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Prevalence of Fungal Phytopathogens on Transgenic Bt Cotton Plant: Isolation and Characterization

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A B S T R A C T

The prevalence of leaf spots and wilt of transgenic Bt cotton were surveyed in several cotton fields of North Maharashtra region, India. In this study, total four phytopathogenic fungal strains from the affected cotton plants were isolated and characterized. The pathogenicity of fungal strains was confirmed by Koch's postulates. The pathogenicity tests showed that all the isolated fungal strains are phytopathogen of the Bt cotton plant. Among the four fungal isolates, the prevalence of *Fusarium chlamydosporum* as potential phytopathogen of cotton is documented for the first time from India.

Introduction

Cotton is one of the most noteworthy and widely cultivated fiber crops. More than 50 countries located at tropical and subtropical regions of the world produces economically important cotton crop. Among them, India has largest area under cotton cultivation constituting about 30% of the global cotton area (WWF-India, 2012). Cotton plays an important role in sustaining the Indian economy as India is the world's second largest cotton producer (Khadi *et al*., 2009). Among the major cotton growing regions of India, North Maharashtra region alone occupies 5 lakh ha (5.23% of total land of India) area under cotton cultivation (Patil and Waykole, 2013) and is known to have high potential for cotton yield (Gopalakrishnan *et al*., 2007).

This important agricultural commodity is sensitive to a number of biotic and abiotic stresses and the diseases. Due to wide distribution and long exposure (5-6 months for planting to harvest) to the changing climatic condition, number of diseases are prevalent on cotton crop which affects the final yield (Bell, 1999). According to Agrios (2005), the estimated 36.5% average of total losses includes - 14.1% caused by diseases (fungi, bacteria and viruses), 10.2% by insects and 12.2% by weeds. Largest crop loss (14.1%) is by phytopathogens alone.

Among the phytopathogens, over 30 species of fungi can cause cotton plant infections (Farrell and Johnson, 2005). The present investigation emphasizes – isolation and identification of the phytopathogenic fungi associated with the transgenic Bt cotton plant cultivated in North Maharashtra region of India.

Materials and methods

Field survey and sampling

A rigorous and periodical field surveys were carried out in severely affected cotton fields cultivated with Bt-cotton variety (RCH- 2) from North Maharashtra region (Dhule, Jalgaon and Nandurbar Districts) in the year 2012- 2014. During this survey, the cotton leaves, stem and developing cotton bolls were noticed with leaf spots. Another noticeable symptom was wilting of cotton plant. The majority of cotton plants showed reduced growth and less vigor and vitality.

Another survey was conducted to compare whether the cotton seed sown in May or June has contributed in inducing the wilt and foliar disease. Total 15 different cotton fields located in North Maharashtra region were examined for appearance of disease symptoms. The five random micro plots of 25 × 25m^2 area with at least 50 plants/micro-plot were examined for diseased plants. The diseased plant parts (leaves, root, stem and developing cotton bolls) were collected in airtight polyethylene bags as specimen for fungal isolation. These specimens were stored at 4°C until further use.

Isolation and identification of the fungal strains

The collected plant specimens - root, stem, leaves, and bolls were washed under running tap water and then blotted dry using sterile filter paper. These specimens were further surface sterilized with 0.5% sodium hypochlorite (for 1 min) followed by washing with sterile distilled water (Zheng *et al*., 2011). The appropriate blocks (5 × 5 mm) of infected tissue of root, stem, leaves and bolls were cut and placed on a modified Czapek dox agar (CDA) plates having the composition (g/L): Sucrose, 30; $NaNO_3$, 0.5; K_2HPO_4, 0.5; $MgSO_4$, 0.5; KCl, 0.50; $FeSO_4$, 0.01; Streptomycin, 0.05; Agar, 20; pH 7.2. The plates were incubated at 28 ± 2°C for 7 days. The isolated strains were sub-cultured several times to ensure the purity and maintained on CDA medium at 4°C.

The morphological characteristics of all the isolated fungal strains were studied by slide culture assembly. The morphological features (colony characteristics, mycelial growth pattern, radial growth pattern, spore forms, size of the conidia) were scientifically examined as per key described by Alexopoulos *et al*., 1996; Williams-Woodward, 2001; Dugan, 2006. The identification of isolated fungal strains was further verified at National Fungal Culture Collection of India, Agharkar Research Institute (NFCCI-ARI), Pune (India).

Pathogenicity test

The isolates were corroborated for their ability to induce infection/disease on cotton plant. The pathogenicity test was conducted on cotton plant as well as cotton seed. The re-isolation of inoculated fungi from symptomatic plants was conducted to confirm the pathogenicity of isolates by Koch's postulates.

Pathogenicity test on cotton plant

Three week old (four-leaf stage of plant) healthy Bt cotton (RCH- 2) plants; grown in 13 × 11 cm plastic pots containing an

autoclaved soil were selected for the experiment. During the experiment, five pots each containing two plants were infected by respective fungal isolate. Similarly, five pots (each with two plants) were kept as control. Experiments were carried out in quadruplets. The spore suspension prepared in sterile distilled water amended with 0.1 % Tween 80 (total spore count - 10^6spores/ml) was employed in the infectivity assay. The control plants were treated with sterile distilled water containing Tween 80 (0.1%). The plants were grown in at 30/25 ℃ (day/night temperature) with a 16 h of photo period in green house and watered on alternate days. The disease progression in each plant was assessed by observing signs and symptoms in plant up to 45 days. The disease symptoms of each plant were rated by analyzing the root browning, vascular discoloration and vigor.

The 0-4 scale was used for analysis of stem and root infection consists: 0 = no symptoms, 1 = <10% root browning with the over ground part having no symptoms, 2 = 11–50% root browning & plant with less than 50% of its crown with wilt; 3 = >50% root browning & plant with greater than 50% of its crown with wilt, and 4 = browning of most of the root, plant dead (Zhao *et al*., 2014).

The plants showing leaf infection were rated by detecting (i) round or irregular shaped spots (2 to 5 mm), (ii) dark brown to grey in coloration and (iii) bright yellow margin on dry leaf. The 0-4 scale was used for analysis of leaf infection: 0 = no symptoms, 1 = <10% leaf spot, 2 = 11–50% leaf spot; 3 = >50% leaf spot and 4 = leaf spot all over the leaf (Shtienberg, 1996). The disease severity index (DSI) ranged from 0 (no disease) to 100 (plants death or complete diseased plant) and calculated as per Zhao *et al*., 2014.

$$DSI = \sum \frac{(\text{Disease severity scale point's} \times \text{number of plants at each scale point})}{(\text{Total number of plants survyed} \times \text{disease severity scale of the highest scale point observed})} \times 100$$

Analysis of variance (ANOVA) was conducted on the DSI data to determine the overall effect of the isolates and their interactions with cotton plant. The comparisons of the mean DSI data were made using Dunnets multiple range test ($P < 0.05$).

Pathogenicity test on seed

The pathogenicity of fungal isolate on cotton seed (Bt RCH- 2) germination was evaluated by calculating percent seed germination and by observing the plants vigor index. The virulence of fungal isolate to attack on seed germination was analyzed by (i) seed treatment method and (ii) soil treatment method. For both the experiments all the cotton seeds were surface sterilized using 1% (v/v) sodium hypochlorite followed by rinsing with sterile distilled water for three times.

For evaluation of seed infectivity by seed treatment method**,** forty seeds were soaked in 20 ml of spore suspension (10^6 spores/ml) of respective fungal isolate for 8 hours while controls were treated with sterile distilled water. The treated seeds were then incubated onto sterile moist filter paper containing plate (10 seeds/plate) at 28℃. On next day, the seeds were transferred to pot containing autoclaved soil (4 seeds/pot) (Shin *et al*., 2014). Similarly, for the evaluation of seed infectivity by soil treatment method**,** the seeds were sown (4 seeds/ pot) in 10 pots containing autoclaved soil thoroughly mixed with respective fungal isolates having spore density 10^6 spores/ml. The control sets were treated with distilled water (Islam and Borthakur, 2012).

The inoculated pots were grown in a greenhouse at 30/25℃ day/night temperature with a 16 h of photo period and watered on alternate days. The number of seeds germinated was recorded as seedling with coleoptiles length>1 cm at 7 days of incubation. Germination percentages were recorded as, the number of germinating seeds inoculated with respective fungal isolate divided by the number of germinated control seeds.

Plant vigor index

The vigor index of all the experimental cotton plants (emerge by seed and soil treatment method) were determined at the end of experiment (45 days) as per Lee *et al*. (2008). The vigor index was calculated by formula-

Vigor index = (Mean of root length + Mean of shoot length) × Percentage of seed germination.

Results and Discussion

Isolation and identification of the fungal pathogens

Based on field survey of major cotton growing regions of North Maharashtra it was observed that the incidence of fungal infection was highly prevalent on cotton plant. Several irrigated cotton fields cultivated with Bt (cotton-Rasi 2 variety) were witnessed as severely infected which is characterized by slow growth, less vigor and vitality, wilting and foliar infection during the month June to September in the year 2012- 2014.

Another survey was conducted to compare the variation of wilt and foliar disease symptoms according to seed sowing time (May-June). The results of survey showed that there was variation in disease symptoms according to seed sowing time.The average minimum/ maximum temperature and humidity of the month May was recorded as 27.13/40.87℃ and 30.27/71.2 respectively. While the average minimum/ maximum temperature and humidity of the month June was recorded as 26.57/38.27℃ and 32.8/75.93 respectively. The data presented in Table1 showed that the disease incidences of wilting symptoms are higher (28.27%) in cotton plants which were sowed in the month of May as compare to the cotton plants which were sowed in June (23.47) (after rain). Similarly the disease incidences (28.13%) for foliar disease symptoms were highly associated with plants that were sowed in June rather than sowed in May.

Total four fungal strains L1, L2, L4 and L8 were isolated during the study. Table 2 represents the identification of fungal strains studied in this investigation. The fungal strains L1, L2, L4 and L8 were identified and deposited at National Fungal Culture Collection of India, Agharkar Research Institute (NFCCI-ARI), Pune (India).

All the fungal isolates were grown on Czapek dox agar plates at 28 ± 2°C and were studied for their cultural and morphological characteristics. Table 3 summarizes the morphological feature and cultural characteristics. Fig.1 shows morphological observation of fungal isolates.

Pathogenicity of fungal isolates

Pathogenicity on cotton plant

The result of the pathogenicity test of all the four isolated fungi showed significant visible disease symptoms on cotton plant (Fig.2). Among the foliar disease causing fungi *Corynespora cassicola* (NFCCI 2952) showed maximum DSI as compare with *Alternaria* sp. (NFCCI 2955). *Corynespora*

cassicola and *Alternaria* sp. showed statistically significant DSI value when compared with control. While both the *Fusarium* species showed significant DSI value as compare with control cotton plant. *Fusarium chlamydosporum* (NFCCI 2948) showed slightly higher DSI than *Fusarium moniliforme* (NFCCI 2949) (Table 4).

Pathogenicity was also determined by measuring the vigor index of test and control plant. All the tested fungal isolates showed reduction in vigor index (Table 4). *Fusarium moniliforme* was the most virulent isolate showing 68.56% reduction in vigor index over control.

Seed germination

Fungal isolates were also studied for their effect on seed germination. All the studied fungal strains showed a considerable reduction of seed germination (Fig.3). In overall seed germination assay soil treatment method showed more reduction in seed germination as compare with seed treatment method. Among all the four fungal isolates *Fusarium chlamydosporum* and *Fusarium moniliforme* showed maximum reduction in cotton seed germination.

Similarly, *Corynespora cassicola* and *Alternaria* sp. also showed a profound (near about 50% reduction in seed germination) effect on reduction in seed germination (Fig.4). Plants emerged from remaining germinated seeds of these experiment were studied for vigor index. All the four isolates contributed in reduction of plants root and shoot length.

Table.1 Wilt and foliar Disease incidences on cotton

Field No.	Seed sowing in May				Seed sowing in June			
	Temperature ℃ (Min/Max)	Humidity (Min/Max) (%)	Wilt disease symptoms (DI)	Foliar disease symptoms (DI)	Temperature ℃ (Min/Max)	Humidity (Min/Max) (%)	Wilt disease symptoms (DI)	Foliar disease symptoms (DI)
1.	26/41	24/75	24	14	26/40	32/78	18	32
2.	28/40	17/70	40	16	27/39	26/79	20	30
3.	26/42	15/73	16	12	26/38	34/76	26	34
4.	28/40	19/72	28	20	27/40	31/72	24	28
5.	29/41	21/64	36	24	28/37	34/78	18	26
6.	28/43	18/78	26	18	26/38	37/79	30	34
7.	27/41	30/73	28	14	27/37	30/80	12	20
8.	27/40	32/68	20	12	28/40	32/76	24	22
9.	26/41	37/71	20	16	26/38	36/75	22	30
10.	26/40	53/81	32	22	26/39	34/74	32	36
11.	25/39	30/72	30	18	25/38	32/71	34	30
12.	29/42	19/69	26	16	27/39	31/69	26	30
13.	26/39	35/74	26	12	28/38	30/75	18	22
14.	26/42	53/65	38	30	25/37	37/78	34	28
15.	30/42	51/63	34	26	26/36	36/79	14	20
Mean	27.13/40.87	71.2/30.27	28.27	18	26.57/38.27	32.8/75.93	23.47	28.13

(DI- Disease incidence)

Table.2 Isolation and identification of pathogenic fungal strain from different parts of cotton plant

Sr. No.	Isolate	Name	Accession no.	Isolated from
1.	L1	*Fusarium fusarioides* (syn.= *chlamydosporum*)	NFCCI 2948	Stem
2.	L2	*Fusarium moniliforme*	NFCCI 2949	Root
3.	L4	*Corynespora cassicola*	NFCCI 2952	Leaves
4.	L8	*Alternaria* sp.	NFCCI 2955	Leaves

Table.3 Visual characteristics of fungal isolates on Czapek dox agar medium after 7 days incubation

Characteristics	*Fusarium chlamydosporum*	*Fusarium moniliforme*	*Corynespora cassicola*	*Alternaria* sp.
Growth rate (mm/d)[a]	11.67 ± 5.16	12.67 ± 3.14	9 ± 2.28	15.2 ± 2.39
Colony diameter (mm) Mean ± SD	79.67 ± 1.53	86.33 ± 1.53	56.33 ± 0.58	89.67 ± 0.58
Mycelia growth pattern	Good growth, aerial, floccose, Circular growth	Good growth, aerial, floccose to powdery circular growth	Moderate growth, circular, flat textured, smooth margin	Fast growth, Circular, thin, flat, rough margin
Colony colour	Top- White Bottom- Light orange	Top- White Bottom- Pale yellow, tinged with purple	Top - Grey Bottom- Black	Top- Olivaceous greenish black Bottom- Black
Conidia	Thick walled and moderately curved. Unequal dorsiventral curvature, *i.e.*, the upper wall is curved and the lower wall is almost straight. Short and pointed apical cell	Hyaline, thin walled, formed in chains, straight to slightly sickle shaped.	Hyaline, long, straight to slightly curved, Obclavate to cylindrical and apex obtuse	Ovoid or ellipsoid in the larger initial units, then progressively broader ovoid, then smaller ovoid in terminal regions of the chain. Multicellular, are produced in straight chains, or branching chains. The end of the conidium has a beak-like appearance
Conidia size[b]	32.12 µm long and 4.35 µm wide	13.44 µm long and 2 µm wide	56.23 µm long and 7.47 µm wide	47.16 µm long and 13.49 µm wide
Septa	septate (2-4)	septate (3-6)	Pseudo septa (4-12)	Vertical (1-2) and Transverse septa (6-7)
Sporulation[c]	Good	Moderate	Abundant	Moderate

a Growth rate was taken after 7 days of incubation at 28°C.
b mean values of length and width of 50 randomly picked macro conidia ± standard deviation.
c Sporulation was measured after 7 days of incubation at 28 °C.

Table.4 Pathogenicity test on cotton plant. Disease severity index and vigor index when healthy plant was artificially inoculated with four fungal isolates

Fungal isolate	Disease severity Index (DSI)*	Vigor Index	Percentdecrease of vigor index over control
Corynespora cassicola	43.63a	2219.38	44.96
Alternaria sp.	42a	2476.29	38.59
Control	0.25	4032.34	-
Fusarium chlamydosporum (=fusarioides)	40.25a	1397.43	65.93
Fusarium moniliforme	39.00a	1289.45	68.56
Control	2.50	4102.32	-

DSI = ∑ (disease severity scale point's × number of plants at each scale point)/ (total number of seeds surveyed × disease severity scale of the highest scale point observed) × 100.

Mean in each coloum followed by the same letter are not significantly different within treatment group calculated by one way ANOVA using Dunnets multiple range test ($P > 0.05$). $P < 0.05$ Vs Control after treatment

Table.5 Pathogenicity test on cotton seed. Disease severity index and vigor index when cotton seeds were artificially inoculated (Seed inoculation and Soil inoculation method) with four fungal isolates

Fungal isolate	Disease severity Index (DSI)*		Vigor index		Percent decrease of vigor index over control	
	Seed inoculation	Soil inoculation	Seed Treatment	Soil Treatment	Seed Treatment	Soil Treatment
Corynespora cassicola	45.63a	51.25a	2505.28	2383.15	37.73	40.24
Alternaria sp.	45a	50.63a	2111.85	2314.73	47.51	41.96
Control	0.25	0.5	4023.64	3988.21	-	-
Fusarium chlamydosporum (=fusarioides)	41.25a	46.25	1285.38	1179.6	67.45	72.11
Fusarium moniliforme	39.38a	40.63	1286.95	1185.93	67.41	71.96
Control	2.50	1.88	3948.88	4229.1	-	-

DSI = ∑ (disease severity scale point's × number of plants at each scale point)/ (total number of seeds surveyed × disease severity scale of the highest scale point observed) × 100.

Mean in each coloum followed by the same letter are not significantly different within treatment group calculated by one way ANOVA using Dunnets multiple range test ($P > 0.05$). $P < 0.05$ Vs Control after treatment

Fig.1 Fig. 1 Growth pattern of fungal isolates after 7 days of incubation at 28 on Czapek Dox agar (a) *Fusarium chlamydosporum*, (b) *Fusarium moniliforme*, (c) *Corynespora cassicola*, (d) *Alternaria* sp.
Morphological features of fungal hyphae observed under microscope (40×objective) (e) *Fusarium chlamydosporum*, (f) *Fusarium moniliforme*, (g) *Corynespora cassicola*, (h) *Alternaria* sp.(Scale bars = 25 µm)

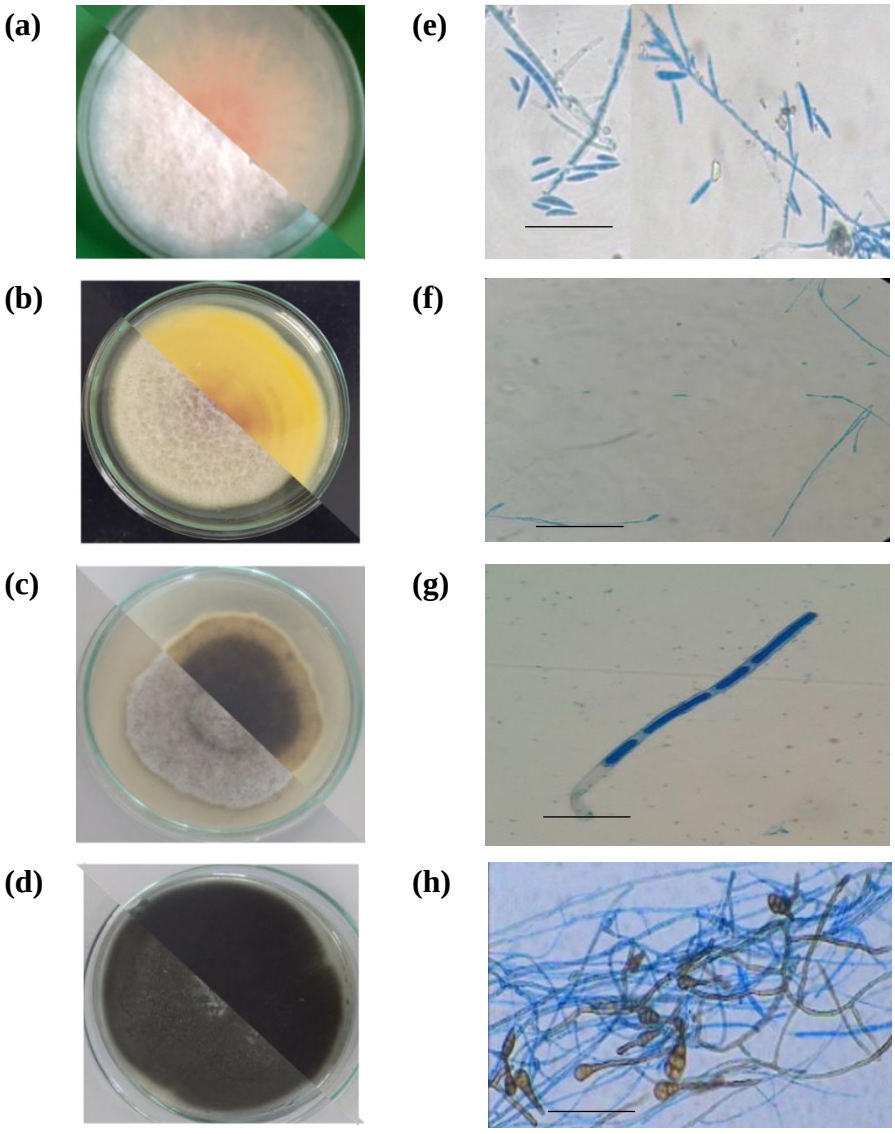


Fig.2 Fig.2 Pathogenicity test of isolated fungi on cotton plant (a)Control, (b)*Fusarium chlamydosporum*, (c) *Fusarium moniliforme*,(d)*Corynespora cassicola* and (e)*Alternaria* sp.

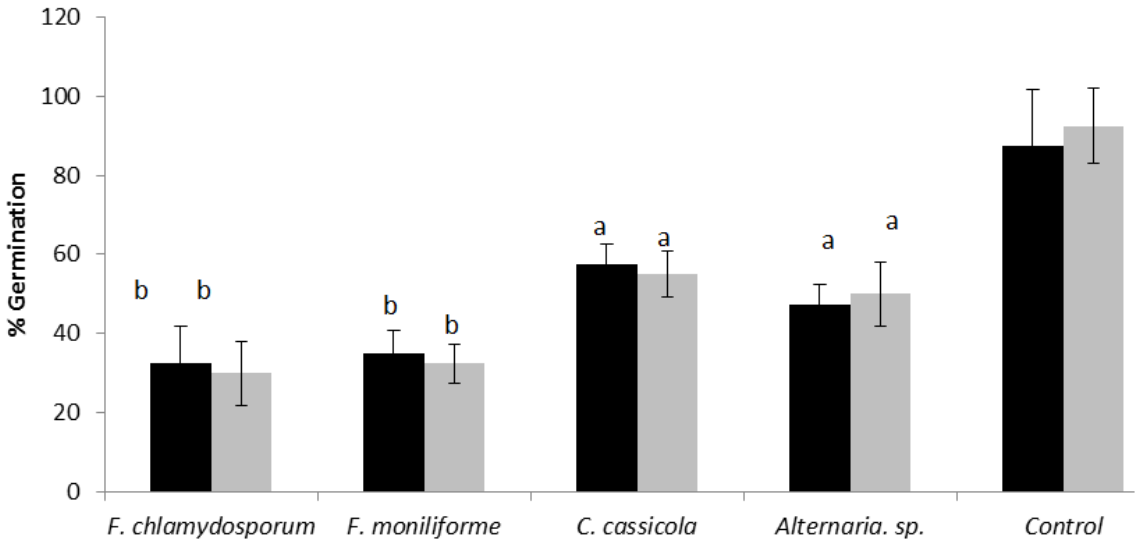
(a) **(b)** **(c)** **(d)** **(e)**

Fig.3 Pathogenicity test of isolated fungi on cotton seeds (a) Control (b) *Fusarium chlamydosporum* (c) *Fusarium moniliforme* (d) *Corynespora cassicola* (e) *Alternaria* sp.

(a) **(b)** **(c)**

(d) **(e)**

Fig.4 Effect of fungal isolates on cotton seed germination (■ seed treatment, ■ soil treatment). Bars with the same letter are not significantly different. [a]P>0.05 Vs. control after treatment; [b]P<0.05 Vs. control after treatment



The vigor index of cotton plant in soil treatment method was consistently lower than seed treatment method. The *Fusarium chlamydosporum* and *Fusarium moniliforme* have contributed in maximum reduction in plant vigor. Similarly *Corynespora cassicola* and *Alternaria* sp. also showed considerable reduction in cotton plant vigor compared to control (Table 5).

The extensive field surveys of various regions of North Maharashtra indicated the prevalence of various fungal infections on Bt cotton plant. During survey cotton plants with wilting, leaf spot of brown, black & pale yellow color, reduced height, less vigorous growth of plantand boll infection were considered symptoms. Total 4 fungal strains - *Fusarium fusarioides* (syn. =*chlamydosporum*) (NFCCI 2948); *Fusarium moniliforme* (NFCCI 2949); *Corynespora cassicola* (NFCCI 2952); *Alternaria* sp. (NFCCI 2955) were isolated from different plant parts indicating that different fungi targets different part of plant for their successful colonization in plant. Among these isolates, *Fusarium chlamydosporum* and *Fusarium moniliforme* are known to cause seedling disease complex causing pre and post-emergence damping off and seedling root rot diseases (Abd-Elsalam *et al*., 2006, Costa *et al*., 2005); while *Alternaria* sp. and *Corynespora cassicola* were reported to cause foliar diseases (Bashan *et al*., 1991, Galbieri *et al*., 2014).

The wilt disease incidence on cotton plants sowed in May was higher than cotton plants sowed in June. This may be due to the favorable climate for fungi causing wilt disease (like *Fusarium* species). In irrigated fields, due to adequate/excess supply of water and higher temperature (above 40℃) elevate the humidity which promotes the growth of fungi causing wilt disease. On the other hand foliar disease incidences are less (18%) on cotton plants sowed in May than cotton plants sowed in June. This might be due to foliar disease causing fungi cannot

tolerate the heat of May and hence could not develop at younger stage.

The pathogenicity test on cotton plant stated that all the isolates are potential phytopathogens which can lead to severe crop damage. The pathogenicity of 4 different fungi attributed to 4 different genera was tested against cotton plant. All the four fungal species were positive and potentially infect to the cotton plant by inducing various symptoms like root browning, wilting and leaf spots. *Fusarium* isolates showed direct influence on reduction of plant height, vigor, and root length. Among the other pathogens *Corynespora cassicola* was the aggressive one and causes leaf spot disease. *Alternaria* sp. also showed effective disease severity index.

Majority of fungal pathogen attacks during the seed germination (the fungal pathogen may be seed borne/soil borne). Among the isolated fungal pathogens, *Fusarium chlamydosporum* and *Fusarium moniliforme* showed a greater impact in reduction of cotton seed germination. Even the germinated seeds (treated with *F.chlamydosporum* and *Fusarium moniliforme*) after emergence up to the entire plantlet showed reduced vigor, vitality, boll formation and overall reduced plant health.

Although *Corynespora cassicola* and *Alternaria* sp. did not show much impact in reduction of seed germination, but the foliar disease symptoms were observed at mature stage showed a moderate effect in reduction of cotton seed germination. From these results it can be concluded that the isolated fungal strains are potential phytopathogens and have the capacity to induce infection to plantlet as well as during germination of cotton seed.

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