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Assessment of arbuscular mycorrhizal population and its diversity in Bt-cotton at Jalgaon, Maharashtra

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Abstract

Cotton is one of the important fiber plant grown worldwide commercially over 70 nations. India is the only country where all the cultivated species and certain hybrid combinations are cultivated commercially. Cotton occupies only five percent of the arable land and supports sixty million people having a direct bearing on the country's economy. Cotton growers have so many constraints for cotton production but the most important is its high vulnerability to insects. Particularly to the larvae of *Lepidopteran* pests, which are impacting cotton production. During the entire growth period of *Bt*-cotton, *Bt* toxin is introduced into the soil through root exudates, decomposing plant material and/or pollen deposition. Deposition *Bt* toxin in soil may pose a potential risk to soil microorganisms such as plant growth promoting rhizobacteria, fungal population, Total bacterial population and arbuscular mycorrhizal (AM) fungi, a kind of non-target soil microorganisms fundamental for soil fertility and plant nutrition. It has been reported that *Bt* toxin leach out in the rhizosphere and inhibits the growth of soil microbes. Therefore, the present study was undertaken to study the impact of *Bt*-cotton and non-*Bt*-cotton cultivation on soil microflora at seedling stages of cotton plants in different sites of Jalgoan, Maharashtra. The obtained results revealed that *Bt*-cotton crop adversely affected the Arbuscular mycorrhizal population and percentage of root bit infection.

Keywords: arbuscular, mycorrhizal, population, Maharashtra

Introduction

Cotton is one of the most significant plant fiber crops commercially grown worldwide in over 70 nations within temperate and tropical climates, covering a total area of 33 million hectares (Smith 1999) [21]. It is also known as 'white gold' and the premier commercial cash crop in India. Cotton (*Gossypium hirsutum* L.) is a member of the family Malvaceae, subfamily Malvoideae, and tribe Gossypieae. Cotton growers have so many constraints to cotton production but the most important is its high vulnerability to insects. Particularly to the larvae of lepidopteran pests, which are adversely affecting productions of cotton. Use of chemicals for the suppression of the cotton pests is proving ineffective for the reason that these pests have a high level of resistance. Such a high level of resistance requires repeated application of insecticides leading to heavy expenditure, crop failures, and vicious cycle of debt for farmers. Therefore, many reasons are behind for their adoption to *Bt*-cotton that could help in the protection of crop against potentially and most destructive bollworms. *Bt*-cotton is the first genetically modified crop to be grown in India. Initially, it was introduced or approved in India on March 26th, 2002 for commercial cultivation. Cotton containing the *Bt* gene contains the *Cry1Ac* gene that was extracted from the soil bacterium *Bacillus thuringiensis* var. *kurstaki*, common soil bacteria, thus resulting in the development of the *Cry1Ac* protein, which gives bollworm resistance (Barwale *et al*., 2004) [1]. The production of cotton in India increased rapidly with the introduction of *Bt*-cotton in the year 2002. Among the top, five cotton producer countries are India (28 million bales), China (26.80 million bales), the United States (18.3 million bales), Brazil (13.2 million bales), and Pakistan (5.7 million bales). Rhizospheric soil is a diverse and dynamic ecosystem in which microorganisms manage the majority of biological processes. Soil microorganisms have several beneficial impacts, including nitrogen fixation, organic matter deposition, breakdown of metabolic wastes, and increased availability of phosphates, sulphates, nitrates, and critical metals (Bridge and Spooner, 2001) [3]. Microorganisms that reside on plant roots can have an impact on plant development, both positively and negatively (Liljeroth and Baath, 1988) [11]. Genetically modified plants have the ability to impact soil microbial populations, hence altering essential ecosystem activities such as carbon cycling, nutrient solubilization, and the prevalence of soil-borne plant disease (Sarkar *et al*., 2009; Beura and Rakshit, 2013) [17, 2]. *Bt* toxin is released into the soil through root exudates, decomposing plant waste, and/or pollen deposition throughout the entire growth period of *Bt* cotton (Icoz and Stotzky, 2008) [7]. To resist microbial degradation, *Bt* toxin binds to clays and humic compounds, and its insecticidal activity can be maintained in the soil for several months (Saxena and Stotzky, 2001) [18]. *Bt* toxin deposition in soil may endanger soil microorganisms such as plant growth-promoting rhizobacteria, fungal populations, total bacterial populations, and arbuscular mycorrhizal (AM) fungi, which are non-target soil microorganisms important for soil fertility and plant nutrition. Among all the soil microorganisms, AM fungi are the most important component of natural and agricultural ecosystems belonging to the ancient phylum Glomeromycota (Schüßler *et al*., 2001) [19]. AM fungi plays an important role in association with and for host plants. AM fungi multiply the absorptive surface of roots by interacting with fine roots on the one hand and spreading over a large region of soil. Increased absorptive surface improves nutrient and water absorption, resulting in improved host plant performance. AM fungi improve activity of nitrogen fixing organisms, i.e., plant growth promoting rhizobacteria (PGPR) and help to survive plant under stress condition i.e, drought conditions (Oyewole *et al*., 2017) [13], nutrient deficient soils, alkaline/saline soils (Porras-Soriano *et al*., 2009; Kehri *et al*., 2016) [15, 8], acidic soils (Seguel *et al*., 2013; Muthukumar *et al*., 2014) [20, 12], mining overburden dumps (Wang, 2017) [22] and heavy metal polluted soils (Gaur and Adholeya, 2004; Khan, 2005; Göhre and Paszkowski, 2006) [4, 9, 6]. In view of the above fact, the present study has been undertaken to understand the impact of *Bt* crops on AM fungi and focusing on how *Bt* plants affect AM fungi colonization and symbiotic growth at seedling stage.

Material and Methods

Selection of Survey Site

For comparative studies of *Bt* and non-*Bt* cotton arbuscular mycorrhizal population and diversity from rhizospheric soil of both cottons were taken. For our research, I chose only the different villages of Jalgaon which come under north Maharashtra (Nashik division). The goal of this study was to investigate the role of below-ground AM population and diversity in *Bt* and non-*Bt* cotton from the rhizospheric zones. AMF spore population was investigated on the basis of their morpho taxonomy.

Collection of Soil Samples

An extensive survey was carried out to access the biodiversity of soil microflora (AMF spore and mycorrhization). The soil samples were collected from rhizosphere soils of *Bt* cotton and non-*Bt*-cotton plants from Diksai, Anjan vihire, Ridhur, Pathrad, Jalgaon, Maharashtra, India. Collected soil samples were properly placed in sterile zip-lock bags, transported to the laboratory, and kept at 4˚ C until further analysis. Samples were collected in the month of Aug-Sep (seedling stage).

Extraction and estimation of arbuscular mycorrhizal spores from rhizospheric soil

For the isolation of AM fungi wet sieving and decanting method of Gerdemann and Nicolson, (1963) [5] was used. Ten grams of air-dried rhizospheric soil was dissolved in 100 ml of tap water. The mixture stirred properly with the help of a glass rod and was kept for some time till all debris and soil particles settled down. The supernatant was decanted through a series of sieves arranged in descending order of mesh size (1 mm, 500, 300,100 and 50 µm). The suspension of each sieve was separately filtered through Whatman No.1 filter paper. The filter papers were placed in the Petri plate and were taken to ensure that they remain moist. The contents of the filter papers were examined for spores and sporocarps under the Stereobinocular microscope under appropriate magnification (4X, 10X, 40X, 100 X) according to the method given by Gaur and Adolaya (1994).

Mounting of AM spores

A mixture of polyvinyl alcohol lacto-glycerol (PVLG) was used to mount intact and crushed spores on a glass slide (Koske and Tessier, 1983) [10]. Take drop of PVLG and spores were put in drops with the help of needle or paint brush. The mountant was allowed to set for 3-5 minutes to become more viscous. The spores mounted in PVLG were used to study the gross spore morphology. The spores mounted in PVLG were crushed carefully applying pressure on the coverslip using the blunt end of a needle. The slides were then placed in an oven at 60°C for 24 hours to remove air bubbles. The slides were labeled and stored in flat boxes. Names of identified species, localities of occurrence, and data regarding their distribution were recorded.

Identification of AM fungi

Collected AM spores from soil samples were mounted in PVLG and Meltzer's reagent was used for staining the spores. External features of the spore like colour, size and shape, number of wall layers, visible contents and shape of the subtending hypha were made using a light microscope. Studies of spore wall layers were done applying gentle pressure on coverslips and kept in the oven at 45ºC for at least 24 hours. Identification of AM spore was done by using phylogeny and taxonomy of Glomeromycota Schüßler and Walker (2001) [19] and for confirmation of the identity of AM spore was done by using the world largest mycorrhizal collection site that is INVAM. Website

Frequency, Density and Abundance of AM Spores

$$\text{Frequency (\%)} = \frac{\text{No. of plants in which AM species was observed}}{\text{Total no of AM spores in plants}} \times 100$$

$$\text{Abundance} = \frac{\text{Total no. of AM sp. Presents in all plants}}{\text{No. of plants where AM species occurred}}$$

$$\text{Density} = \frac{\text{Total no. of AM sp. Presents in all plants}}{\text{total no. of plants}}$$

AM species diversity was calculated by following formula

Shanon-Weiner diversity index = - $\sum_{i=1}^{s}(\text{Pi} \ln \text{Pi})$

Pi = proportion of individual of species i

Simpson diversity index = $1 - \sum_{i=1}^{s}(\text{Pi})^2$

Pi = proportion of individual of species i

Species eveness is calculated by Pielou's index = $\frac{H'}{H'\text{max}}$

H´ = Shanon-Weiner diversity index

H´max = maximum Shanon-Weiner diversity index

AM colonization

Collected tertiary root samples were properly washed and with the help of tap water and processed by using Phillips and Hayman, (1970) [14] staining method. Properly cleaned collected root samples were cut into 1.0 cm root bits and kept in 10% KOH (potassium hydroxide) for 24 hours at room temperature or at 90°C for 1 hr. Root bits were washed again with the help of tap water to remove excess KOH. Then root bits kept into 4% NaOCl (sodium hypochlorite) for bleaching for 10 minutes. After this, roots bits were washed with tap water and 1% HCl was added in the test tube to soak the root pieces for 3-4 minutes, and then solution was poured off. The root segments were stained in 0.05% trypan blue (prepared in lactic acid, glycerol, and water (2:2:1) and kept 24 hours at room temperature, or this was also achieved by heating at 90°C for 1 hour. Excess stain removed by using a destaining solution (lactic acid, glycerol, and water (2:2:1). Root segments were mounted in destain and examined under the microscope for AM colonization. Total 100 root-pieces were observed for calculation of the percentage of mycorrhization was as follows-

Measurement of root colonization by AM fungi

Root segments (1cm long) stained in 0.1% trypan blue were mounted on microscope slides. The presence or absence of AM colonization (arbuscules / vesicles / hyphae) was scored. Percentage of root colonization was calculated using the formula of (Read *et al*., 1976) [16].

$$\textbf{\% Root colonization} = \frac{\text{No. of roots bits infected}}{\text{Total no. of root bits observed}} \times 100$$

Result and Discussion

Physico-chemical Properties of Soil

The data analysis of soil samples collected from the different survey sites (Diksai, Ridhur, Anjan Vihire and Pathrad) of Jalgaon, Maharashtra.The pH, electrical conductivity and nutrient status of soil varied according to the sites, cotton variety as well as rhizospheric and non rhizospheric soil. The pH of soil of selected sites was slightly alkaline and ranges from 7.4 to 7.8. The electrical conductivity was 0.26-0.50 ms/cm. The nutrient status of selected sites was more in rhizospheric soil in comparison to non rhizospheric soil for both Bt cotton plants and non-Bt cotton plants. The organic carbon ranged from 0.24 to 0.77%. Available phosphorus estimated in terms of P_2O_5 and potassium content estimated in terms of K_2O, was ranged from 13-23 kg/h and 258-269 kg/h respectively. Sulphur amount was 18-28 ppm in soil samples of selected sites. Average essential micronutrients in the soil were also recorded, Boron amount 0.5-0.9 ppm, Iron 0.80-10.92 ppm, Manganese 10.60-13.04 ppm, Zinc 0.56-1.18 ppm and Copper 0.62-2.20 ppm.

Arbuscular Mycorrhizal Status

The arbuscular mycorrhizal status of both *Bt*-cotton and non-*Bt* cotton collected from different sites located at Jalgaon, Maharashtra was estimated in the terms of mycorrhizal infection in the roots and spore population in the rhizospheric and non-rhizospheric soils at their seedling stage.

Mycorrhization

All the root samples collected from different sites showed mycorrhizal infection. However, the magnitude of infection and spore population varied with the site, cotton variety and stage of plant growth. Average root bit infection ranged from 34% to 52% in non *Bt*-Cotton plants and 23% to 29% in *Bt*-cotton plants (Table 1; Plate 1). Maximum root bit infection was recorded at Diksai site while minimum at Pathrad site in both *Bt*-cotton and non *Bt*-cotton plant.

Table 1: Mycorrhizal colonization in roots of non-*Bt* cotton growing at Jalgaon, Maharashtra, India

	Mycorrhizal Infection (%MI)	
Site/Stages	**Non-*Bt*-Cotton**	***Bt*-Cotton**
Diksai	52±3.1	29±3.0
Ridhur	50±3.0	27±3.2
Anjan Vihire	44±2.7	27±2.7
Pathrad	34±2.7	23±2.7

*: means ± standard deviation with same letters is not significantly different at P = 0.05 (n=3)

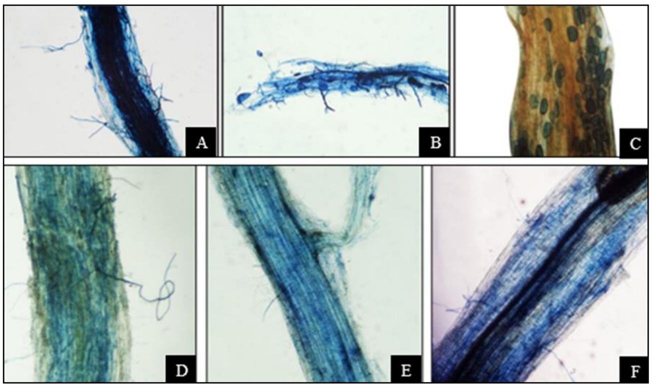

Plate 1: A, B, C Mycorrhizal colonization showing in roots of Non-*Bt* cotton plants
D, E, F Mycorrhizal colonization showing in roots of *Bt* cotton plants

AMF Spores Population

A variety of AMF spores were isolated from the rhizospheric and non- rhizospheric soils of both *Bt*-cotton and non-*Bt* cotton plants from survey sites has been is depicted in Tables 2 & 3. They were identified with the help synoptic keys of Trappe (1982), Schenck and Perez (1990), Morton and Benney (1990), Oehl *et al* (2011) and INVAM species guide.

The average spore population in rhizospheric soil ranged from 49 to 68 spores/10 g air dried soil in non-*Bt*-cotton at seedling stage while in *Bt*-cotton average spore population ranged from 35 to 48 spores/10 g air dried soil (Table 2) and the average spore population in non-rhizospheric soil ranged from 24 to 33 spores/10 g air dried soil in non-*Bt* cotton at seedling stage while in *Bt*-cotton average spore population ranged from 17 to 28 spores/10 g air dried soil (Table 3). Maximum AMF spore population was recorded at Diksai site while minimum at Pathrad site in both rhizospheric soils for both *Bt*-cotton and non *Bt*-cotton plants.

Diversity, Density, Frequency and Abundance of AM Fungi in the Rhizospheric and non-rhizospheric Soils of the cotton crops at seedling stage

AMF spore population, density, frequency, abundance, diversity and species evenness in the rhizospheric and non rhizospheric soil of the non- *Bt*-cotton and *Bt*-cotton plants were presented in (Table 4-7; Plate. 2). Mostly they were the species of *Glomus, Acaulospora, Scutellospora* and *Claroideoglomus.* The AMF spore diversity, frequency and density were varied with the site, cotton. Maximum diversity was recorded in non-*Bt*-cotton as compare to *Bt*-cotton in both rhizospheric and non rhizospheric soil. *Glomus* was the most dominant among them *Claroideoglomus* was very rare.

Table 2: AMF spore population in the rhizospheric soil of non-*Bt* and *Bt*-Cotton cotton growing at Jalgaon, Maharashtra, India

AM Spore Population 10 $^{g-1}$ of airdried soil) Rhizospheric		
Site/Stage	**Non-*Bt*-Cotton**	***Bt*-Cotton**
Diksai	68±5.2	42±4.5
Anjan Vihire	57±7.4	35±4.1
Ridhur	61±8.7	48±5.4
Pathrad	49±5.2	37±5.5

All data shows average of 3 sampling (n=3)

Table 3: AMF spore population in the non- rhizospheric soil of non-*Bt* and *Bt*-Cotton cotton growing at Jalgaon, Maharashtra, India

AM Spore Population 10 $^{g-1}$ of airdried soil) non -rhizospheric soil		
Site/Stage	**Non-*Bt*-Cotton**	***Bt*-Cotton**
Diksai	33±6.3	22±3.9
Anjan Vihire	29±2.7	17±3.4
Ridhur	28±3.1	28±3.3
Pathrad	24±3.9	21±3.4

All data shows average of 3 sampling (n=3)

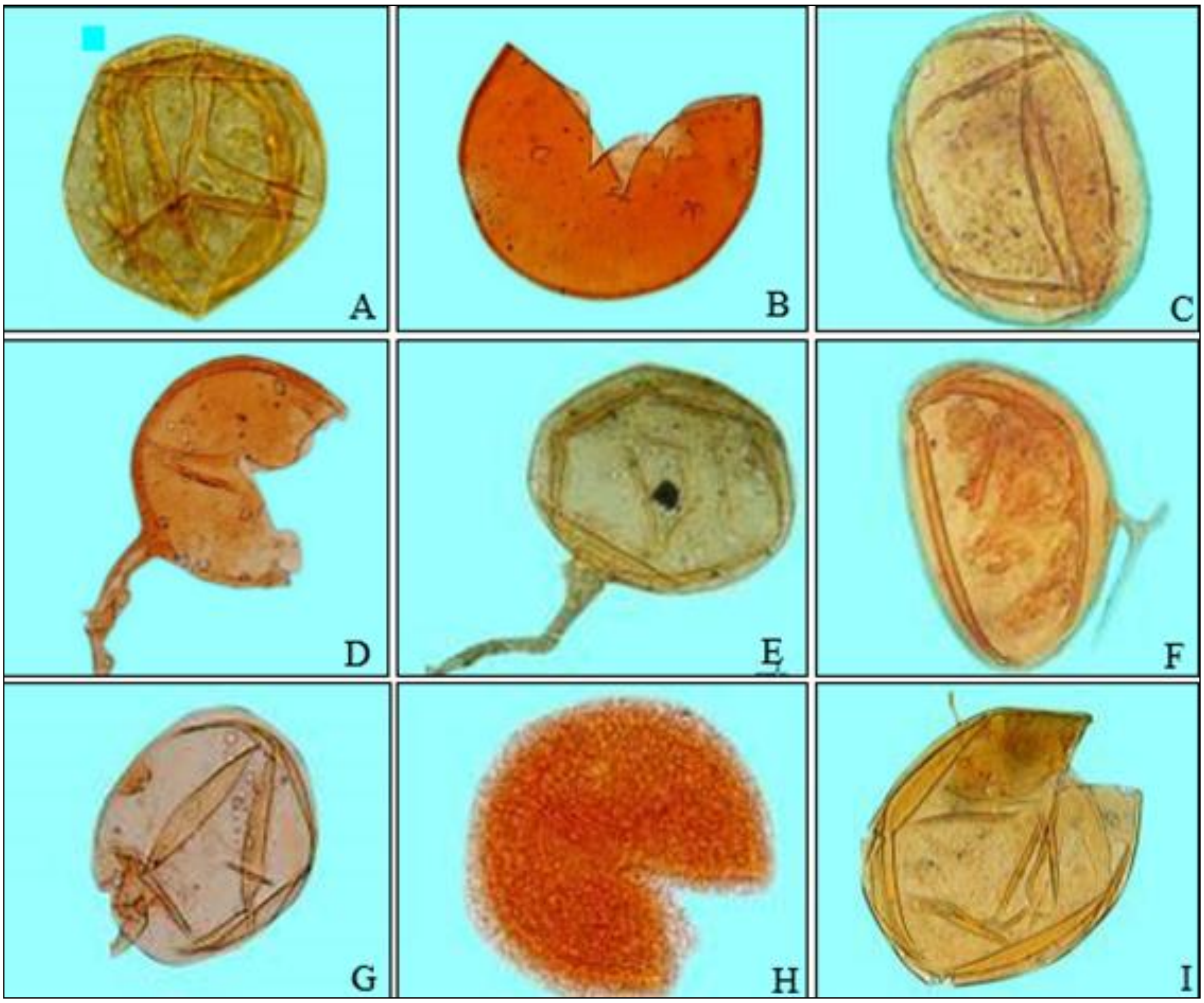

Plate 2: AMF spores isolated from the rhizospheric and non-rhizospheric soils of *Bt*- cotton and Non-*Bt* cotton plants:
A: *Acaulospora laevis,* **B:** *Acaulospora sp.,* **C:** *Acaulospora longula,* ***D:*** *Claroideoglomus etunicatum,*
E: *Glomus albidium,* **F:** *Glomus intraradices,* **G:** *Glomus mosseae,* **H:** *Glomus tortuosum* & **I:** *Glomus* sp.

Table 4: Community structure of AM fungi during Seedling stage of non-*Bt* cotton (rhizospheric soil) at Jalgaon, Maharashtra

Community composition of AM fungi	**Diksai**	**Anjan Vihire**	**Ridhur**	**Pathrad**	**Frequency**	**Abundance**	**Density**
Acaulospora laevis	5	-	3	6	75	4.6	3.5
Acaulospora scrobiculata	2	8	-	2	75	4.0	3.0
Acaulospora sp.1	3	-	-	2	50	2.5	1.2
Glomus aggregatum	9	2	17	9	100	9.2	9.2
Glomus fasciculatum	9	7	16	3	100	8.7	8.7
G. mosseae	15	21	7	10	100	13.2	13.2
Glomus tortuosum	9	5	-	-	50	7.0	3.5
Glomus sp.1	7	3	-	4	75	4.6	3.5
Glomus sp.2	2	-	2	6	75	3.3	2.5
Glomus sp.6	4	5	-	4	75	4.3	3.2
Glomus sp.7	-	-	9	3	50	6.0	3.0
Scutellospora sp.1	3	6	7	-	75	5.3	4.0
Total No. of Spores	68	57	61	49			
Dominance_D	0.1263	0.201	0.1981	0.1295			
Shannon diversity index H	2.212	1.837	1.747	2.162			
Simpson diversity index 1-D	0.8737	0.799	0.8019	0.8705			
Evenness_e^H/S	0.8302	0.7851	0.8192	0.8689			

Table 5: Community structure of AM fungi during seedling stage of *Bt* cotton (rhizospheric soil) at Jalgaon, Maharahtra

Community composition of AM fungi	Diksai	Anjan Vihire	Ridhur	Pathrad	Frequency	Abundance	Density
Acaulospora sp.1	2	1	1	1	100	1.2	1.2
Acaulospora sp.3	1	3	1	7	100	3.0	3.0
Acaulospora scrobiculata	8	6	6	2	100	5.5	5.5
Claroideoglomus etunicatum	2	2	2	-	75	2.0	1.5
Glomus aggregatum	9	5	5	-	75	6.3	4.7
Glomus clarum	-	5	2	1	75	2.6	2.0
Glomus *constrictum*	1	2	2	-	75	1.6	1.2
Glomus mosseae	18	6	20	17	100	15.2	15.2
Glomus sp.1	-	3	1	3	50	3.5	1.7
Glomus sp.2	-	-	-	4	25	4.0	1.0
Glomus sp.3	1	4	-	-	50	2.5	1.2
Total No. of Spores	42	37	40	35			
Dominance_D	0.2721	0.1205	0.2975	0.3012			
Shannon diversity index H	1.566	2.192	1.617	1.498			
Simpson diversity index 1-D	0.7279	0.8795	0.7025	0.6988			
Evenness_e^H/S	0.5984	0.8952	0.5598	0.6389			

Table 6: Community structure of AM fungi during seedling stage of non-*Bt* cotton (Non-rhizospheric soil) at Jalgaon, Maharashtra

Community composition of AM fungi	Diksai	Anjan Vihire	Ridhur	Pathrad	Frequency	Abundance	Density
Acaulospora scrobiculata	-	2	5	3	75	3.3	2.5
Acaulospora sp.1	1	1	-	6	75	2.6	2.0
Glomus aggregatum	6	-	5	2	75	4.3	3.2
Glomus fasciculatum	7	4	3	-	75	4.6	3.5
Glomus mosseae	4	13	8	5	100	7.5	7.5
Glomus tortuosum	2	3	-	-	50	2.5	1.25
Glomus. sp.1	9	-	-	-	25	9.0	2.25
Glomus.sp.2	4	3	2	3	100	3.0	3.0
Glomus sp.6	-	2	2	4	75	2.6	2.0
Glomus sp.7	-	-	3	1	50	2.0	1.0
Scutellospora sp. 1	-	1	-	-	25	1.0	0.25
Total No. of Spores	33	29	28	24			
Dominance_D	0.1864	0.2533	0.1786	0.1736			
Shannon diversity index H	1.781	1.703	1.829	1.831			
Simpson diversity index 1-D	0.8136	0.7467	0.8214	0.8264			
Evenness_e^H/S	0.8477	0.6866	0.8895	0.8918			

Table 7: Community structure of AM fungi during seedling stage of *Bt*-cotton (Non-rhizospheric soil) at Jalgaon, Maharashtra

Community composition of AM fungi	Diksai	Anjan Vihire	Ridhur	Pathrad	Frequency	Abundance	Density
Acaulospora sp.1	2	2	1	1	100	1.5	1.5
Acaulospora sp.3	-	3	-	-	25	3	0.7
Acaulospora scrobiculata	5	-	4	2	75	3.6	2.7
Claroideoglomus etunicatum	-	-	2	-	25	2	0.5
Glomus aggregatum	2	4	2	4	100	3	3
Glomus clarum	1	3	3	2	100	2.25	2.2
Glomus constrictum	-	-	-	1	25	1	0.2
Glomus mosseae	9	5	11	5	100	7.5	7.5
Glomus sp.1	2	2	2	1	100	1.7	1.7
Glomus sp.2	1	2	2	1	100	1.5	1.5
Glomus sp.3	-	-	1	-	25	1	0.2
Total No. of Spores	22	21	28	17			
Dominance_D	0.2479	0.161	0.2092	0.1834			
Shannon diversity index H	1.637	1.885	1.876	1.871			
Simpson diversity index 1-D	0.7521	0.839	0.7908	0.8166			
Evenness_e^H/S	0.7345	0.9412	0.7255	0.8115			

Conclusion

A significant difference in Rhizosphere and non-Rhizosphere soil AM spore between *Bt* and non-*Bt*-cotton clearly demonstrated the transgenic influence of *Bt*-cotton on soil flora. Microbiological abundance in non-*Bt* soil revealed the non-toxication effect while the toxic effect of the *Cry1AC* gene reduced the microbial population. Therefore, *Bt* may not be safe for the non-targeted microbes including rhizosphere and non-rhizosphere AM spore population.

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