



Development of a model system to assess the impact of genetically modified corn and aubergine plants on arbuscular mycorrhizal fungi

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Received 5 February 2003. Accepted in revised form 9 July 2003

Key words: arbuscular mycorrhizal fungi, *Bt* toxin, environmental impact, plant defensins, root exudates of transgenic plants, test system

Abstract

We developed an experimental model system to monitor the impact of generically modified (GM) plants on arbuscular mycorrhizal (AM) fungi, a group of non-target soil microorganisms, fundamental for soil fertility and plant nutrition. The system allowed us to study the effects of root exudates of both commercial *Bt* corn and aubergine plants expressing Dm-AMP1 defensin on different stages of the life cycle of the AM fungal species *G. mosseae*. Root exudates of *Bt* 176 corn significantly reduced pre-symbiotic hyphal growth, compared to *Bt* 11 and non-transgenic plants. No differences were found in mycelial growth in the presence of Dm-AMP1 and control plant root exudates. Differential hyphal morphogenesis occurred irrespective of the plant line, suggesting that both exuded *Bt* toxin and defensin do not interfere with fungal host recognition mechanisms. *Bt* 176 affected the regular development of appressoria, 36% of which failed to produce viable infection pegs. Our experimental model system represents an easy assay for testing the impact of GM plants on non-target soil-borne AM fungi.

Introduction

Plants genetically modified (GM) for resistance to pests represent a potential environmentally safe alternative to pesticides and could lead to decreased chemical contamination of groundwater, soil and air. However, the introduction of this new agrotechnology may pose risks to natural and agricultural ecosystems as shown by reports on direct non-target effects on beneficial and native organisms. For example, corn pollen expressing *Bacillus thuringiensis* (*Bt*) insecticidal toxin caused a high mortality of non-target monarch and black swallowtail caterpillars (Zangerl et al., 2001). Other studies reported that the composition and diversity of soil and rhizospheric microbial communities were modified in the presence of GM

potato lines producing *Galanthus nivalis* agglutinin and of glyphosate resistant *Brassica napus* (Griffiths et al., 2000; Siciliano and Germida, 1999).

An important group of non-target microorganisms, fundamental for soil fertility and plant nutrition is represented by arbuscular mycorrhizal (AM) fungi, which establish mutualistic symbioses with the roots of most plant species (Smith and Read, 1997). AM fungi are strongly affected by agricultural practices, including treatments with chemical fertilizers and pesticides, and by changes in soil characteristics, thus representing potential key non-target microorganisms to be monitored in studies on environmental impact of GM plants (Fontanet et al., 1998; Giovannetti and Avio 2002; Helgason et al., 1998; Johnson and Pflieger, 1992).

In this work we developed an experimental model system to investigate the potential effects of two GM

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plants, *Bt* corn and defensin expressing aubergine plants, on AM fungi.

Bt corn has been designed to express the *CryIAb* gene and produces an insecticidal endotoxin to control *Ostrinia nubilalis* (European corn borer), a major pest in Europe and North America. *In vitro* and *in situ* studies showed that the toxin is released into rhizosphere soil in root exudates (Saxena et al., 1999; 2002; Saxena and Stotzky, 2000) and in plant residues incorporated into soil after crop harvest (Tapp and Stotzky, 1998). Since *Bt* toxin is able to bind to clays and humic acids and to maintain its activity for 180 days, it may represent a potential risk also to non-target soil microorganisms (Saxena and Stotzky, 2001).

Dm-AMP1 aubergine plants were produced by genetic transformation to express the Dm-AMP1 defensin from *Dahlia merckii* (Turrini et al., submitted). Defensins are antimicrobial compounds capable of inhibiting the growth of phytopathogenic fungi by reducing hyphal elongation (Broekaert et al., 1995, 1997; Terras et al., 1995). Dm-AMP1 protein was expressed in all tissues of our transgenic aubergines, which showed foliar resistance to *Botrytis cinerea*, and was released into root exudates, which reduced the growth of the root pathogen *Verticillium albo-atrum* (Turrini et al., submitted).

In this work we developed an experimental model system for *in vivo* assessment of the impact of *Bt* 176 and *Bt* 11 corn plants and Dm-AMP1 aubergines on the life cycle of non-target AM fungi. We investigated (i) the effects of root exudates of transgenic plants on pre-symbiotic growth and host recognition responses of *Glomus mosseae*, and (ii) the ability of this AM fungal species to establish mycorrhizal infection with the two transgenic plants.

Materials and methods

Fungal material

The AM fungal species *G. mosseae* (Nicol. and Gerd.) Gerdemann & Trappe (IMA 1) maintained in the pot cultures collection of the Department of Chemistry and Agricultural Biotechnology, University of Pisa, Italy, was used. Sporocarps were extracted from pot-culture soil by wet-sieving and decanting, down to a mesh size of 100 μm (Gerdemann and Nicolson, 1963). Fungal material retained on sieves was flushed into Petri dishes and stored at 4 °C until used.

Plant material

The following transgenic plants were used to test the impact of GM plants on AM fungi: (a) transgenic *Bt* corn hybrids (transformation events *Bt* 11 and *Bt* 176) genetically modified to express the *CryIAb* gene from *B. thuringensis* and the non-transgenic maize NK4640, kindly provided by Prof. G. De Fago, ETH Zurich; (b) transgenic aubergines genetically transformed to express the Dm-AMP1 defensin from *Dahlia merckii* and the isogenic non transgenic aubergine.

Experimental model system

The experimental model used represents an implementation of the 'sandwich system' devised for investigating the early stages of AM fungal life cycle (Giovannetti et al., 1993). Briefly, *G. mosseae* sporocarps were manually collected with forceps under a dissecting microscope (Wild, Leica, Milano, I) and placed on 47-mm diameter cellulose ester MilliporeTM membranes (0.45 μm diameter pores). Two membranes, each containing 10 sporocarps, were superposed as showed in Figure 1, covered with an empty membrane and buried in sterile acid washed quartz grit (2–5 mm diameter) at 25°C in the dark. After 10 days the membranes were removed from the grit, opened and checked for sporocarp germination. Each plant root system was inserted between the first two MilliporeTM membranes in contact with germinated sporocarps, and the second membrane containing the other 10 germinated sporocarps was maintained in place to complete the double sandwich, as shown in Figure 1. In this way, germinated sporocarps growing on the external membrane were exposed to root exudates, whereas those in contact with roots could differentiate infection structures, the appressoria, and establish mycorrhizal symbiosis. To assess mycorrhizal colonization, single sandwiches were set up (Giovannetti et al. 1993). Each experimental plant, with sandwiched root system, was placed into a 10-cm diameter pot, buried in sterile, acid washed quartz grit and maintained under controlled conditions (18–24°C, 16–8-h photoperiod of irradiance 100 $\mu\text{E m}^{-2} \text{s}^{-1}$, 60% RH). Ten and three replicates for each plant line and for each assay were used for corn and aubergines, respectively. Plants with double sandwiches were harvested after 10 days, and both internal and external membranes bearing sporocarps were stained with 0.05% Trypan blue in lactic acid to assess pre-symbiotic fungal growth and hyphal differential morphogenesis.

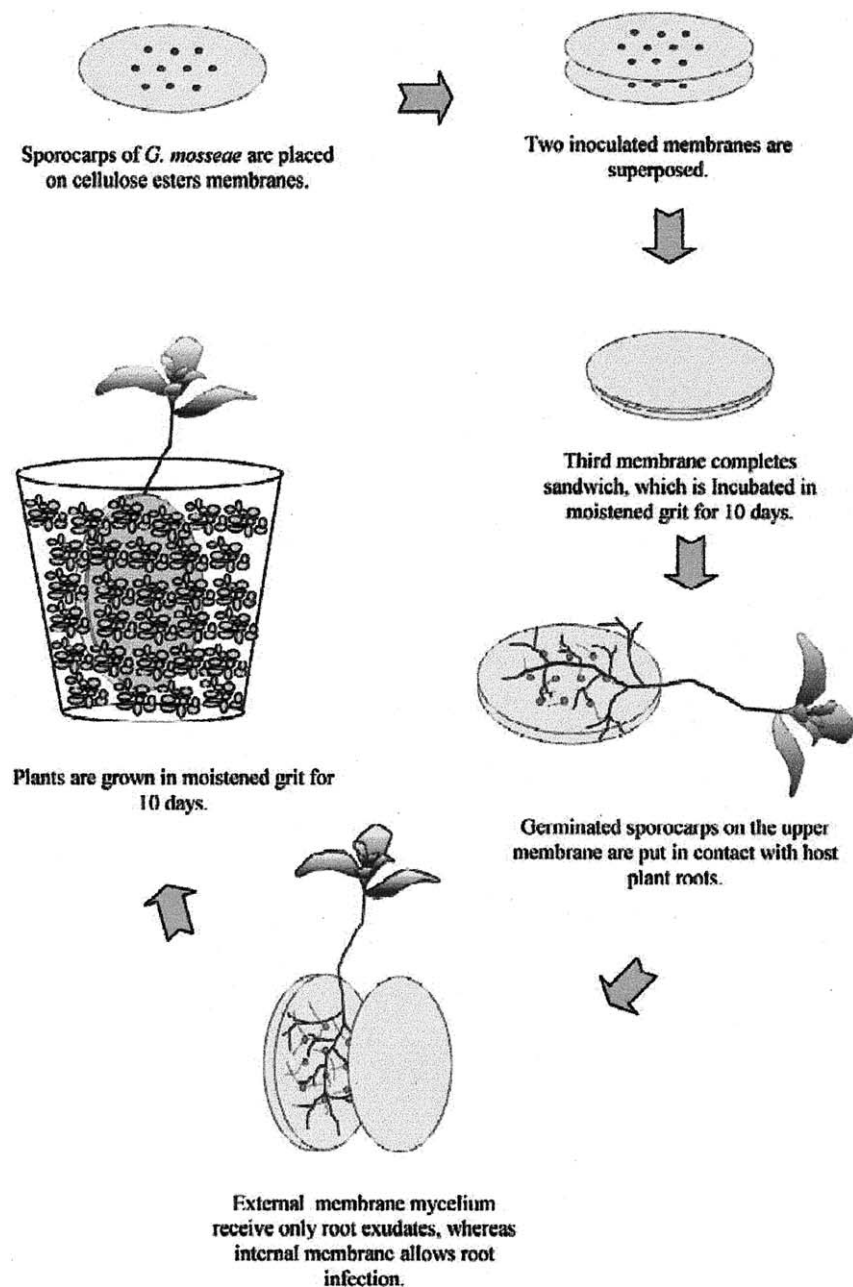


Figure 1. Scheme of the experimental model system used to study the effects of GM plants and their exudates on different stages of *G. mosseae* life cycle.

Mycelial length was measured by using the grid-line intersect method (Giovannetti and Mosse, 1980). Plant roots were cleared in KOH and stained in Trypan blue in lactic acid (0.05%) (Phillips and Hayman,

1970). Infected root pieces were mounted on microscope slides and observed under a Reichert-Jung Polyvar light microscope in order to count the number of appressoria and infection units. Plants with single

sandwiches were harvested after 35 days and roots were cleared and stained in Trypan blue as above. Infected root length was estimated by using the gridline intersect method. The data were submitted to one-way ANOVA.

Results

Effects of root exudates of Bt corn and Dm-AMP1 aubergine on G. mosseae pre-symbiotic growth and host recognition responses

Bt corn

The length of *G. mosseae* mycelium in the presence of transgenic *Bt* corn 176 was 961.01 ± 92.79 mm, significantly lower ($P=0.04$) than that detected in the presence of *Bt* 11, 1341.37 ± 77.66 mm, or non-transgenic plants, 1149.89 ± 123.72 mm.

Differential hyphal morphogenesis, the first detectable sign of host recognition by AM fungi, was elicited by root exudates of both *Bt* and control plants. A densely branched hyphal net developed on membrane areas overlying the roots of both GM and control corn (Figure 2A). Percentages of sporocarps whose mycelium showed differential growth pattern were 20.2, 27.0 and 29.0% in presence of root exudates of *Bt* 11, *Bt* 176 and non transgenic maize, respectively.

Dm-AMP1 aubergines

Hyphal growth of *G. mosseae* in the presence of defensin-expressing aubergines was 1131.7 ± 150.9 mm, value not statistically different from that of control plants, 1079.3 ± 61.1 mm. Also differential hyphal morphogenesis did not differ between Dm-AMP1 and control aubergines. The percentage of sporocarps showing differential growth pattern was very high, namely 96.3 and 75.0% in Dm-AMP1 and control plants, respectively.

Infection structures formation on Bt corn and Dm-AMP1 aubergine roots

Bt corn

The impact of transformation events *Bt* 11 and 176 on the establishment of mycorrhizal symbiosis by *G. mosseae* was studied. Microscopic analysis revealed that infection structures were formed on transgenic and control plant roots. However, many appressoria formed on *Bt* 176 corn roots were not able to develop

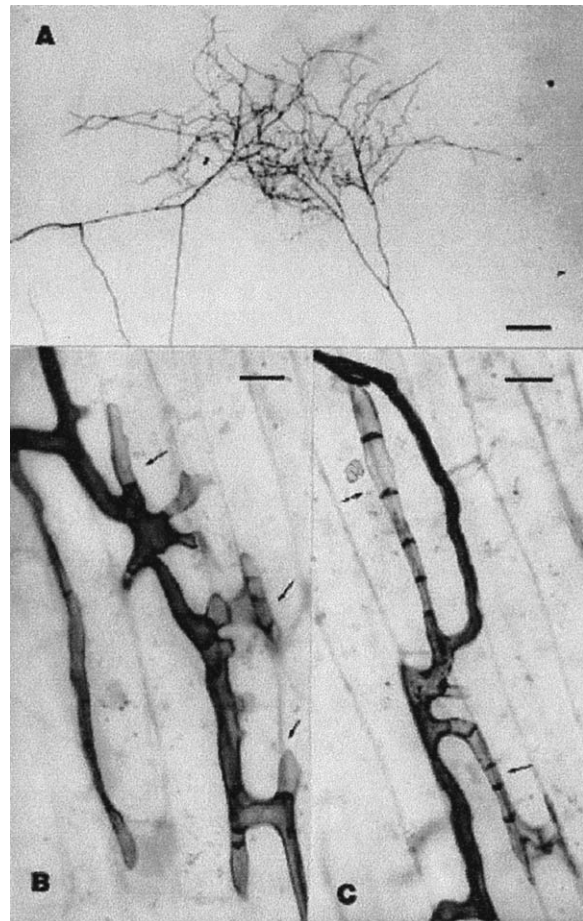


Figure 2. Light micrographs showing hyphal morphogenesis and root infection structures of the AM fungus *G. mosseae* in the presence of *Bt* plants. (A) Differential morphogenesis developed on membranes overlying *Bt* 176 corn roots. Scale bar = $150 \mu\text{m}$. (B) Multiple appressoria developed on *Bt* 176 corn roots showing many abortive infection pegs (arrows). Scale bar = $15 \mu\text{m}$. (C) Appressorium showing infection pegs septate and devoid of protoplasm (arrows). Scale bar = $15 \mu\text{m}$.

infection units, producing infection pegs which became septate and devoid of protoplasm (Figure 2B,C).

Table 1. Analysis of appressorium development on *Bt* and non-*Bt* corn roots

Corn lines	Number of appressoria not	
	Total number of appressoria (mean \pm SE)	developing infection units (mean \pm SE)
<i>Bt</i> 176	36.1 ± 5.2	13.8 ± 3.1
<i>Bt</i> 11	25.3 ± 8.0	2.1 ± 0.6
NK4640	30.0 ± 5.1	3.4 ± 1.1

The development of infecting hyphae by appressoria was significantly affected ($P=0.04$) by presence of *Bt* 176, where 35.7% of appressoria were not able to colonise roots (Table 1). On the contrary, the percentage of appressoria which did not develop intraradical infection, with respect to all infection structures formed, was similar in *Bt* 11 and control roots, 10.6 and 9.1%, respectively.

Infected root length percentages were 14% and 25% in *Bt* and wild type corn lines, respectively ($P=0.08$).

Dm-AMP1 aubergines

The analysis of infection structures developed on Dm-AMP1 and control aubergine plants, reported in Table 2, showed that there were no statistically significant differences in total number of appressoria and in the number of appressoria which did not develop infection units. The percentage of appressoria unable to develop infecting hyphae and arbuscules was very low in Dm-AMP1 aubergines (2.5%) and in controls (3.7%), showing that the transformation event did not interfere with the capacity of *G. mosseae* of establishing mycorrhizal symbiosis. These data were consistent with results of infected root length, which ranged from 30 to 60%, showing no differences between transformed and control lines.

Discussion

Our experimental model system allowed us to monitor the impact of GM plants on pre-symbiotic mycelial growth, host recognition responses and root infectivity of the AM fungus *G. mosseae*. Our results showed that modulation of AM hyphal growth and infectivity may be evidenced in the presence of GM plant roots expressing two different biopesticide proteins when using the test system developed here.

The use of a physical barrier between fungal mycelium and plant roots hindered the establishment of mycorrhizal symbiosis and made it possible to assess fungal growth and hyphal differentiation in the presence of GM or control root exudates. It has been observed that both *Bt* corn and Dm-AMP1 aubergine plants released the transgenic proteins in root exudates (Saxena et al., 1999; Turrini et al., submitted). Different studies have reported that exudates containing toxins or antimicrobial compounds might affect non-target soil organisms and microbial soil communities composition (Griffiths et al., 2000; Siciliano and Ger-

mida, 1999). A preliminary study on the impact of *Bt* plants on soil organisms demonstrated that the *CryIAb* toxin released by *Bt* plants had no apparent toxicity to earthworms, nematodes, protozoa and saprophytic soil fungi, but specific analyses on beneficial fungi were not performed (Saxena and Stotzky, 2001).

Our results showed that root exudates of *Bt* 176 corn plants significantly reduced pre-symbiotic hyphal growth of *G. mosseae* with respect to *Bt* 11 and non-transgenic plant root exudates, whereas no differences were found in mycelial growth when exudates from Dm-AMP1 and control plant roots were compared. Since the growth of AM fungal mycelium is affected by many compounds occurring in the environment (Fontanet et al., 1998), this fungal parameter could be used as an indicator of the impact of different biological or chemical substances on AM fungi.

The experimental system used allowed the detection of differential morphogenesis in the presence of GM plant root exudates, showing that fungal recognition responses occurred irrespective of the plant tested. Host recognition by AM fungi is an early step in the process of symbiosis establishment, and its occurrence is revealed in this system by a differential growth pattern of mycelium in membrane areas overlying roots (Giovannetti et al., 1993). The results obtained suggest that compounds exuded by both *Bt* corn and defensin-expressing aubergine did not interfere with fungal recognition mechanisms. The fungus maintained the ability of perceiving and responding to host derived factors, as previously observed when AM fungi were challenged with non mycorrhizal (*myc*⁻) mutants of different host plant species (Giovannetti and Sbrana, 1998).

The recognition of a putative host is a prerequisite for infection structures formation, since AM fungal mycelium differentiate appressoria only on host plants roots (Giovannetti et al., 1994). Our results showed that *Bt* 176 corn line affected the regular development of appressoria, 36% of which failed to produce viable infection pegs. Instead, the pegs appeared devoid of protoplasm and septate and did not lead to intraradical colonization. Abortive penetration hyphae were also observed in AM fungal mycelium differentiating appressoria on the roots of resistant pea mutant P2 (*myc*⁻¹), where resistance responses of epidermal cells hindered intracellular development of hyphae originating from appressoria, which became empty and septate (Gollotte et al., 1993). Nothing is known of the mechanisms involved in the reduction of root colonisation observed in *Bt* 176 roots. Further investigations

Table 2. Analysis of appressorium development on Dm-AMP1 and wild type aubergine roots.

Aubergine plants	Total number of appressoria (mean \pm SE)	Number of appressoria not developing infection units (mean \pm SE)
Dm-AMP1 aubergine	81.0 \pm 8.3	2.0 \pm 0.6
Wild type	80.5 \pm 28.5	3.0 \pm 1.0

could provide deeper insight into cellular interactions between roots and appressoria in this GM line. On the contrary, no alterations in the infection pattern were observed when AM fungal mycelium grew in the presence of *Bt* 11 corn and Dm-AMP1 aubergine roots, where almost all appressoria gave rise to complete infection units.

Infected root length percentages were lower in *Bt* corn lines than in controls, though not statistically different. New experiments will be performed to obtain further data on mycorrhizal infection in *Bt* lines. By contrast, mycorrhizal colonization was not affected by the presence of Dm-AMP1 in aubergines, consistently with results obtained for pre-infection and early infection events.

G. mosseae was able to establish mycorrhizal infection in roots of *Nicotiana sylvestris* constitutively expressing tobacco chitinases and of *Nicotiana tabacum* transformed to express different pathogenesis-related proteins, at the same extent as in control plants (Tahiri-Alaoui et al., 1994; Vierheilig et al. 1993, 1995). In our work, mycorrhizal mycelium was not affected by the plant defence compound Dm-AMP1 defensin in aubergines, though constitutively expressed. AM fungal ability to tolerate molecules such as chitinases, glucanases, phytoalexins and phenolics, which are transiently accumulated by root cells during early stages of AM fungal colonization may provide further explanations to our results (Dumas-Gaudot et al., 1996; Gianinazzi-Pearson et al., 1992; Morandi, 1996; Volpin et al., 1994).

AM fungal behaviour differed in the presence of the two GM corn lines, although both plants were expressing *Bt* toxin. No correlation can be done between the observed alterations in fungal-plant interactions and the transformation event. However it is interesting to note that a higher level of *CryIAb* toxin expression was reported for *Bt* 176 than for *Bt* 11 (80.63 and $<0.55 \mu\text{g CryIAb/g}$ protein, respectively). Moreover, in contrast with other *Bt* corn lines, pollen from *Bt* 176

corn caused significant mortality and larval mass reduction in monarch and black swallowtail caterpillars (Zangerl et al., 2001).

In conclusion, the experimental system used allowed us to assess GM plant impact on soil beneficial non-target AM fungi, evidencing their differential behaviour in the presence of different GM lines. Our results are consistent with the relevant literature, and suggest that a case-by-case study, involving different test organisms and experimental approaches, may be useful when evaluating environmental risks of GM plants.

Acknowledgements

This work was partly supported by ISPESL, Italy (Istituto Superiore per la Prevenzione e la Sicurezza del Lavoro, convention PF/DIPIA/UO4/99) and CNR (Consiglio Nazionale della Ricerche, Italy).

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