with 0.1% $HgCl_2$, washed thrice with sterile distilled water and then sown as per experimental design.

Fungal isolate

Previously identified, sequenced and immunologically characterised single isolate of*Bipolaris sorokiniana* (WH.PBW.IP.04) was used for the assessment of Spot blotch disease in sorghum plant.

Microbial strains and soil application

The bioresources selected for the present investigations were six strains of PGPR[*Bacillus methylotropicus* (NAIMCC-B-01492), *B. symbiont* (NAIMCC-B-01489), *B. altitudinus*(NAIMCC-B-01484),*B. megaterium* (NCBI JX312687), *B. pumilus* (NAIMCC- B-01483) and *Paenibacillus polymyxa* (NAIMCC-B-01491)] , *Glomus mosseae* (AMF) *and Trichodermaasperellum* (BCA). *T. asperellum* (T561) isolated from rhizosphere soil of tea growing in hill region was mass multiplied in wheat bran medium. In case of PGPR isolates, six previously sequenced and characterised rhizobacteria were used for foliar spray. For the preparation of suspension the bacterial isolates were cultured in Nutrient Broth. After 48 hours of inoculation the culture was centrifuged (5,000 rpm for 15 mins). Supernatant was discarded and the pellet was dissolved in equal amount of distilled water. Few drops of tween 20/ carboxy methyl cellulose was added to it as adhesive. The aqueous suspension was then sprayed to the plant at seedling stage. Repeated application was done at 20 days interval. Another bioinoculant used was *Glomus mosseae* (AMF); in this case spores of AM fungi were obtained following wet sieving and decanting method from the rhizosphere soil of mature tea plants grown in field conditions. Surface sterilized sorghum seeds were sown in soils treated with BCA (500 g/ row). Prior to the seed sowing AMF spore mass was also inoculated in the soil; the same amount of spore was again inoculated to the rhizosphere of one month old seedlings.

Evaluation of plant growth Pathogen inoculation

Selected isolate of *Bipolaris sorokiniana* was used for screening of sorghum cultivar against Spot blotch. The fungal culture was grown on potato dextrose agar medium (PDA) in petriplates. Sorghum plants at one month old stage were inoculated by spraying conidial suspension(1×10^3 conidia/ml) of 7 day old culture of *B. sorokiniana*.

Assessment of Spot blotch disease

Disease assessment was done after 12, 24, 48, 72 and 96 h of inoculation on the basis of appearance of infection on leaves in field condition of one month old sorghum plants. The disease severity was measured in terms of lesion number per leaf and percentage disease index was calculated by following the method of Adlakha *et al,* (1984). The following formula was used for percent disease index (PDI) calculation -[(class rating x class frequency)/(total no. of leaves x maximum rating)] x 100. The mean PDI was transformed into disease reaction as: 0%= no infection/ immune; 0-10%= resistant response (R); 10.1-20.0%= moderately resistant (MR); 20.1-30.0%=moderately susceptible (MS); 30.1-50.0%= susceptible (S) and >50.0%= highly susceptible (HS).

Biochemical analyses of leaves Determination of total soluble protein

Leaf protein was extracted using phosphate buffer (pH 7.2) and protein content was determined following the method as described by Lowry *et al*, using BSA as standard.

Extraction and estimation of phenol

Phenol content was determined following the method as described by Mahadevan and Sridhar (1982) using caffeic acid as standard.

Assessment of defense enzymes in leavesExtraction

By using suitable buffers and liquid nitrogen enzymes were extracted from life tissues. For extraction of chitinase and β-1,3-glucanase 0.1M sodium acetate buffer (pH=5) was used.Phenylalanine ammonia lyase was extracted using 0.1M sodium borate buffer (pH=8.8) and peroxidase was extracted using 0.1 M sodium phosphate buffer (pH=8.8).

Assay

Chitinase (CHT, EC 3.2.1.14) activity was measured according to the method described by Boller and Mauch (1988). The enzyme activity was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg N-acetyl glucosamine (GlcNAc) released/min/g fresh wt.Assay of β-1, 3-glucanase (β- GLU, EC 3.2.1.38) activity was done by following the laminarin dinitrosalicylate method described by Pan *et al.* (1991). The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissue.Phenylalanine ammonia lyase (PAL-EC.4.3.1.5) was assayed following the method described by Chakraborty *et al.,* (1993) with modifications. PAL activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The enzyme activity was expressed as µg cinnamic acid produced min-1 g-1 fresh tissueAssay of peroxidase (POX, EC1.11.1.7) activity was done following the method of (Chakraborty *et al*, 1993). The reaction mixture contained 1 ml of 0.2 Na-phosphate buffer (pH 5.4), 1.7 ml dH_2O, 100 µl crude enzyme, 100 µl O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2.

Immunological assays

As described by Chakraborty and Purkayastha (1983), fungal antigen was prepared using 0.05 M sodium-phosphate buffer (pH 9.6).Polyclonal antibodies were raised against fungal pathogen (*B. sorokiniana*) in white, male rabbit following the procedure described by Alba and Devay (1985). Before immunization, normal sera were collected from rabbit. Following injection schedule with antigens, blood samples were collected and kept at 37^0C for 1 h for clotting, followed by centrifugation at 5000 rpm for 10 min at room temp.Dot immunobinding assay was carried out using PAb raised against *B. sorokiniana*following the procedure suggested by Lange *et al,* (1989).

Fluorescence antibody staining and microscopy

PAb of *B.sorokiniana* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC) were used for indirect immunofluorescence study. Observations were made using a Biomed microscope (Leitzz) equipped with an I-3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Moticam Pro 285B.

HPLC Analysis of phenolic compounds

Fresh leaves of sorghum plant were chopped into pieces and soaked overnight in methanol in the ratio 1:3 (w/v), filtered through Buchner's funnel and the solvent was evaporated. The dried powder was finally mixed in HPLC graded methanol and stored at 4^OC for further analysis. HPLC analysis of phenolic compounds present in extracts was done using SPD-10A VP Shimadzu UV-VIS Detector. A flow rate of 1 ml/min, and gradient elution of acetonitrile-water-acetic acid (5.93.2, v/v/v) [solvent A] and of acetonitrile-water-acetic acid (40:58:2, v/v/v) [solvent B], 0-50 min solvent B from 0to 100%; and injection volume of 20 µl were applied; the separation of compounds was monitored at 280 nm (Pari *et al,* 2007).

RESULTS AND DISCUSSION

After artificial inoculation with *B.sorokiniana*, the plants were kept under observation for development of Spot blotch disease. Disease index were computed and results have been presented in Table 1. It was observed that in all bioinoculants treated plants disease intensity was significantly reduced in comparison to the untreated control.Estimation of protein contents in all the treated and control set of plants revealed enhanced protein content in treated inoculated plants rather than control set of plant and plant treated with bacteria, of which highest accumulation was obtained in plants treated with *Bacillus methylotropicus (*NAIMCC-B-01492) and inoculated with *Bipolaris sorokiniana*.Maximum protein content was

Table 1 : Disease Index of sorghum plants inoculated with *Bipolaris sorokiniana*

Treatments	PDI (%) Days after inoculation	
	7	15
Untreated inoculated	62.83	78.00
Treated inoculated		
Bacillus methylotrophicus	23.43	27.41
B.symbiont	27.01	31.42
B.altitudinus	29.65	30.55
B.megaterium	31.42	44.70
B.pumilus	26.98	31.42
Paenibacillus polymyxa	38.37	44.70

Percent Disease Index (PDI) among treatments after 7 and 15 days of inoculation were significant at p=0.05.

Table 2 : Total soluble protein and phenol content of sorghum leaf following application with plant growth promoting rhizobacteria and inoculated with *B. sorokiniana*

Treatments		Protein content(mg/gt)	Total phenol content (mg/gt)	Ortho phenol content (mg/gt)
Control	UH	20.0±0.53	1.6±0.014	0.50±0.066
	UI	24.6±0.20	2.6±0.023	0.90±0.023
B. methylotropicus	TH	51.5±0.14	2.7±0.079	1.50±0.015
	TI	53.2±0.41	5.0±0.026	2.25±0.040
B. symbiont	TH	28.5±0.44	2.0±0.160	0.75±0.026
	TI	32.0±0.24	2.7±0.030	2.25±0.043
B. altitudinus	TH	27.5±0.28	2.0±0.120	1.50±0.059
	TI	34.0±0.42	3.2±0.079	1.75±0.020
B .megaterium	TH	47.5±1.14	2.3±0.056	0.75±0.008
	TI	52.5±0.20	2.6±0.058	2.00±0.068
B. pumilus	TH	22.0±0.57	2.0±0.022	1.25±0.008
	TI	34.5±0.66	3.7±0.095	1.50±0.037
P.polymyxa	TH	40.0±0.65	2.0±0.120	0.75±0.029
	TI	50.5±0.77	2.8±0.154	1.75±0.040

Values are replicates of three experiments, ± standard error; UH=untreated healthy, UI=untreated inoculated with *B.sorokiniana*; TH= PGPR treated healthy; TI= PGPR treated inoculated with *B.sorokiniana*

53.2±0.41 mg/g tissue (Table 2).In case of total and ortho phenol content also treated inoculated plants showed increased amount of accumulation with the highest being in plants treated with *Bacillusmethylotropicus (*NAIMCC-B-01492) and *B. megaterium* (NCBI JX312687) (Table.2). Results revealed that significant reduction of disease was achieved by treatment with any of the bioinoculants both 7days and 14days after inoculation (Fig. 1 A and B)The result is in agreement with the previous study of Shirinzadeh *et al,* (2013) who found positive effect of seed priming with PGPR on agronomic traits and yield of barley cultivars. Total soluble protein, total phenol content when estimated were found in increased amount in treated inoculated plants in comparison to control

Fig.1: Appearance of disease symptoms in leaves of Sorghum plant (A and B). Germinating spores of *Bipolaris sorokiniana* under microscopic view (C and D)

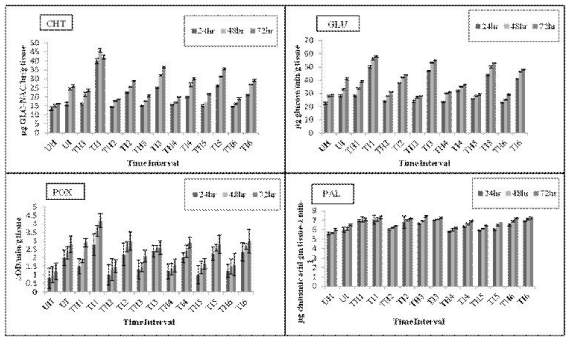

Fig. 2: Time course accumulation of - chitinase, â-1,3-glucanase, phenylalanine ammonia lyase and peroxidase.
[CHT= chitinase, GLU=â-1,3-glucanase, PAL=phenylalanine ammonialyase, POX=peroxidise, UH= untreated healthy, UI=untreated inoculated, TH=PGPR treated healthy, TI=PGPR treated inoculated with *B.sorokiniana* (TH1,TI1= *B. methylotropicus;* TH2,TI2= *B. Symbiont*; TH3,TI3= *B. altitudinus;* TH4,TT4= *B .megaterium*; TH5,TI5= *B. pumilus*; TH6,TI6= *P. polymyxa*]

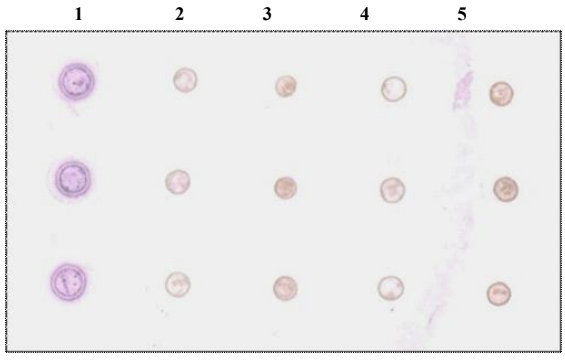

Fig. 3: Dot immunobinding assay of leaf antigens of healthy, treated and inoculated sorghum plants probed with PAb of *Bipolaris sorokiniana*.
[Lane 1**:** Mycelial antigen of *Bipolaris sorokiniana;* Lane 2**;** Untreated healthy leaf antigen; Lane 3 : untreated inoculated leaf antigen ; Lane 4: *Bacillus methylotrophicus* treated healthy leaf antigen: Lane 5: *Bacillus methylotrophicus* treated inoculated leaf antigen]

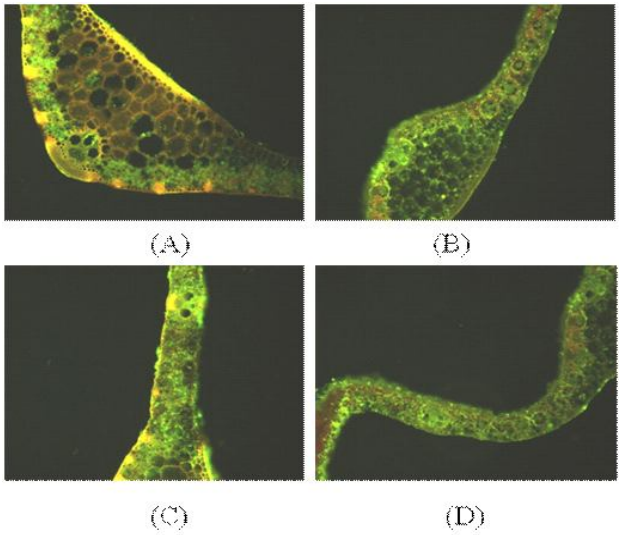

Fig.4: Immunofluorescence of healthy (A) and infected (B,C,D) sorghum leaf labelled with antibody of *B. sorokiniana*

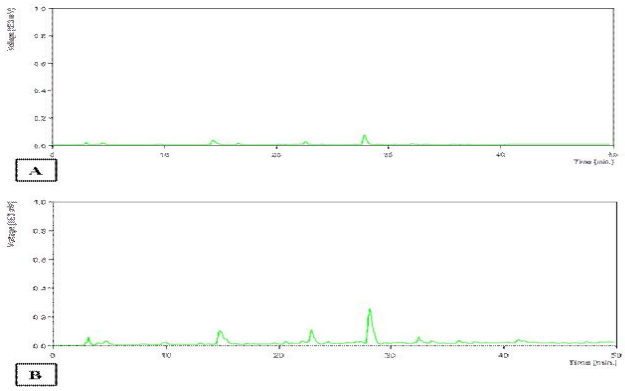

Fig.5: HPLC analysis of phenolic compounds identified in healthy and infected sorghum leaf A, B:1. Chlorogenic acid, 2. Ferulic acid

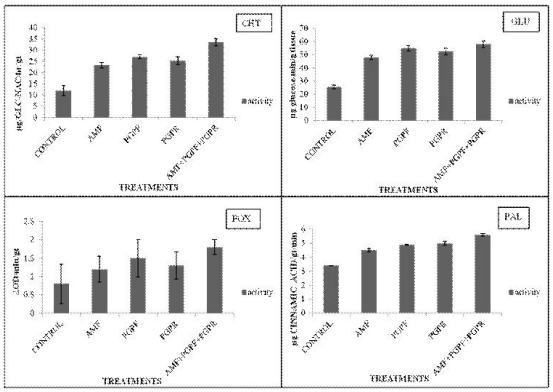

Fig.6: Activities of defense enzymes (A=CHT, B=GLU, C=POX, D=PAL) of control and bioinoculant treated sorghum plants.
[CHT=chitinase,GLU=â-1,3-glucanase,POX=peroxidise, PAL=phenylalanine ammonialyase; AMF=arbuscular mycorhizal fungi (*Glomus mosseae*), PGPF= *Trichoderma asperellum*, PGPR= Plant growth promoting rhizobacteria]

Table 3 : Total soluble protein and phenol content of sorghum leaves treated with different bioinoculants.

Treatments	Protein content (mg/gt)	Total phenol content (mg/gt)	Ortho phenol content (mg/gt)
Control	12.00±0.02	1.40±0.008	0.75±2.46
AMF	23.25±0.08	1.64±0.023	1.24±0.01
PGPF	27.00±0.12	2.10±0.058	1.80±0.05
PGPR	25.23±0.09	1.80±0.070	1.67±0.02
AMF+PGPF+PGPR	33.5.0±0.12	2.25±0.044	1.90±0.06

Values are replicates of three experiments, ± standard error; AMF=arbuscular mycorhizal fungus (*Glomus mosseae*), PGPF= *Trichoderma asperellum*, PGPR= Plant growth promoting rhizobacteria (*Bacillus methylotrophicus*)

Table 4 : Dot immunobinding assay of leaf antigens of Sorghum plants treated with *Bacillus methylotrophicus* and inoculated with *B.sorokiniana*

Antigen Source	DIBA Colour intensity of dots*
Leaf Antigens	
Untreated healthy	++
Untreated inoculated	+++
Treated Healthy	+
Treated Inoculated	++
Mycelial Antigen	
B. sorokiniana	++++

PAb dilution: 1:1000; Alakaline phosphatase dilution:1:10,000; *Colour intensity- Pinkish red- ++++; Bright pink- +++; Pink- ++; Light pink- +; No colour

set of plant (Table 3). Similar result was found in study of previous workers in the experiment on plant growth promoting rhizobacteria mediated improvement of health status of tea plants (Chakraborty *et al,* 2013). Determination of time course accumulation of all the four enzymes (CHT,GLU,PAL,POX) showed increased activity in treated inoculated plants than control set, among which TI1 showed highest accumulation from 24-48 hr. But in 72 hr CHT activity reduced in TI1 sample than previous hr treatments (Fig. 2). Susceptible and resistant reactions were further confirmed by dot blot using leaf antigens of treated healthy and treated inoculated leaf samples, probed with PAbs of *B. sorokiniana*from 3rd bleed. In dot blot, intensity of dots were high in homologous binding, whereas in treated healthy sample dot colour intensity was slightly low in comparison to treated inoculated sample (Fig. 3; Table 4)In the present study indirect immunofluorescence of healthy and infected leaf was carried out with antibody of *B. sorokiniana* and reacted with fluorescein isothiocyanate (FITC) labelled antibodies of goat specific for rabbit globulin. Healthy leaf tissue segement treated with PAb of of *B. sorokiniana* showed autofluorescence of the cuticle layer of the segment that indicates that pathogen is not present in the healthy section. Whereas in case of infected leaf section, apple green fluorescence is seen in small quantities throughout the infected tissue indicating the spread of infection in the leaf (Fig.4). Acharya *et al,* (2015) reported similar result in their study on serological detection of *Pestalotiopsis disseminata*in *Persea bombycina* causing grey blight disease.HPLC analysis was done for the detection of phenolic compounds in the sorghum plants treated with *Bacillus methylotropicus* showing the lowest PDI percentage and control set of plant (UH). The results showed high amount of accumulation of phenolic compound in response to the disease following the treatments (Fig.5A and B).The most efficient PGPR *Bacillus methylotropicus* among the six was then taken for field trial with other two poteintial bioinoculants PGPF (*T. asperellum* 561) and AMF (*Glomus mosseae*)singly and with combination. In this set of experiment plants treated with all the three treatments (PGPR, PGPF, AMF) showed increased accumulation of protein and phenol rather than other treatments and control set of plants. In case of defense enzymes also the activity was higher in plants treated with all the bioinoculants (Fig. 6).

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