Endophytic fungi associated with transgenic and non-transgenic cotton

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Transgenic Bt cotton expresses the Cry1Ac protein from Bacillus thuringiensis, which could influence the plant's capability to host endophytic fungi. The diversity of endophytic fungi in leaves, stems and roots from transgenic (Bt) and its isoline (non-Bt) cotton was evaluated during different plant developmental stages to investigate possible non-target effects of genetically modified cotton on endophytic fungal communities. A total of 17 genera of endophytic fungi were isolated. The most frequently isolated species were Phomopsis archeri from leaves and stems and Phoma destructiva from roots. While the Bt modification had no effect on endophytes, the cotton tissue and the plant developmental stage significantly influenced the diversity and composition of the fungal community. These results represent the first evaluation of the composition of endophytic fungi associated with transgenic cotton plants.

Keywords: diversity; endophyte; Bt cotton; Cry1Ac protein; Gossypium

Introduction

Cotton (Gossypium spp.) is one of the most economically important crops in Brazil occupying 856,000 ha and yielding 1.25 million tons in 2009 (CONAB 2009). Bt cotton is the third most cultivated genetically modified crop worldwide representing approximately 7% of the area cultivated by biotechnology-derived crops. Several countries, including Brazil, now allow Bt cotton to be commercially planted (James 2009).

The high demand for cotton makes it one of the most financially stable commodities. Processing of the cotton crop requires a significant investment in specialized ginning facilities, and the crop itself attracts a variety of damaging insect pests, especially lepidopteran larvae. Approximately US$ 3–5 billion are spent on pesticides each year, and of that, US$ 645 million goes toward cotton production (Santos et al. 2003; Martins et al. 2007). One alternative to pesticides is the use of genetically modified plants that are pest resistant, for example Bt plants. However, it is important to evaluate and understand the potential effects of these crop modifications on their associated microorganisms.

The genome of genetically modified Bt cotton incorporates a gene from the entomopathogenic bacterium Bacillus thuringiensis (Bt). This gene encodes the protein Cry (crystal protein), which is toxic to many lepidopteran pests (Crickmore et al. 1998). The cultivation of Bt cotton has been considered a beneficial technology reducing the need for insecticides. It is a pest-control technology that can be used by large or small producers under a variety of cultivation or pest-management practices (Torres et al. 2009).

The Cry proteins, including Cry1Ac which is encoded by the cry1Ac gene, are constitutively expressed in genetically engineered plants and are therefore produced continuously throughout the plant's life cycle. This protein is actively accumulated in plant cells where it may directly interact with associated microorganisms (Sticher et al. 1997). Consequently, the expression of the Cry protein in the cotton plant could adversely affect the composition and environmental functions of the associated microbial community, which includes soil microorganisms, nitrogen-fixing bacteria and epiphytic and endophytic fungi (Donegan et al. 1995; Saxena and Stotzky 2000; Bruinsma et al. 2003).

Endophytic microorganisms include bacteria and fungi that inhabit, for at least part of their life cycle, the interior of plants without harming the hosts, and in fact, they may be beneficial to the host (Petrini et al. 1992). Endophytic fungi may promote the health of the host plant, enhancing the plant's ability to adapt to environmental stresses. It is known that endophytic fungi promote plant resistance to abiotic (Redman et al. 2002; Bae et al. 2008) and biotic stresses (Azevedo et al. 2000; Arnold et al. 2003). This
The symbiotic relationship may confer greater fitness to plants thus allowing them to fully develop, as demonstrated by their higher rates of germination and rooting, and increased tissue biomass and seed production under adverse conditions (Schardl and Phillips 1997; Liu et al. 2001; Faeth 2002; Selosse et al. 2004; Azevedo and Araújo 2007). Therefore, the present study aimed to investigate possible changes in the density and composition of endophytic fungal communities associated with Bt cotton compared to non-Bt cotton.

Materials and methods

Plant materials

The study was conducted with a single variety of genetically modified Bt cotton (Acala 90B), and its non-Bt isolate, Acala 90. From May to November 2008, plants were grown in an experimental field at the Department of Agronomy, Federal Rural University of Pernambuco (UFRPE), Recife-PE, Brazil (14 m altitude; 08°01’009”S and 34°56’694”W). Seeds were provided by Monsanto of (Sao Paulo, Brazil Ltd) and the local soil was characterized, at pH 6.6, 0.125 cmol·dm⁻³ potassium, 0.078 cmol·dm⁻³ sodium and 1100 cmol·dm⁻³ phosphorus. Soil preparation, planting and weed control were performed manually.

Bt and non-Bt cotton were planted in the same area, with one row per isolate, and 1.0 m spacing between rows. The plants received only one fertilizer treatment formulated with 10 g of NPK 4-14-8 per plant. No chemical pest control was applied during the experiment.

Plants were sampled at four developmental stages (Souza and Beltrão 1999): pre-flowering (20 days after emergence), flowering (60 days after emergence), apple formation (100 days after emergence) and boll opening (140 days after emergence). Ten plants (5 Bt and 5 non-Bt) were sampled and analysed at each time point. At each stage, the plants were removed from the soil using a trowel, placed in plastic bags and transported to the laboratory where healthy leaves, stems and roots were collected individually and washed under running tap water.

Three leaf samples per plant were obtained: one from the top, one from an intermediate node and one from the base of the plant. Sampled stems were collected by cutting a section (5–15 cm long by 0.5–2.0 cm diameter, depending on the age and size of the plant) from the top, middle and basal sections of each sampled plant. The main and secondary roots were also sampled.

Isolation and identification of endophytic fungi

Isolation

Fungi were isolated from healthy leaves, stems and roots after surface disinfection by serial washing in 70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl⁻) for 3 min, 70% ethanol for 30 s followed by two rinses in sterilized distilled water (Araújo et al. 2005). Previous analyses have shown that the same surface disinfection protocol may be used for all plant tissues. The efficiency of surface disinfection process was confirmed by plating aliquots of the sterile distilled water used in the final rinse on potato dextrose agar (PDA). After dis-infection, samples were cut into 5 × 5 mm pieces and aseptically transferred onto plates (6 fragments per plate) containing PDA supplemented with 100 mg l⁻¹ of chloramphenicol to suppress bacterial growth. A total of 540 fragments were sampled per sample time, 270 from Bt cotton and 270 from non-Bt cotton, which corresponded to fragments from each tissue type across both genotypes. Plates were incubated at 28°C and monitored for up to 30 days to capture emergence of slow-growing fungi. The frequency of colonization (FC, %) was measured as the ratio between the number of fragments with microbial colonies and the total number of fragments (Araújo et al. 2005). Emerging fungal colonies were selected, purified and stored at 4°C on PDA.

Identification

Fungi were identified by microscopic observation of typical fungal structures and compared with the literature (Booth 1971; Ellis 1971; 1976; Button 1980; Barnett et al. 2000; Halin 2000; Lacaz et al. 2002). Representative isolates of fungi were deposited at the Culture Collection URM (Universidade Recife Micologia), Brazil.

Cry1 Ac protein quantification

The same fresh cotton tissues that were used for the isolation of endophytic fungi were also sampled and stored at −25°C for subsequent quantification of the Cry1 Ac protein levels by enzyme-linked immunosorbent assay (ELISA) (Torres and Ruberson; 2008).

All frozen material was thawed, weighed, macerated with phosphate-buffered saline solution (PBST= 0.8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4, 1 L H2O, pH 7.2) and Tween 20 (0.5% v v⁻¹) and homogenized, transferred to 10 ml tubes at a dilution of 1:10 (w v⁻¹) for leaves and 1:5 (w v⁻¹) for stems and roots, and centrifuged at 5000 × g for 1 min. The extracted supernatants were transferred to 1.5 ml centrifuge tubes and stored at −25°C until the ELISA assay was completed (1–2 weeks later). For protein assays, samples were thawed at room temperature and centrifuged at 4000 × g for 1 min and loaded at a rate of 100 µl per test well containing antibody coated for Bt Cry1 Ac ELISA with a kit using peroxidase enzyme conjugate. Standards of Cry1 Ac at 0.625, 1.25, 2.5, 5, 10, 20 and 40 ng ml⁻¹ were serially titrated to plot a standardized optical density curve for estimating the protein content of the tested material. Reactions were stopped by adding 50 µl of...
3 M sulphuric acid. Absorbance at 450 nm was measured with an ELx808 microtitre plate reader. The Cry1Ac protein concentrations in each sample were estimated from the standard curve.

Statistical Analysis

The data for colonization frequency of fungi in plant parts at different developmental stages were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Bartlett’s test). Data were subsequently examined by analysis of variance using a $2 \times 3 \times 4$ factorial design with two treatments (Bt and non-Bt cotton) using three plant parts and four developmental plant stages as the main factors. Initially, cotton, the main factor, was insignificant, and therefore it was removed from further analysis and mean comparisons. Means of the factors with significant results were compared using Tukey’s HSD (studentized range test) at a 5% significance level. All analyses were conducted using SAS Software (SAS Institute, Cary, NC, USA; 1999–2001). Species richness and the Simpson indices were estimated using the program EstimateS (Colwell et al. 2004). Correlations between individual isolates and environmental variables (plant genotype, plant tissue and stage of development) were determined by multivariate analysis using the software Canoco 4.5 for Windows (Biometris, Wageningen, The Netherlands), following previously described procedures (Andreote et al. 2009a; Ramette 2007; Ter Braak and Smilauer 2002). Briefly, detrended correspondence analysis (DCA) was performed first to calculate the gradient distribution of the “species” in all evaluated treatments. At a normal distribution of “species” in fungal community identification (gradient in the first axis < 4.0), data were analysed by redundancy analysis (RDA). For statistical analyses of the correlations between “species” and “environmental” factors, a Monte Carlo permutation test was included, based on 499 unrestricted permutations. In addition to $P$-values, values of Lambda 1 were obtained as a quantification of the amount of variance explained by each environmental factor.

Results

Isolation of endophytic fungi

The FC from each community of endophytic fungi associated with Bt cotton and non-Bt cotton were similar ($F_{1,4} = 1.38, P = 0.3050$), indicating that expression of the Bt Cry1Ac protein did not affect the degree of fungal colonization in either plant type. However, the FC increased with increasing plant age in all tissues (root, stem and leaf), indicating a significant relationship with the cotton plant developmental stages that was independent of cotton genotypes ($F_{3,12} = 94.94, P < 0.0001$, Figure 1). Furthermore, the FC of endophytic fungi among the different plant tissues differed significantly ($F_{2,8} = 100.08, P < 0.0001$). At all developmental stages, the leaves showed a higher FC (Figure 1). The community of endophytic fungi associated with roots, stems and leaves consisted of 17 genera: Acremonium (A. fusidioides and A. kiliense), Cladosporium (C. cladosporioides), Colletotrichum (C. gloeosporioides), Curvularia (C. lunata var. aeria), Fusarium (F. lateritium, F. moniliforme and F. oxysporum), Glomerella (G. cingulata), Guignardia (G. bidwellii), Lecanicillium (L. lecanii), Nigrospora (N. sphaerica), Pestalotiopsis (P. maculans), Phoma (P. destructiva, P. cupyprena and P. glomerata), Phomopsis (P. archeri), Rhizopus (R. microsporus and R. stolonifer), Rhodotorula (R. glutinis), Talaromyces sp., Tritirachium (T. oryzae) and Xylaria sp. Bt cotton and non-Bt cotton harboured a similar abundance, richness and diversity of fungi (Table 1). The groups of identified endophytes found were Zygomycota (8.70%), Ascomycota (17.4%), and anamorphic fungi (73.90%). Other fungi were categorized as non-sporulating fungi because they did not produce reproductive structures and therefore could not be identified by their morphological characteristics.

Among the species of endophytic fungi found in both cotton genotypes, the most frequent were P. destructiva from roots (11.4%) and P. archeri Sutton from stems (16.8%) and from leaves (22.9%).

With respect to the prevalence of species during the different plant developmental stages, the most frequent species were P. maculans during the pre-flowering stage (29.3%), followed by P. archeri during flowering (35.1%) and opening of bolls (10.5%) and C. gloeosporioides during apple formation (16.8%).

Regarding the specificity of colonization by the plant part surveyed, the following species were found in both
Table 1. Species of fungi associated with Bt and non-Bt cotton and frequency of colonization (FC, %) in different plant parts (leaves, stems and roots) collected during four different developmental stages (pre-flowering, flowering, apples formation and boll openings).

| Endophytic fungi                              | Bt cotton | Non-Bt cotton |
|-----------------------------------------------|-----------|---------------|
|                                              | N   | L  | S  | R       | N   | L  | S  | R       |
| **Acremonium fusidioides** (Nicot) Gams      | 1   | 0  | 100.0 | 0  | 2   | 0  | 100.0 | 0  |
| **Acremonium kiliense** (Grütz)              | 9   | 33.3 | 22.2 | 44.4  | 8   | 25.0 | 0  | 75.0  |
| **Cladosporium cladosporioides** (Fresen) Vries | 4   | 0  | 25.0 | 75.0  | 5   | 0  | 20.0 | 80.0 |
| **Colletotrichum gloeosporioides** (Penz.) Penz. & Sacc | 36  | 72.2 | 22.2 | 5.5  | 37  | 56.8 | 35.1 | 8.1  |
| **Curvularia lunata var. aeria** (Lima & Vasconc.) Ellis | 30  | 46.6 | 30.0 | 23.3  | 33  | 48.5 | 33.3 | 18.2 |
| **Fusarium lateritium** Nees                  | 13  | 30.7 | 61.5 | 7.6  | 19  | 21.1 | 47.4 | 26.3 |
| **Fusarium moniliforme** Sheld                | 0   | 0  | 0  | 0      | 2   | 100.0 | 0  | 0  |
| **Fusarium oxysporum** * Schldl               | 13  | 30.7 | 53.8 | 15.4  | 18  | 33.3 | 55.6 | 11.1 |
| **Glomerella cingulata** (Stoneman) Spauld & Schrenk | 4   | 0  | 50.0 | 50.0  | 5   | 80.0 | 0  | 20.0 |
| **Guignardia bidwellii** (Ellis) Viala & Ravaz | 2   | 100.0 | 0  | 0      | 4   | 75.0 | 25.0 | 0  |
| **Lecanicillium lecanii** (Zimm.) Zare & Gams | 2   | 50.0 | 0  | 50.0  | 4   | 25.0 | 0  | 75.0 |
| **Nigrospora sphaerica** (Sacc.) Mason        | 5   | 80.0 | 0  | 20.0  | 12  | 50.0 | 0  | 50.0 |
| **Pestalotiopsis maculans** (Corda) Nag Raj   | 23  | 47.8 | 34.7 | 17.4  | 35  | 48.6 | 40.0 | 11.4 |
| **Phoma destructiva** * Plowr                 | 14  | 7.1  | 35.7 | 57.1  | 16  | 12.5 | 25.0 | 62.5 |
| **Phoma eupyrena** Sac.                       | 1   | 0  | 0  | 100.0  | 8   | 50.0 | 12.5 | 37.5 |
| **Phoma glomerata** (Corda) Wollenw & Hochapfel | 5   | 60.0 | 0  | 40.0  | 6   | 83.3 | 0  | 16.7 |
| **Phomopsis archeri** Sutton                  | 44  | 61.3 | 27.2 | 11.4  | 56  | 60.7 | 32.1 | 7.1  |
| **Rhizopus microsporus** Tiegh                | 4   | 0  | 75.0 | 25.0  | 5   | 20.0 | 0  | 80.0 |
| **Rhizopus stolonifer** (Ehrenb.) Vuill       | 14  | 7.1  | 21.4 | 71.4  | 12  | 8.3  | 25.0 | 66.7 |
| **Rhodotorula glutinis** (Fresen) Harrison    | 4   | 100.0 | 0  | 0      | 5   | 100.0 | 0  | 0  |
| **Talaromyces** sp.                           | 10  | 0  | 60.0 | 40.0  | 16  | 0  | 50.0 | 50.0 |
| **Trichothecium oryzae** (Vincens) de Hoog    | 4   | 100.0 | 0  | 0      | 8   | 75.0 | 25.0 | 0  |
| **Xylaria** sp.                               | 1   | 100.0 | 0  | 0      | 3   | 100.0 | 0  | 0  |
| **Non-sporulating fungi**                     | 20  | 35.0 | 20.0 | 45.0  | 23  | 21.7 | 17.4 | 60.9 |
| **Total number of fungal isolates**           | 261 | 118 | 78  | 65    | 342 | 148 | 101 | 93    |
| **Simpson index (1/D)^a**                     | 6.8 ± 2.05 | 9.1 ± 1.73 |
| **Richness (S)^b**                            | 23 ± 5.9 | 18 ± 4.8 | 15 ± 5.1 | 17 ± 3.7 | 24 ± 6.4 | 21 ± 6.2 | 15 ± 5.1 | 18 ± 3.3 |

Notes: N= total number of fungi isolated; L= leaves; S= stems; R= roots.

aSimpson index for species diversity with error estimated by Jackknife (t = 1.56, P = 0.2159).
bAverage species richness at 95% probability of the average estimates by the Jackknife method.

*Species cited as pathogenic to the cotton plant.

Cotton genotypes and in all parts of the plant: C. gloeosporioides, C. lunata var. aeria, F. lateritium, P. maculans, P. destructiva, P. glomerata and P. archeri.

DCA analysis of species distribution revealed gradient lengths of 1.84 in size justifying the use of RDA for categorizing fungal communities. Tissue type was the most important factor influencing the composition of the fungal communities in all plant stages and genotypes as demonstrated by the closest correlation of this vector with the first axis of the ordination diagram (Figure 2A). This effect was significant and Lambda 1 values were 21 and 20 for leaves and roots respectively (Figure 2B), demonstrating that most of the variance in fungal species composition can be explained by the tissue type from which they were isolated. No clear effect of plant genotype (Bt and non-Bt) on fungal species composition was found in isolates made across all tissue samples and sample times.

**Quantification of Cry1Ac**

The levels of Cry1Ac protein varied among the different plant tissues; the highest level of protein was detected in the leaves at all the plant developmental stages surveyed (Figure 3). The level of Cry1Ac in the non-Bt cotton Acala 90 was used as a control for comparisons.

**Discussion**

Since the introduction of genetically modified plants, the potential for effects on microbial populations resulting from heterologous protein expression, such as in Bt plants,
Figure 2. Multivariate analysis of the isolated fungal community from transgenic and non-transgenic cotton plants at different times and different tissues. (A) Ordination diagrams represent all environmental variables influencing each isolated fungal community. Values represent the percentage of the correlation species-environmental variable explained in each axis. (B) Statistical parameters calculated by multivariate analyses with inclusion of a Monte Carlo permutation test. Values for Lambda 1 indicate the amount of variance explained by each individual environmental parameter. Variables are statistically significant at $P < 0.05$ according to the Monte Carlo permutation test.

Figure 3. Expression of Cry1Ac protein in different tissues of Bt cotton plants during the indicated plant developmental stages. The error bars represent the standard errors of the means (SEM).

has been controversial (Shelton and Sears 2001; Azevedo and Araújo 2003). Overall, the results of this study which were confirmed by multivariate analysis suggest that the expression of the Cry1Ac protein in transgenic cotton plants does not affect endophytic fungal communities. Multivariate analysis combines different measurements from the same sample and can recognize correlations and interactions between factors, and therefore, is a powerful tool to understand external influences on biodiversity (Andreote et al. 2009a). For this reason, many studies aiming to quantify the effects of transgenic plants on microbial-associated communities have utilized this important instrument (Andreote et al. 2008 2009b).

The results presented here were consistent with a previous study on bacteria from the rhizosphere of the Bt cotton variety NuCOTN99B (Rui et al. 2005), in which toxin levels were uncorrelated with the number of associated bacteria. Additionally, a similar finding was demonstrated during a study of transgenic Bt maize where the release of Cry3Bb proteins into the rhizosphere did not cause variations in the fungal communities between isogenic Bt and non-Bt maize (Devare et al. 2004).

The differences in the fungal communities described in this study were associated with plant age and tissue type and were independent of protein expression. The increase in the frequency of endophytic fungi throughout plant developmental stages was consistent with other studies, a result that is likely associated with the prolonged exposure time of older plants to fungal spores and with physiological changes in tissues (Taylor et al. 1999; Photita et al. 2001). The community of fungi was affected in transgenic imidazolinone-tolerant sugarcane plants; however, these effects were also related to other factors, such as age (Stuart et al. 2010).

Many studies have also observed higher frequency of endophytic fungi in leaves relative to other parts of plants throughout all stages of plant development (Stone 1987; Espinosa-Garcia and Longenheim 1990; Kinkel 1991; Lappalainen et al. 1999). Increased colonization may be explained by the fact that leaves have natural stomatal openings and thus are the first port of entry for airborne or water-dispersed spores (Fröhlich et al. 2000). From the endophytic species identified (Table 1), the 8 most prevalent species, with more than 20 isolates each, were found in leaves, stems and roots from both Bt and non-Bt plants. The remaining 13 species were isolated from both transgenic and non-transgenic plants, but not from all plant organs. This distribution can be interpreted in some cases as a result of the low frequency of isolates from each species. Additionally, effects from method of sterilization on the frequency of isolation of some fungi cannot be discounted. Furthermore, it has been shown that different
species are common to multiple plant organs while others are found preferentially in roots or aerial parts such as leaves (Sánchez Márquez et al. 2010; Herrera et al. 2010).

Of the 17 genera isolated from both Bt and non-Bt cotton, some are commonly found as cotton endophytes in tropical regions, such as *Cladosporium, Fusarium, Guignardia, Pestalotiopsis, Phoma, Phomopsis* and *Xylaria* (Wang et al. 2007). Two fungal species, *F. oxysporum* and *P. destructiva* (*P. exigua*) are reported to be pathogens of cotton. They may occur as latent pathogenic endophytes in tropical regions, such as non-Bt cotton, some are commonly found as cotton leaves (Sánchez Márquez et al. 2010; Herrera et al. 2010). Others are found preferentially in roots or aerial parts such as *A. kiliense*, *C. lunata* var. *aeria*, *F. lateritium*, *Talaromyces* sp., *T. orysae* and *Xylaria* sp. These species are commonly found in other plants, but approximately 50% of the species isolated in this study had not previously been reported as endophytes of cotton.

In summary, the composition of endophytic fungi associated with Acala 90B and Acala 90 cotton plants were characterised and found not to differ significantly, and therefore, the fungal endophyte community appears to be unaffected by the expression of Cry1Ac protein from *B. thuringiensis* in transgenic plants.

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