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A comparison of endophyte assemblages in transgenic and non-transgenic cotton plant tissues

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Seed, root, stem, petiole and leaf tissues of *Bt* and non-*Bt* cotton (Krishidhan variety) were screened for the presence of symptomless endosymbiotic fungi (endophytes). All the tissue types of both *Bt* and non-*Bt* plants harboured endophytes. Although the number of endophyte species isolated from the two types of plant did not vary much, the number of isolates of endophytes was higher in all non-*Bt* tissues when compared with the respective *Bt* tissues. The lower infection frequency observed for the *Bt* cotton tissues may not be due to a direct effect of *Bt* gene insertion, but possibly due to the *Bt* plant warding off insect pests.

Keywords: *Bt* cotton, fungal endophytes, infection frequency, plant tissues.

THE efficiency of the insecticidal protein (Cry1Ab protein) produced by *Bacillus thuringiensis* (*Bt*) in killing several lepidopteran herbivorous pests has led to the development of transgenic crop plants carrying the *Bt* gene, such that they are insect-resistant *ab initio* in the field. Among such genetically modified crops, *Bt* cotton is most popular and is being cultivated all over the world[1]. In India, about 7.6 million hectares (m ha) was under *Bt* cotton cultivation during 2009, accounting for nearly 80% of the total area under cotton cultivation[2]. To evaluate the effects of transgenic plants on agroecosystems, several studies have been conducted on the influence of *Bt* cotton on target and non-target insects[3–5]. Relatively fewer studies address the effect of *Bt* gene integration on microorganisms associated with cotton plants. Sarkar *et al.*[6] and Chen *et al.*[7] studied the impact of *Bt* cotton on the soil enzymes and rhizosphere bacteria respectively. Wang *et al.*[8] looked at the changes in the diversity of leaf surface microorganisms of *Bt* cotton. However, except for the recent work of Vieira *et al.*[9], there are no studies on the effect of *Bt* gene integration on the endophyte status (viz. diversity, tissue preference and density of infection) of cotton plants. Here we compare the endophyte assemblage of different tissues of *Bt* cotton plant with those of non-*Bt* cotton plant to assess the influence of the genetic modification of host on endophyte colonization and to provide baseline data for further detailed studies.

Endophytes are an ecological group of fungi, mainly belonging to the Ascomycotina, which reside inside living tissues of plants without producing any visible disease symptoms[10]. They are ubiquitous[11], and may enhance the fitness of their hosts by protecting them against insect pests[12] and pathogens[13]. Plants infected with endophytes tolerate abiotic stress better than those that are endophyte-free[14]. The interactions between host plants, herbivorous insects and the endophytes colonizing these plants are complex and little understood[12,15].

Seedlings of Krishidhan variety of *Bt* and non-*Bt* cotton raised from seeds of *Gossypium hirsutum* (Central Institute of Cotton Research, Nagpur, India) were sampled. Mature, green and symptomless leaves, petioles, main stems (5 cm above soil level) and roots (5 cm below soil level) were collected from 60-day-old plants grown in open garden, and screened for endophytes. We did not study older plants since Cry protein levels are reported to be low in older seedlings[16]. Healthy tissues were collected from 10 *Bt* or non-*Bt* plants, washed in tap water, cut into 0.5 sq. cm segments, and surface sterilized using ethanol and bleach[17]. Surface-sterilized *Bt* and non-*Bt* seeds were also cut into 0.5 cm^2 segments and screened for endophytes. The different tissue segments (100 segments for each tissue type) were plated on antibiotic (chloramphenicol 150 mg l^{-1}) amended potato dextrose agar medium and incubated in a light chamber at 26°C for 1 month to isolate the endophytes[17]. To test the efficacy of surface-sterilization, the surface-sterilized tissue segments were gently pressed onto the agar medium and removed. Such petri dishes were incubated and observed

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Table 1. Number of species, infection frequency (IF%, ± = standard error) and dominant endophyte species isolated from *Bt* and non-*Bt* cotton plants

Organ	Cotton	No. of species	IF %	Dominant endophyte species
Seed	Non-*Bt*	7	76 ± 4.37	*Aspergillus* sp. 6
	Bt	9	12 ± 1.73	*Aspergillus* sp. 1
Leaf	Non-*Bt*	9	35 ± 1.33	*Phomopsis* sp.
	Bt	7	22 ± 1.33	*Colletotrichum* sp. 1
Petiole	Non-*Bt*	10	23 ± 1.67	*Aspergillus niger*
	Bt	6	8 ± 0.67	*Chaetomium* sp. 1
Stem	Non-*Bt*	20	42 ± 1.20	*Phomopsis* sp.
	Bt	13	28 ± 1.53	*Phomopsis* sp.
Root	Non-*Bt*	14	25 ± 0.33	*Aspergillus* sp. 5
	Bt	8	10 ± 1.45	*Phomopsis* sp.

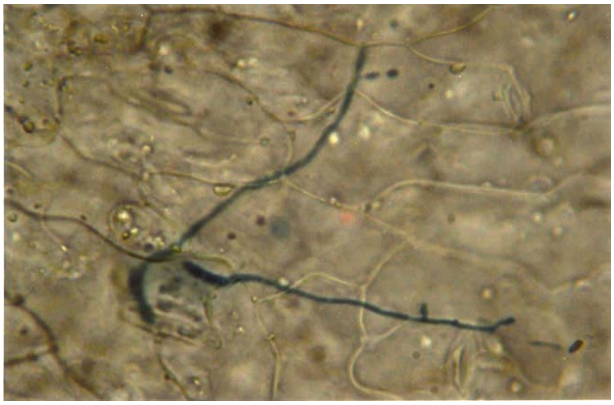

Figure 1. Endophytic *Colletotrichum* sp. spores germinating on *Bt*-cotton leaf.

for the growth of fungi. The absence of growth of any fungus from such tissue impressions proved the efficacy of the sterilization procedure[18]. The infection frequency (IF) of endophytes was calculated as follows.

$$\text{IF\%} = \frac{\left(\begin{array}{c}\text{Number of segments colonized}\\ \text{by endophyte species}\end{array}\right)}{\text{Total number of segments screened}} \times 100.$$

To observe the germination of conidia of an endophyte isolate on *Bt* leaf surface, a drop of spore suspension in water (10^6 ml^{-1}) was placed on a detached and washed *Bt* leaf and incubated in a moist chamber for 48 h. After this, the leaf was fixed and cleared by autoclaving for 1 min at 103 kPa in lactophenol : ethanol (1 : 2 v/v) mixture and stored at 26°C. Cleared leaves were stained with 0.05% trypan blue in lactophenol for 40 min at 60°C and destained in warm lactophenol for observation under the microscope[19]. Agar imprints of washed and uninoculated *Bt* leaves were made simultaneously and incubated. The absence of *Colletotrichum* colony in these plates proved that any conidial germination observed is not a result of pre-existing conidia on the leaf surface.

All the tissues of the cotton plants screened harboured endophytes, although their infection frequency varied; seeds had maximum endophyte infection followed by stem and leaf (Table 1). The number of endophyte species isolated from the respective *Bt* and non-*Bt* cotton tissues did not vary to great extent. However, for all the tissue types screened, the number of endophyte isolates was higher for the non-*Bt* tissue than for the *Bt* tissue (Table 1). *Aspergillus* spp., *Aureobasidium pullulans*, *Bartalinia* sp., *Chaetomium* spp., *Colletotrichum* sp., *Drechslera* sp., *Geomyces* sp., *Lasiodiplodia theobromae*, *Nigrospora oryzae*, *Nodulisporium* sp., *Paecilomyces* sp., *Penicillium* sp., *Phomopsis* sp., *Pseudogymnoascus* sp. and *Trichoderma* sp. could be isolated from both *Bt* and non-*Bt* tissues. *Alternaria* sp., *Cladosporium cladosporioides* and *Phyllosticta capitalensis* were present only in non-*Bt* tissues. These are common endophytic fungi with a wide host range[11] and could have been isolated from *Bt* tissues with increased sampling. *Myrothecium* sp. and *Pyrenochaeta* sp. were isolated only from *Bt* tissues. The IF% of the above fungi which occurred exclusively in *Bt* or non-*Bt* tissues was very low (1–2). Increased sampling is required to confirm such tissue specificity. *Aspergillus* spp., *Phomopsis* sp., *Colletotrichum* sp. and *Chaetomium* sp. were dominant in various tissues of cotton (Table 1). Vieira *et al.*[9] also observed these genera of fungi in *Bt* and non-*Bt* cotton tissues. Although Vieira *et al.*[9] report that the integration of *Bt* gene in cotton has no influence on the frequency of endophyte colonization, our results show that at least in the early stages of the plant, the IF% of endophytes in the various tissues of *Bt* plants is less when compared to those of non-*Bt* plants (Table 1). Although the *Bt* gene produces Cry protein in all tissues of the *Bt* plant[20], the reduction in endophyte number in transformed plant tissues may not be due to the Cry protein since, according to Vieira *et al.*[9], the Cry protein does not influence endophyte colonization. The non-protein metabolites of the *Bt* tissue also may not affect the endophyte colonization, since we found that the growth of *Phomopsis* and *Colletotrichum* endophytes of cotton was not affected by *Bt* leaf extracts. Furthermore, we observed that the conidia of *Colletotrichum* endophyte germinated readily and produced appressoria (infection structures) on *Bt* leaves (Figure 1). Since our earlier studies show that a sampling of 100 tissue segments (0.5 sq. cm size each) is adequate

for endophyte survey[21], it is likely that the low levels of endophyte infection in *Bt* tissues is not due to the direct effect of Cry protein on the fungi, but due to an indirect effect of the *Bt* gene incorporation. We suggest that this is a result of reduced insect damage to these plants. *Bt* cotton plants are known to support lesser diversity of natural insect pests[22]. Wounds caused by phytophagous insects increase susceptibility of plants to fungal pathogens[23,24]. The incidence of insect-mediated fungal diseases has been shown to be much less in *Bt* plants[25]. Our hypothesis that *Bt* gene insertion influences endophyte colonization indirectly by reducing insect visitations raises a few questions. (i) Do phytophagous insects play a role in the acquisition of endophytes by plants? (ii) Since the presence of some endophytes can improve biotic[13] and abiotic[14] stress tolerance of their hosts, how would young *Bt* plants with lesser endophyte infection perform under such stresses? (iii) As endophyte colonization imposes a cost on the plant host[26], is the better yield by *Bt* plants also, at least in part, a result of lesser endophyte colonization? Studies involving different varieties of Bt cotton under different stress conditions can throw more light on the multitrophic interactions among *Bt* plant, their endophytes and insect herbivores.

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