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Toxigenic diversity of *Alternaria* spp. infecting Bt-cotton and induction of systemic resistance to Alternaria leaf spot

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The culture filtrates of fourteen isolates *Alternaria* spp. obtained from Bt-cotton differed in their toxigenic ability to produce the phytotoxic symptoms on cotton seedlings at various concentrations. The maximum inhibition of seed germination and shoot and root length was noticed in culture filtrates of A_1, A_2 and A_3 isolates. Least inhibition of shoot length and root length was noticed in isolate A_{10}. Among different resistance inducing chemicals tested, salicylic acid (10mM) was effective in inhibiting the mycelial growth of *A. macrospora* (84.15%). The least inhibition of mycelial growth was observed in potassium dihydrogen phosphate (2.86 per cent). The resistance inducing chemicals, plant extracts and bio-agents when tested *in vivo*, with challenge inoculation of *A. macrospora, Bacillus subtilis* was found effective in suppressing the pathogen and resulted in good germination (88.88%) and higher vigour index (3113.52) which was followed by salicylic acid and *Allium sativum* bulb extract. The higher vigour index showed in these treatments is mainly due to their support for increased germination, good root and shoot growth by the systemic resistance inducing agents.

Key words: *Alternaria macrospora,* bio agents, cotton, culture filtrate inducing chemicals, systemic resistance and toxigenic potential

INTRODUCTION

Leaf spot of cotton caused by *Alternaria* spp. (*Alternaria macrospora* Zimm. and *Alternaria alternata* Keissler) is an important foliar disease which causes yield loss to the extent of 26.0 per cent (Chattannavar *et al.*, 2001). However, the outbreak of disease in central and south zones was very significant, especially in certain Bt-cotton hybrids (Anonymous, 2005). The disease symptom mainly manifested on the leaf surface as minute brown spots, which were initially round later became round to irregular, expanded up to one cm diameter with purple margin around the spot having dry grey centre. With the introduction of Bt-cotton, there is spurt in incidence of few diseases, of which Alternaria leaf spot is one among. Several *Alternaria* spp., are known to produce a variety of toxins even though they are not required for their normal growth and reproduction. In *Alternaria*, many non-host specific toxins have been studied in detail with respect to *Alternaria alternata* f. sp. *lycopersici* (AAL toxin), *A. alternata* f. sp. *citri tangerine* (ACT toxin), *A. alternata* f. sp. *kikuchiana* (AK toxin), *Alternaria alternata* infecting sunflower (AS toxin), *A. brassicae* (Destruxin B) and *A. helianthi* (Deoxyradicin and radicinin) (Thomma, 2003). Where as the toxin produced by *A. macrospora* inhibited seed germination of cotton upto 100 per cent (Padmanaban and Narayanasamy, 1977) and was characterized as phenolic acid (Balasubramanian and Bhama, 1977). This paper makes an effort to understand the diversity in toxigenic potential of fourteen isolates of *Alternaria* spp., infecting Bt-cotton and thus might give an explanation for increased Alternaria leaf spot incidence of Bt-cotton.

The non toxic synthetic chemicals and some bio agents are known to act as strong elicitors of plant defense reaction leading to induced resistance and

enhanced broad spectrum of resistance against pathogens (Meteraux *et al.*, 1991). Hence, an attempt was made to induce the defense system in susceptible cultivar through seed treatment with inducing agents which might provide a reasonable solution to the non availability of resistant cultivars against specific pathogen.

MATERIALS AND METHODS

Collection of isolates of Alternaria spp.

The isolates of *Alternaria* spp. were obtained from the infected leaves of Bt-cotton by using standard tissue isolation technique. The pure culture of the fungus was obtained by hyphal tip isolation (Viviana *et al.*, 2007). The source and identity of fourteen isolates *Alternaria* spp. obtained from Bt-cotton are presented in Table 1.

Extraction of culture filtrate

The effect of culture filtrates of isolates of *Alternaria* spp. were studied in Czapeck's broth medium in order to find out the presence of phytotoxic metabolites (Padmanabhan and Narayanasamy, 1977). Fifty ml of Czapeck's broth was poured in 250 ml conical flask. After sterilization, one cm disc from the periphery of seven days old culture of the respective *Alternaria* spp. were inoculated and incubated at 27 ± 1° C for 15 days. Later mycelial mat was separated from the broth culture by filtration through cheese cloth and filtered by using Whatman No. 42 filter paper. The extract was examined through microscope to confirm that there was no spore in the filtrate. The culture filtrate thus obtained was used for bioassay.

Effect of culture filtrate on cotton seedlings

Different dilutions of culture filtrates of fourteen isolates *viz.*, 1:0, 1:1 and 1:3 were prepared by adding sterile distilled water and their effect was tested on cotton seedlings. The healthy 21 days old seedlings of cotton were placed in culture filtrate of 10 ml solution and suitable control was maintained using distilled water. Effect of culture filtrates on these plants was recorded after 24 h and 48 h, by observing the necrosis, chlorosis, epinasty and drooping symptoms.

Effect of culture filtrate on cotton seeds

Sixty seeds of cotton were soaked in 100 ml culture filtrates of each of fourteen isolates separately for 24 h. They were then spread on moistened blotting paper and each treatment was replicated thrice containing 60 seeds. Equal number of healthy seeds were soaked in sterile distilled water, which served as control. Observations on germination percentage of cotton seeds were recorded after seven days. The per cent inhibition of seed germination and vigour

Table 1. Sources and identity of fourteen isolates of *Alternaria* spp. infecting Bt-cotton

Isolates	District	Locality	Identification
A_1	Raichur	Attanur	*Alternaria macrospora*
A_2	Gulbarga	Bheemarayanagudi	*Alternaria macrospora*
A_3	Raichur	Dinni	*Alternaria macrospora*
A_4	Raichur	Farmers field	*Alternaria alternata*
A_5	Raichur	RARS, Raichur	*Alternaria macrospora*
A_6	Raichur	Jakkala Dinni	*Alternaria alternata*
A_7	Raichur	Kakarakallu	*Alternaria macrospora*
A_8	Raichur	Matamari	*Alternaria macrospora*
A_9	Raichur	Nelhal	*Alternaria alternata*
A_{10}	Raichur	Rampura	*Alternaria macrospora*
A_{11}	Maharashtra	Parbhani	*Alternaria macrospora*
A_{12}	Raichur	Sunkeswarala	*Alternaria alternata*
A_{13}	Raichur	Sunkeswarala	*Alternaria alternata*
A_{14}	Raichur	Uttur	*Alternaria macrospora*

index of seedlings treated with culture filtrate of each of fourteen isolates was calculated by following formula given by Abdulbaki and Anderson (1976).

Vigour index = (Shoot length + Root length) X Germination percentage

Induced systemic resistance

Effect of resistance inducing chemicals on growth of A. macrospora

Resistance inducing chemicals such as mannitol, magnesium sulphate, potassium dihydrogen phosphate, potassium nitrate, salicylic acid, sodium nitrate and sucrose were evaluated on the mycelial growth of *A. macrospora* which is a predominant species causing leaf spot of cotton. The fungus, *A. macrospora* was grown on PDA medium for 10 days prior to setting up of experiment. The PDA medium was prepared and melted. The resistance inducing chemicals were added to the melted medium to obtain the desirable concentration based on the molecular weight of the chemical. Twenty ml of this medium was poured in each sterilized Petri-plate. Suitable check was maintained without addition of resistance inducing chemical. Five mm mycelial disc taken from the periphery of the 10 days old colony was placed in the centre of Petri-plate and incubated at 27 ± 1° C for nine days. Three replications were maintained for each treatment. The diameter of the colony was measured in two directions and average was recorded and the per cent inhibition of growth was calculated by using the formula given by Savitha *et al.* (2004).

In vivo testing of induced resistance

The efficacy of different bio agents *viz., Bacillus subtilis* (E) *Pseudomonas fluorescens* (E), *Trichoderma harzianum* (E), *Trichoderma viride* (E) (1 X 10^8 cfu/ml), botanicals *viz.*, garlic and onion bulb extracts and *Prosopis juliflora* leaf extract at 10 per cent and resistance inducing chemicals, salicylic acid and magnesium sulphate (1%) were used for induction of resistance. The seeds of cotton NCS-145Bt were soaked in the above mentioned treatments for four hrs followed by 30 minutes shade drying and the seeds were inoculated with spore suspension (1 x 10^6 spores/ ml) of *A. macrospora*. The seeds treated with the fungus alone and distilled water was maintained for comparison. Twenty seeds were sown separately in pots having sterilised soil for each treatment. The seed germination and seedling vigour were calculated 20 days after sowing by using the formula given by Abdulbaki and Anderson (1976).

RESULTS AND DISCUSSION

Toxigenic potential of culture filtrate on cotton seedlings

A preliminary incication of toxin production by any fungus *in vitro* is usually provided by a number of bioassay methods *viz.*, bioassay methods of plant cutting, seed germination bioassay and roots and shoot elongation bioassay (Anahosur, 1976). The culture filtrate obtained from fourteen isolates of *Alternaria* spp. were subjected to above bioassay methods to know the toxigenic variable potency of different isolates. The cotton seedlings, which were placed in pure culture filtrate of all fourteen isolates showed varied toxigenic effect of metabolites. As the concentration decreased, expression of wilting and other symptoms on seedlings was also delayed. On the contrary, the seedlings placed in distilled water remained healthy even beyond 48 hrs. The isolates A_1, A_2 and A_3 showed drooping, wilting and necrosis within 24 hrs and showed severe wilting and browning within 48 hrs (Table 2). The effect of metabolites of isolates A_1, A_2, A_3, A_4, A_5, A_8, A_{11} and A_{14} on cotton seedlings was seen at all concentrations tested at both 24 and 48 hrs after incubation. The isolates A_{10} and A_{12} did not produce any effect on cotton seedling at 1:1 and 1:3 concentrations both at 24 and 48 hrs after incubation. Savitha *et al.*, (2004) also noticed the existence of toxigenic variability of different isolates of *Alternaria* spp. infecting sesame on sesame and tomato seeds.

Effect of culture filtrate on seed germination and seedling growth

All fourteen isolates differed in their ability to inhibit the seed germination, root and shoot length elongation and induction of phytotoxic symptoms on cotton seedlings at various concentrations tested. The results are in agreement with observation of Lu *et al.* (1987) who reported 135 isolates of *A. solani* differing in their ability to inhibit tomato seedlings.

Table 2. Toxigenic diversity of fourteen isolates of *Alternaria* spp. infecting Bt-cotton using culture filtrates

Isolates						
Conc.	1:0	1:1	1:3	1:0	1:1	1:3
A_1	Drooping, necrosis and wilting	Drooping , wilting and necrosis of lower leaves	Partial drooping	Complete wilting and browning	Drooping and necrosis of middle and lower leaves	Drooping and partial wilting
A_2	Drooping, necrosis and wilting	Drooping , wilting and necrosis of lower leaves	Partial drooping	Complete wilting and browning	Drooping and necrosis of middle and lower leaves	Drooping and partial wilting
A_3	Drooping and necrosis on lower leaves	Drooping , wilting and necrosis of lower leaves	Partial drooping	Complete wilting and browning	Drooping and necrosis of lower leaves	Drooping and partial wilting
A_4	Drooping and necrosis on lower leaves	Drooping of lower and middle leaves	Drooping of lower leaves	Drooping, necrosis of lower and middle leaves	Drooping and necrosis lower leaves	Drooping of leaves
A_5	Drooping and necrosis of lower leaves	Partial drooping of lower and middle leaves	Partial drooping of lower leaves	Complete wilting and necrosis	Drooping and necrosis lower leaves	Partial drooping
A_6	Partial drooping of lower and middle leaves	Partial drooping	Healthy	Drooping of entire plant	Drooping of lower and middle leaves	Healthy
A_7	Partial drooping of lower and middle leaves	Partial drooping of lower leaves	Healthy	Complete wilting and necrosis of lower mad middle leaves	Wilting and necrosis of middle and lower leaves	Drooping of leaves
A_8	Partial wilting	Partial drooping of lower leaves	Partial drooping of lower leaves	Complete wilting, browning and necrosis of lower mad middle leaves	Drooping and necrosis of lower leaves	Drooping of leaves
A_9	Partial drooping of lower and middle leaves	Drooping of lower leaves	Healthy	Drooping of leaves	Drooping of middle and lower leaves	Drooping of older leaves
A_{10}	Partial drooping of lower leaves	Healthy	Healthy	Drooping of lower and middle leaves	Healthy	Healthy
A_{11}	Drooping and necrosis on lower leaves	Drooping of lower and middle leaves	Drooping of lower leaves	Complete wilting and necrosis	Drooping and necrosis of lower leaves	Drooping of leaves
A_{12}	Partial drooping of lower leaves	Healthy	Healthy	Drooping of lower and middle leaves	Healthy	Healthy
A_{13}	Partial drooping of lower leaves	Healthy	Healthy	Drooping of entire plant	Drooping of middle and lower leaves	Drooping of older leaves
A_{14}	Partial wilting	Partial drooping and necrosis of lower leaves	Partial drooping of lower leaves	Wilting and necrosis	Partial wilting and necrosis of lower leaves	Partial drooping

The maximum inhibition of cotton seed germination was noticed in A_4 (38.44%) and shoot and root length in A_1 isolate to the extent of 52.14 and 52.45 per cent respectively (Table 3). Least inhibition of seed germination (21.61%) and root and shoot length of 21.61 and 23.11 per cent was noticed by isolate A_{10}. Several workers also established the toxigenic potentials of culture filtrates of *Alternaria* spp. (Ozcelik, 1996 and Maiero and Bean, 1991). The toxigenic potential of culture filtrate of *A. macrospora* on cotton, brinjal and tomato was studied by Vijayalaxmi *et al.* (1996). However, Balasubramanian and Bhama (1977) reported a phytotoxic compound, phenolic acid produced by *A. macrospora*, which was responsible for phytotoxic symptoms production on cotton, *Phaseolis vulgaris* and *Cyamopsis tetragonoloba*.

Table 3. Effect of culture filtrate of fourteen isolates of *Alternaria* spp. on seed germination and seedling growth of Bt-cotton

Isolates	Inhibition of seed germination (%)	Inhibition of seedling growth over control (%)	
		Shoot length	Root length
A_1	33.41	52.14	52.45
A_2	27.01	51.31	46.76
A_3	33.34	47.31	45.19
A_4	38.44	38.17	40.14
A_5	29.84	46.77	42.99
A_6	26.02	30.10	32.18
A_7	22.52	36.55	34.43
A_8	26.84	40.32	43.32
A_9	26.67	37.09	30.42
A_{10}	21.61	23.11	26.81
A_{11}	27.35	43.54	43.95
A_{12}	22.18	30.64	29.00
A_{13}	26.21	35.48	34.07
A_{14}	26.66	32.25	37.93
S. Em ±	0.51	0.61	0.70
C.D. at 1%	1.99	2.39	2.75

Induced resistance

Some of the chemicals are used to induce resistance in host plants, which defend against pathogenic infection. This systemic acquired resistance will spread to untreated part also thus providing protection against pathogens. In the present investigation, results showed that salicylic acid and magnesium sulphate at 10 mM concentration were effective in inhibiting the mycelial growth of *A. macrospora* by 84.15 and 52.50 per cent respectively (Table 4). Vigour index is the true criteria for observing induction of resistance. The efficacy of resistance inducing chemicals, botanicals and bio-agents was further studied *in vivo* condition also. The study indicated that the maximum

Table 4. Efficacy of resistance inducing chemicals in inhibiting the mycelial growth of *A. macrospora*, infecting Bt-cotton

Induced chemicals	Per cent inhibition
Mannitol	10.26
Magnesium Sulphate	52.50
Potassium dihydrogen phosphate	2.86
Potassium nitrate	27.15
Salicylic acid	84.15
Sodium nitrate	37.70
Sucrose	12.28
S. Em±	0.226
C.D. at 1%	0.92

Table 5. Effect of systemic resistance inducing agents on seed germination, seedling growth and vigour of cotton, challenged with *A. macrospora*

Treatments	Germination (%)	Mean shoot length (cm)	Mean root length (cm)	Vigour index
Bacillus subtilis	88.88	12.73	22.30	3113.52
Pseudomonas fluorescens	86.11	15.00	14.90	2574.75
Trichoderma harzianum	88.88	14.70	15.17	2654.72
Trichoderma viride	86.11	15.13	13.63	2476.25
Allium sativum (10%) bulb extract	88.88	13.53	18.23	2822.72
Allium cepa (10%) bulb extract	83.33	12.77	17.67	2536.01
Prosopis juliflora (10%) leaf extract	55.55	13.67	12.60	1459.27
Magnesium sulphate (1%)	52.78	14.27	9.37	1247.64
Salicylic acid (1%)	83.33	14.37	19.50	2822.66
Uninoculated control	91.11	19.00	17.97	3368.50
Inoculated control	52.78	9.20	7.83	898.81
S. Em ±	1.96	0.31	0.21	35.18
C.D. at 1%	7.87	1.251	0.84	141.58

germination per cent was observed in *Bacillus subtilis*, *Allium sativum* and *Trichoderma harzianum* treated seeds (88.88%) (Table 5), the maximum shoot length was observed in *Trichoderma viride* treated seeds (15.13 cm) where as root length in *Bacillus subtilis* treated seeds (22.30 cm). Over all, higher vigour index was noticed in *Bacillus subtilis* treated cotton seeds (3113.52). The efficacy was high in *Bacillus subtilis*, salicylic acid and *Allium sativum* treatments. The higher vigour index showed in these treatments is mainly due to their support for developing good root, shoot growth and increased germination percentage, hence vigour index was more in these treatments. Ratnam *et al.* (2001) also noticed effective control of *A. helianthi* by salicylic acid seed treatment in sunflower. Savitha *et al.* (2004) reported high vigour index of sesame seedlings by use of salicylic acid against *A. sesami*. This indicated the ability of these chemicals in inducing resistance which can also be extended to field for managing disease after working out the dosage and timing of such treatments.

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