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Transgenic *Bt* cotton inhibited arbuscular mycorrhizal fungus differentiation and colonization

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ABSTRACT

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The present study investigated the impact of transgenic *Bacillus thuringiensis* (*Bt*) cotton on several aspects of arbuscular mycorrhizal (AM) fungus *Funneliformis mosseae*. The results showed that *Bt* cotton significantly inhibited spore germination and pre-symbiotic hyphal growth. The appressorium density, arbuscule frequency and colonization intensity in *Bt* roots were also decreased. The statistical analysis demonstrated that the transformation event resulted in the inhibition of hyphal development and colonization. The reduced interaction between AM fungi and plants could affect nutrient uptake and transportation in plant-fungus symbiosis. The mechanism might involve the direct toxicity of *Bt* toxins or the interference of signal perception between AM fungus and *Bt* cotton.

Keywords: inoculation; hyphal branching; nutrient uptake; signal production; ecological functions

Transgenic *Bacillus thuringiensis* (*Bt*) cotton is the main genetically modified crop in China. In 2014, the cultivation area of *Bt* cotton reached 3.8 million hectares, accounting for 96% of the total area of cotton cultivation (James 2015). During the growth period of *Bt* cotton, *Bt* toxins can enter soil via root exudation, pollen release and crop residues (Obrycki et al. 2001, Stotzky 2005, Icoz and Stotzky 2008). The released *Bt* toxins are bound onto clay minerals and humic substances, which leads to resist to microbial degradation and retain insecticidal activity for several months (Tapp and Stotzky 1998, Saxena and Stotzky 2001). The *Bt* toxin residue in soil may pose a potential risk for non-target organisms, including soil microorganisms.

Arbuscular mycorrhizal (AM) fungi are a kind of functional microorganisms in soil, which can form mutualistic symbioses with 80% of terrestrial plant families, facilitating the uptake of water and mineral nutrients to their host plants (Smith and Read 2008). They can also increase the resistance of the host to biotic and abiotic stress and improve soil stability (Wehner et al. 2010). The 450-million-year-old symbiosis is ecologically important mutualism on Earth (Remy et al. 1994). However, AM fungi may be more sensitive to *Bt* crops than other organisms because of the obligately biotrophic relationship. As an unequivocally important microbial community, AM fungi represent potential key non-target microorganisms for environmental risk assessment of *Bt* plants (Liu 2010).

To the best of our knowledge, the report about the impact of *Bt* cotton on the development of AM fungi is very limited. Our earlier findings indicated that three cultivars of *Bt* cotton significantly inhibited the development of AM fungus *Rhizophagus irregularis* (Chen et al. 2016). To in-

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vestigate whether the inhibitive effect of *Bt* cotton on *R. intraradices* is particular or general, a follow-up study on AM fungus species *Funneliformis mosseae* was conducted. Just like *R. intraradices*, *F. mosseae* is another abundant species in diverse ecosystems (Öpik et al. 2003, Jansa et al. 2014), but with different traits, such as spore size, subcellular structure of spore and hyphal wall, germination characteristics (http://invam.wvu.edu/the-fungi/species-descriptions). The two species may behave differently in the differential performance and structural development when exposed to *Bt* cotton, which will represent the potential impact of *Bt* cotton on the fungi and soil ecosystems. This work evaluated (i) the effects of root exudates of four cultivars of *Bt* cotton on *F. mosseae* spore germination and pre-symbiotic growth, and (ii) the host recognition responses and the ability of this AM fungal species to establish mycorrhizal infection with the four transgenic cotton.

MATERIAL AND METHODS

Plant cultivars. The cultivars of *Bt* cotton used were Jin26, GK12, Jin44 and sGK321 expressing *Cry1Ac*. The corresponding non-transgenic isolines were Jin7, Si3, Ji492 and Shiyuan321, respectively.

Similar-sized cotton seeds were delinted in concentrated H_2SO_4 followed by surface-sterilization with 3% NaClO, and then kept for germination at 25°C. The seedlings were transferred into sterile quartz grit and allowed to grow for 10 days before transplanting.

Fungal material. The AM fungus *Funneliformis mosseae* (BGC NM04A) used in the study was maintained in pot culture. The fungal inoculum consisted of mycorrhizal roots, soil containing spores and extraradical mycelium. The root colonization percentage was above 90%, and the density of spores was over 150 per gram.

Experiment 1. Assessment of spore germination and hyphal morphology in the rhizosphere. Spores were extracted manually by wet-sieving (Gerdemann and Nicolson 1963) and surface- sterilization according to Bécard and Fortin (1988). A 'sandwich system' was used to detect the effect of root exudates of *Bt* cotton on spore germination and hyphal differentiation (Turrini et al. 2004). Briefly, 30 spores were placed on a 47-mm diameter cellulose ester Millipore TM membranes (0.45 µm diameter pores) and covered with an empty membrane. The sandwiched spores and the 10-day cotton seedlings were transplanted into sterile quartz grit according to Chen et al. (2016) in the way of spores being exposed to root exudates, but without direct contact with the roots. All pots were randomly arranged in a growth chamber under a 16-h-light (25°C)/8-h-dark (22°C) regime. Sterilized water and 1/10 phosphate strength Hoagland solution (Hoagland and Arnon 1950) was supplied as required. Spore germination, hyphal differentiation were examined separately at 10, 20 and 30 days after transplanting (DAT), with three replicates for each cotton cultivar and each assay. For the assessment of germination percentage and pre-symbiotic fungal differentiation, the membranes bearing spores were stained with 0.05% Trypan blue and observed under a dissecting microscope.

Zeiss Axiocam MRc5 digital camera (Carl Zeiss Inc., Oberkochen, Germany) was used for capturing fungal hyphal morphology. To gain high contrast images needed for quantification, the images of hyphal morphology were imported into Image-Pro Plus, Version 4.03 (Media Cybernetics, Rockville, USA). The hyphal networks were traced using a magnetic pencil tool (Twanabasu et al. 2013).

Experiment 2. Mycorrhizal colonization. The 10-day cotton seedlings were transplanted into pots containing the mixture of 50 g fungal culture collections and 600 g sterile quartz grit, with one plant per pot. The pots were randomly assigned in the same growth chamber as that in experiment 1, supplied with sterilized water and 1/10 phosphate strength Hoagland's solution as required.

Plants were harvested separately at 18, 21, 24, 27, 30 DAT to examine appressorium formation and mycorrhizal colonization, with three replicates for each harvest. Root subsamples were stained according to Phillips and Hayman (1970). The appressoria number and root colonization percentage were determined according to Trouvelot et al. (1986).

Data analysis. Statistical procedures were performed using SPSS 19.0 (Chicago, USA). Significant differences were determined by the Duncan's multiple range test at $P < 0.05$.

RESULTS

Spore germination in rhizosphere. The spore germination rate increased with time. The germi-

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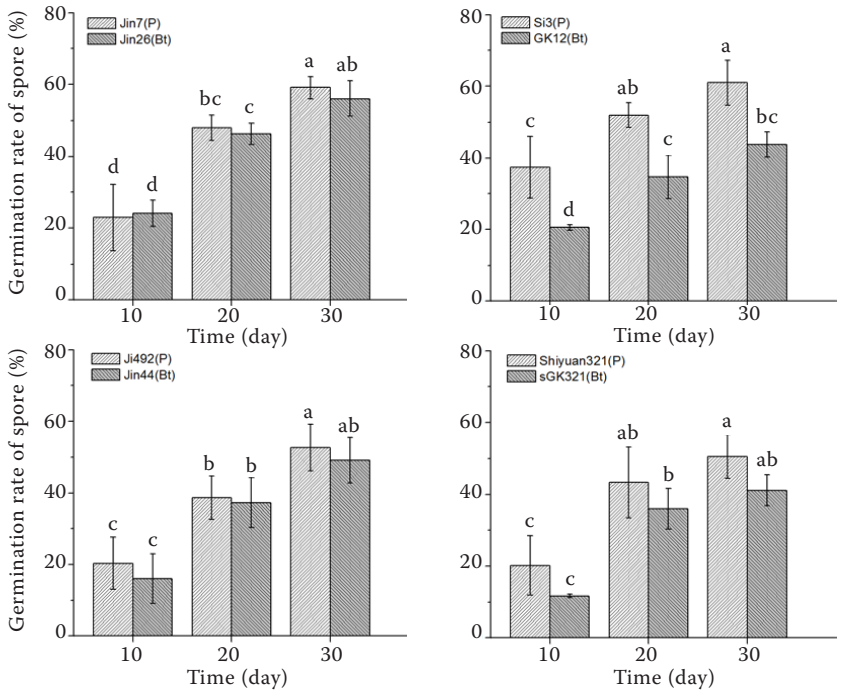

Figure 1. Spore germination percentage in the rhizosphere of *Bacillus thuringiensis* (*Bt*) and non-*Bt* cotton (P). Values are means of three replicates. Bars represent standard errors of the means. Different letters above bars indicate a significant difference ($P < 0.05$)

nation percentage at 30 DAT ranged from 41.1% for *Bt* isoline sGK321 to 61.0% for non-*Bt* isoline Si3, which was significantly higher than those at 10 and 20 DAT (Figure 1). Within each pair of *Bt* and non-*Bt* cotton isolines, the spore germination percentage was lower in the rhizosphere of *Bt* cotton at each time point, with the exception of Jin26 at first harvest, which showed an almost equal percentage with its non-*Bt* line Jin7 (Figure 1).

Hyphal branching. For both *Bt* and non-*Bt* cotton lines the number of hyphal branches increased significantly over time (Figure 2). Within each pair of isolines, the number of hyphal branches in the rhizosphere of *Bt* cotton was significantly lower at 20 DAT and 30 DAT. A reduction in the number of hyphal branches in *Bt* cotton rhizosphere was also observed at 10 DAT although it was not always statistically significant. Figure 3 showed a

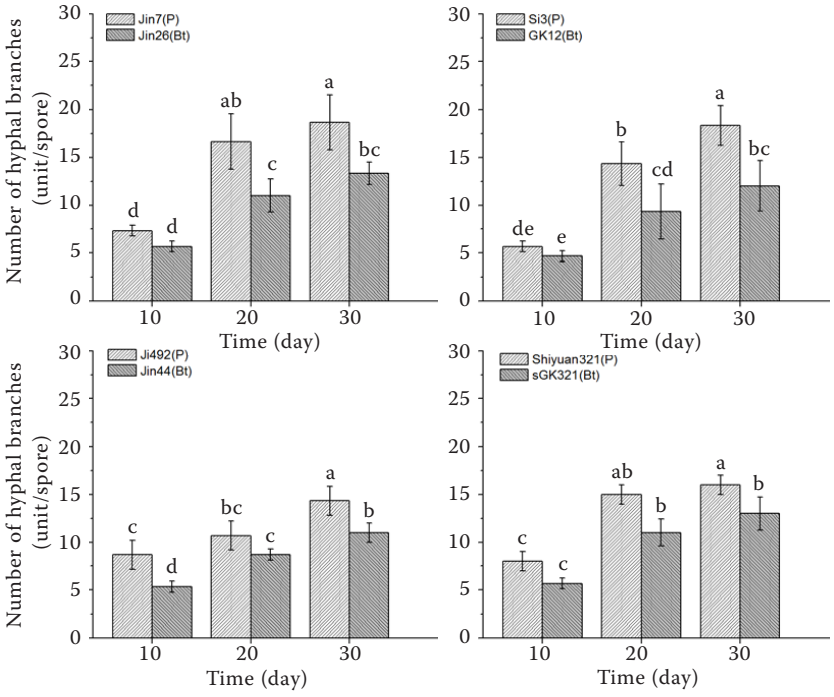

Figure 2. Number of hyphal branches in the rhizosphere of *Bacillus thuringiensis* (*Bt*) and non-*Bt* cotton (P). Values are means of three replicates. Bars represent standard errors of the means. Different letters above bars indicate a significant difference ($P < 0.05$)

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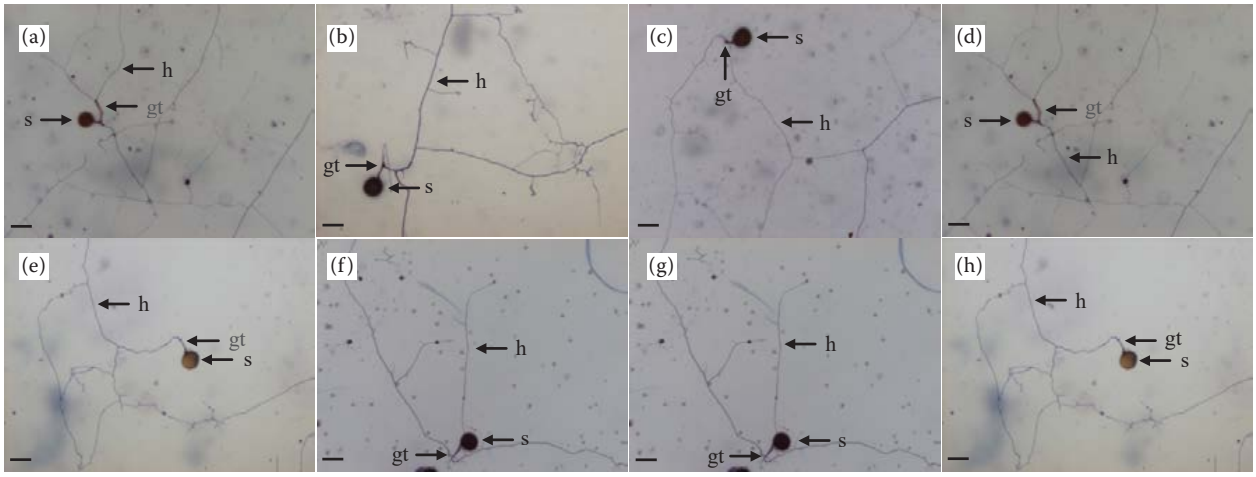

Figure 3. The microphotographs of spore germination and hyphal branching. a–d – non-*Bacillus thuringiensis* (*Bt*) cotton lines (Jin7; Si3; Ji 492; Shiyuan321); e–h – *Bt* cotton lines (Jin26; GK12; Jin 44; sGK321); s – spore; gt – germ tube; h – hyphae. Scale 200 μm

branched hyphal net developed on membrane areas overlying the roots of both *Bt* and non-*Bt* cotton at 30 DAT. The higher hyphal branches for the non-*Bt* cotton lines were observed (Figure 3a–d).

Total hyphal length. Total hyphal length significantly increased over time for all cotton cultivars. At each time point, the hyphal length in the rhizosphere of *Bt* lines was lower than that in the corresponding non-*Bt* line rhizosphere (Figure 4).

Appressorium formation on *Bt* and non-*Bt* roots. By the first harvest at 18 DAT, appressoriums were formed on both *Bt* and non-*Bt* roots. The appressorium density significantly increased over time (Figure 5). There were differences in *F. mosseae* response to *Bt* and non-*Bt* roots plants. At each time point, non-*Bt* cotton showed higher appressorium density than the corresponding *Bt* cotton. By contrast, the *Bt* cotton resulted in a 26, 27, 34 and 20% reduction at 30 DAT for Jin26, GK12, Jin44 and sGK321, respectively (Figure 5).

AM fungal colonization. At the first and second harvests (18 DAT and 21 DAT) sGK321 showed similar colonization intensity with its corresponding non-*Bt* plants, and so did GK12 at 18 DAT, but

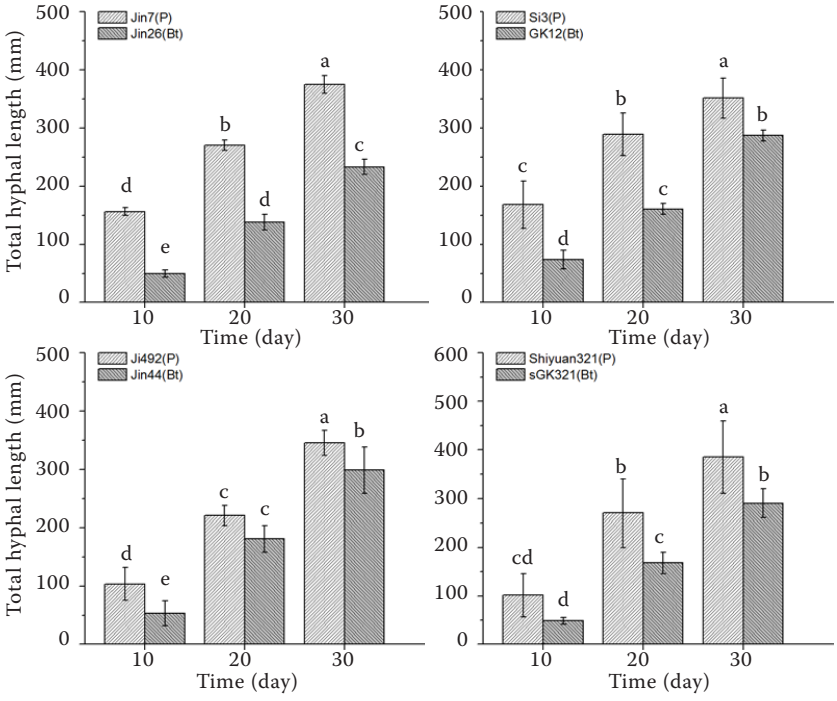

Figure 4. Hyphal length in the rhizosphere of *Bacillus thuringiensis* (*Bt*) and non-*Bt* cotton (P). Values are means of three replicates. Bars represent standard errors of the means. Different letters above bars indicate a significant difference ($P < 0.05$)

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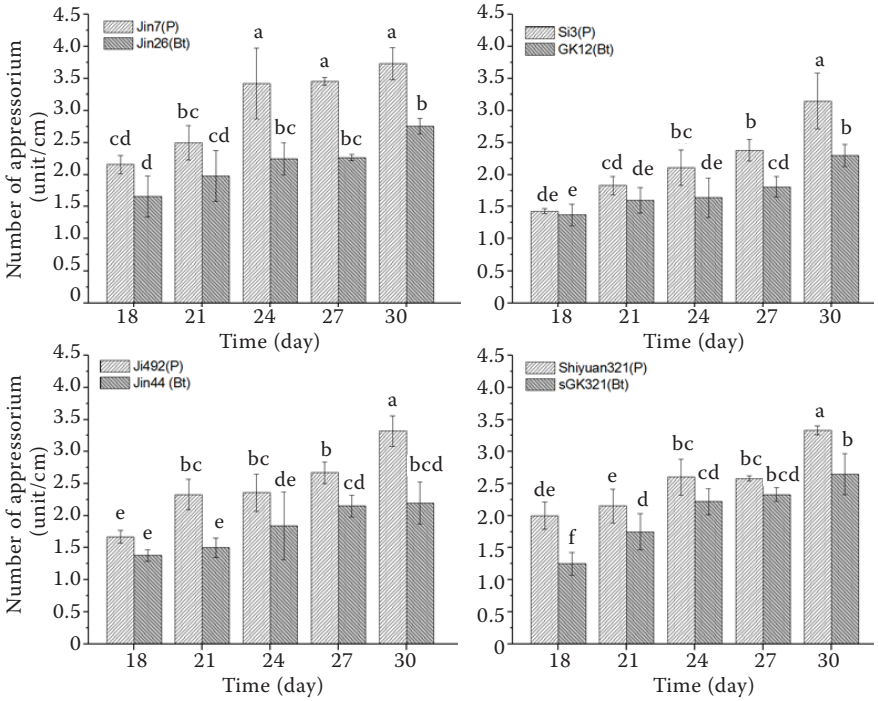

Figure 5. Appressorium density on the root cortex of *Bacillus thuringiensis* (*Bt*) and non-*Bt* cotton (P). Values are means of three replicates. Bars represent standard errors of the means. Different letters above bars indicate a significant difference ($P < 0.05$)

thereafter GK12 and sGK321 showed remarkedly lower colonization level than their non-*Bt* lines (Figure 6). Throughout the experiment, Jin26 and Jin44 showed significantly lower levels of root colonization than their corresponding non-*Bt* plants. At 30 DAT the colonization intensity in *Bt* lines showed a 29.4, 45.0, 29.5 and 21.8% reduction for Jin26, GK12, Jin44 and sGK321, respectively, which was consistent with the lower efficiency in appressorium formation.

Arbuscule frequency. Arbuscule frequency increased significantly with the increase of the culture time (Figure 7). Except that at 18 DAT and 27 DAT, GK12 showed similar levels with its corresponding non-*Bt* line, the *Bt* lines exhibited significantly lower arbuscule frequency (Figure 6),

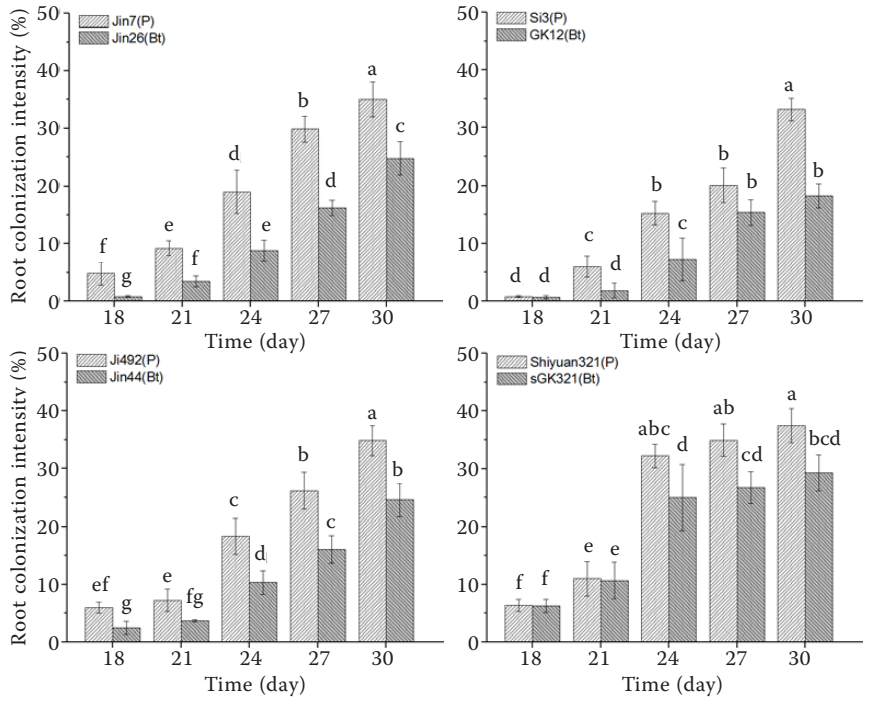

Figure 6. Root colonization intensity of *Bacillus thuringiensis* (*Bt*) and non-*Bt* cotton (P). Values are means of three replicates. Bars represent standard errors of the means. Different letters above bars indicate a significant difference ($P < 0.05$)

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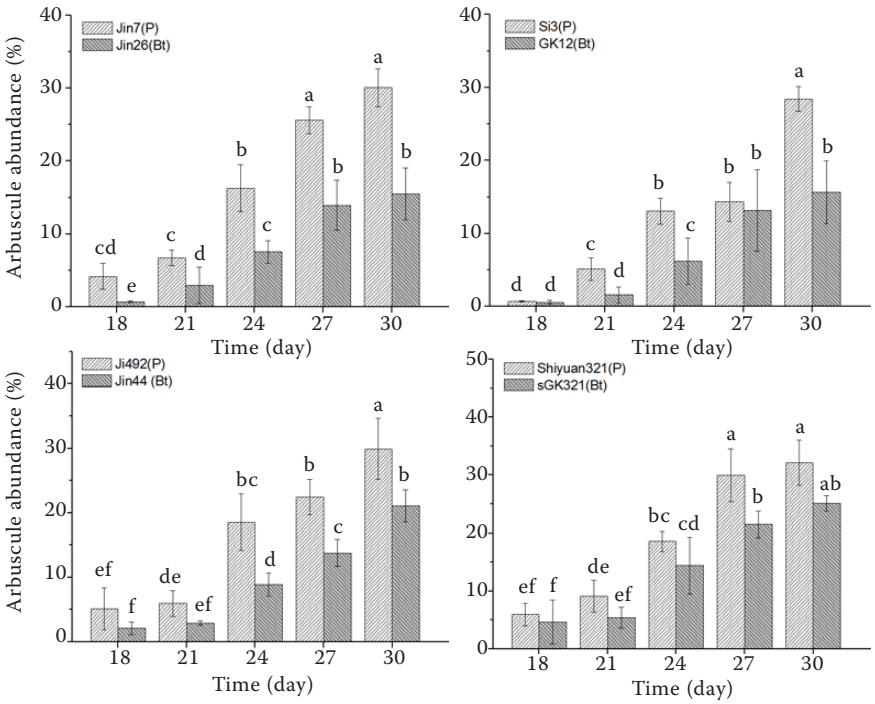

Figure 7. Arbuscule frequency within roots of *Bacillus thuringiensis* (*Bt*) and non-*Bt* cotton (P). Values are means of three replicates. Bars represent standard errors of the means. Different letters above bars indicate a significant difference ($P < 0.05$)

with a 48.4, 45.0, 29.5 and 22.0% reduction at 30 DAT for Jin26, GK12, Jin44 and sGK321, respectively.

The effect of *Bt*-trait on AM fungal development. To identify whether *Bt*-trait or cotton cultivar plays the dominant role in influencing hyphal differentiation and mycorrhiza formation, comparisons were drawn among the averages of variables over all time points. For pre-symbiotic stage, with the exception of germination percentage for Jin26, the statistical averages of germination percentage, hyphal branch numbers and total hyphal length for all *Bt* lines were significantly lower than those for the non-*Bt* lines (Table 1). The inhibitive effects of *Bt* lines on appressorium density, colonization intensity and arbuscule frequency were also significant, except for sGK321 (Table 1).

DISCUSSION

Previous studies have indicated that *Bt* corn affects the early events of mycorrhizal establishment and the development of symbiosis (Turrini et al. 2004, Castaldini et al. 2005). The cellular interactions between *Bt* roots and AM fungal hyphae led to the failure of intraradical colonization in *Bt* corn lines, and thus the decrease of functional infective structures and infected root length. In this study,

Table 1. Means of spore germination percentage, number of hyphal branches, hyphal length, appressorium density, colonization intensity and arbuscule frequency over all time points

Cotton cultivars	Germination percentage (%)	Branch number (unit/spore)	Hyphal length (mm)	Appressorium density (unit/cm)	Colonization intensity (%)	Arbuscule frequency (%)
Jin7 (P)	40.83^{b}	13.00^{a}	267.40^{a}	3.05^{a}	25.08^{b}	22.80^{a}
Si3 (P)	45.09^{a}	12.78^{ab}	269.77^{a}	2.20^{c}	20.77^{c}	18.67^{b}
Ji492 (P)	37.18^{bc}	11.22^{bc}	223.36^{b}	2.47^{b}	24.38^{b}	22.65^{a}
Shiyuan321 (P)	37.63^{bc}	14.22^{a}	252.07^{ab}	2.53^{b}	28.49^{a}	23.67^{a}
Jin26 (*Bt*)	40.26^{b}	10.00^{cde}	140.44^{d}	2.19^{c}	17.29^{d}	14.92^{cd}
GK12 (*Bt*)	34.79^{cd}	8.67^{de}	173.98^{c}	1.74^{d}	15.10^{e}	13.85^{d}
Jin44 (*Bt*)	35.16^{cd}	8.33^{e}	177.55^{c}	1.81^{d}	18.37^{d}	16.84^{bc}
sGK321(*Bt*)	31.99^{d}	10.33^{cd}	170.43^{c}	2.04^{c}	25.44^{b}	22.26^{a}

Values are means of three replicates of each line. Different letters within a column indicate significant difference ($P < 0.05$)

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the spore germination percentage, hyphal growth and colonization were significantly inhibited in *Bt* cotton lines. The results also agree with the earlier findings, which showed the inhibited impact of *Bt* cotton on *R. irregularis* (Chen et al. 2016).

The life cycle of AM fungi begins with the germination of fungal spores, and then the signals of the host induce hyphal growth and branching, followed by hyphal differentiation (Akiyama et al. 2005). The semi-purified root extract has been proved to trigger a typical pre-symbiotic fungal response within a few hours (Buee et al. 2000). The purified root exudates, such as strigolactone, 2-hydroxy fatty acids and flavonoids, can also activate hyphal ramification and asymbiotic fungal growth at extremely low concentration (Bécard et al. 1992, Nagahashi and Douds 2000, Akiyama et al. 2005). Although the root exudates were not determined in this study, the inhibitive effect of *Bt* lines might attribute to the interference of signal production or perception between the fungus and the host.

In this study, *Cry* protein was determined in *Bt* cotton by ELISA kit according to protocal. 112 ± 26 ng/g, 72 ± 19 ng/g, 88 ± 11 ng/g and 65 ± 17 ng/g fresh weight of *Bt* protein were detected in roots of Jin26, GK12, Jin44 and sGK321, respectively. Studies reported that exudates containing toxins or antimicrobial compounds might affect non-target soil organisms and microbial community composition (Siciliano and Germida 1999, Griffiths et al. 2000). It was assumed that *Bt* toxin involved in the inhibitive effect of *Bt* cotton on AM fungi, it might suggest that AM fungi are very susceptible to the *Cry* toxin.

Many researches have conducted studies on the action model of *Bt* toxin cytotoxicity to invertebrates (Carroll and Ellar 1993, Vachon et al. 2012, Melo et al. 2016). The present study does not identify the mechanism of the inhibitive effect of *Bt* cotton on AM fungi. It was deduced that it might be due to the interference in signal perception or the direct cytotoxicity of *Cry* toxins. Regardless of the mechanism, the inhibitive effect has important consequences on our understanding of the potential impact of *Bt* cotton on soil ecosystems.

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