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Review

Belowground environmental effects of transgenic crops: a soil microbial perspective

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Abstract

Experimental studies investigated the effects of transgenic crops on the structure, function and diversity of soil and rhizosphere microbial communities playing key roles in belowground environments. Here we review available data on direct, indirect and pleiotropic effects of engineered plants on soil microbiota, considering both the technology and the genetic construct utilized. Plants modified to express phytopathogen/phytoparasite resistance, or traits beneficial to food industries and consumers, differentially affected soil microorganisms depending on transformation events, experimental conditions and taxa analyzed. Future studies should address the development of harmonized methodologies by taking into account the complex interactions governing soil life.

Keywords: Microbial communities; Transgenic plants; Pleiotropy; Horizontal gene transfer; *Bt* plants

1. Introduction

The cultivation of transgenic plants (or genetically modified plants, GMPs) has prompted scientists to seek greater understanding of their direct and indirect impact on natural and agricultural ecosystems. While GMPs have been assumed to be safe in terms of human health, unforeseen environmental effects have been observed in the field, varying according to the genetic traits of the modified plants, and in space and time, as a result of the complex network of interactions ruling aboveground and belowground ecosystem functioning [1]. Some of the effects reported in the available scientific literature may be directly ascribed to the technology utilized, while others are linked to the nature of the genes introduced in the transgenic plants.

Most transgenic events have been obtained using the cauliflower mosaic virus (CaMV) 35S RNA promoter, which induces constitutive expression of transgenic proteins: some of them act as toxins towards particular groups of organisms and are exuded by the roots [2–4]. This stresses the need to assess the effects of such genetic modification on microbes living in the rhizosphere and in the soil. In such environments plants release up to 25% of the carbon allocated to the roots as root exudates [5], and crop residues are incorporated at the end of production cycles. Other outcomes of the technology used for production of transgenic plants may derive from pleiotropy, a phenomenon leading to development of unexpected phenotypes as a result of insertions of foreign genes in a new genomic context. For example, some GMPs showed increases or decreases in the content of plant secondary metabolism compounds or alterations in crop chemistry not directly linked to the particular genes introduced [6–8], which might affect, directly or indirectly, the soil microbiota.

With regard to the nature of the genes introduced in transgenic plants, the use of marker genes for antibiotic

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resistance and their fate during and after cultivation in the field have been considered critical issues by the World Health Organization [9], as antibiotic resistance genes may be transferred to rhizosphere and soil microbes, and from them to pathogenic bacteria, through horizontal gene transfer (HGT) [10]. In addition, crops modified to tolerate broad-spectrum herbicides like glyphosate have also raised concerns, as glyphosate inhibits Class I EPSPS, a key enzyme in the synthesis of aromatic amino acids occurring in plants, fungi and bacteria [11].

GMPs may directly or indirectly impact the structure, function and diversity of soil and rhizosphere microbial communities, which play key roles in the belowground environment, providing essential ecosystem services, e.g. decomposition of crop residues, completion of biogeochemical cycles within the soil food web, and maintenance of environmental quality and productivity [5]. Rhizosphere microorganisms may be affected by plant genotype [12] and by changes in agricultural management inherent to cultivation of transgenic plants, such as herbicide application. Thus, they represent potential key non-target organisms to be monitored in studies on the environmental impact of transgenic crops (Fig. 1).

In this work, we review available data on direct, indirect and pleiotropic effects of GMPs on the structure and function of soil microbial communities, considering both the technology utilized for production of engineered plants and the nature of the transgenes.

2. Direct, indirect and pleiotropic effects of transgenic plants on soil microbes

2.1. Transgenic plants constitutively producing Bt toxins

Bt plants are engineered with *cry* genes derived from the soil bacterium *Bacillus thuringensis* Berliner to express insecticidal δ-endotoxins (called crystal proteins or Cry proteins), conferring resistance to some insect pests from the orders Lepidoptera, Coleoptera or Diptera [13]. The amounts of *Bt* toxins expressed in plant tissues and released into the environment, ranging from 152 to 183 ng per gram in decomposing root residues, directly derive from the technology utilized to produce transgenic plants constitutively expressing Cry proteins. Such data will deserve attention in the years to come, in particular in multiple *Bt* toxin stacked-trait lines [14]. Indeed, it has long been known that insecticidal *Bt* toxins are exuded by *Bt* maize roots into the soil [2] where, together with those derived from plant residues, they are bound to humic acids and clay soil particles and, protected from microbial degradation, often maintain their activity [13]. Some authors reported that the Cry3Bb and the Cry1Ac toxins may persist for 21 and 56 days in soil microcosm and laboratory experiments, respectively [15,16], and that no *Bt* toxin is retrieved from field soils for 3–6 consecutive years of *Bt* cotton cultivation [17]. Variable persistence has been observed for the Cry1Ab toxin, which was not detected in a nine-year field trial of *Bt*-maize MON810 [18], or else was shown to be still detectable after 4 years in the field [13], possibly depending on soil chemical and physical characteristics.

In an experiment carried out on *Bt* maize plants in relation to soil biota, Saxena and Stotzky found that the CryIAb toxin released into root exudates or directly incorporated into soil exerted no adverse effects on culturable bacteria or saprophytic fungi (nor on earthworms, nematodes or protozoa) [19]. Small or no changes in culturable microflora were detected in the rhizosphere of Cry-expressing cotton and rice and in the composition of microbial communities in the presence of Cry1Ab maize residues compared with control plants (Table 1). Accordingly, two long-term field studies found no consistent differences in soil microbial communities between GMPs and controls or during successive years [15,20]. A significant temporary decrease in saprophytic fungal populations was observed 30 days after sowing *Bt* maize in comparison with the isogenic line [21], and variation in fungal decomposer communities was detected in one out of 16 trials by Xue et al. [22] (Table 1).

Other works, using culture-independent methods, reported no significant or only slight effects of *Bt* maize plants on soil microbial communities, suggesting that plant age, soil type and texture may represent the overriding factors affecting bacterial diversity (Table 1). In contrast, differing fingerprints of soil bacterial communities exposed to *Bt* maize were reported by other authors [23–26]. Castaldini et al. [25] also observed that microbial activity, assessed by measuring soil respiration, changed in soils amended with *Bt* plant residues, in agreement with other reports [27,28] (Table 1).

In the majority of the cited studies, it is impossible to distinguish between effects that can be directly ascribed to the toxins and indirect and non-specific outcomes of transgenic events (pleiotropy). However, an interesting work highlighted the occurrence of pleiotropic effects which were not linked to the products of the inserted genes, but resulted from transformation technology [16]: the cultivation of *Bt* cotton affected soil microbial populations, while the purified *Bt* toxin showed no effect. These data were corroborated by results detailed in Naef et al. [29], who found that purified Cry1Ab toxin did not inhibit growth of *Fusarium graminearum* or *Trichoderma atroviride*, while *Bt* and non-*Bt* maize residues affected fungal growth in vitro (Table 1).

A pleiotropic effect of *cry1Ab* transgenic plants - alteration in the shikimic acid pathway leading to a higher lignin content in the stem - was detected in several transformation events of *Bt* maize lines [6,8] and also in *Bt* canola, cotton, potato, rice and tobacco [27]. However, the harm or benefits of the slower degradation rate of *Bt* plant residues and putative resulting shifts in microbial community composition remain to be verified. A field study [30] found that *Bt* maize decomposed significantly faster than non-*Bt* maize in winter in bags with 20 and 125 μm mesh sizes, which excluded macrofauna but allowed microflora (bacteria, fungi) and mesofauna activity. Such results were explained by the higher amount of proteins in the plant matrix (20% of *Bt* toxin still present), which stimulated growth of soil microbial populations. Conversely,

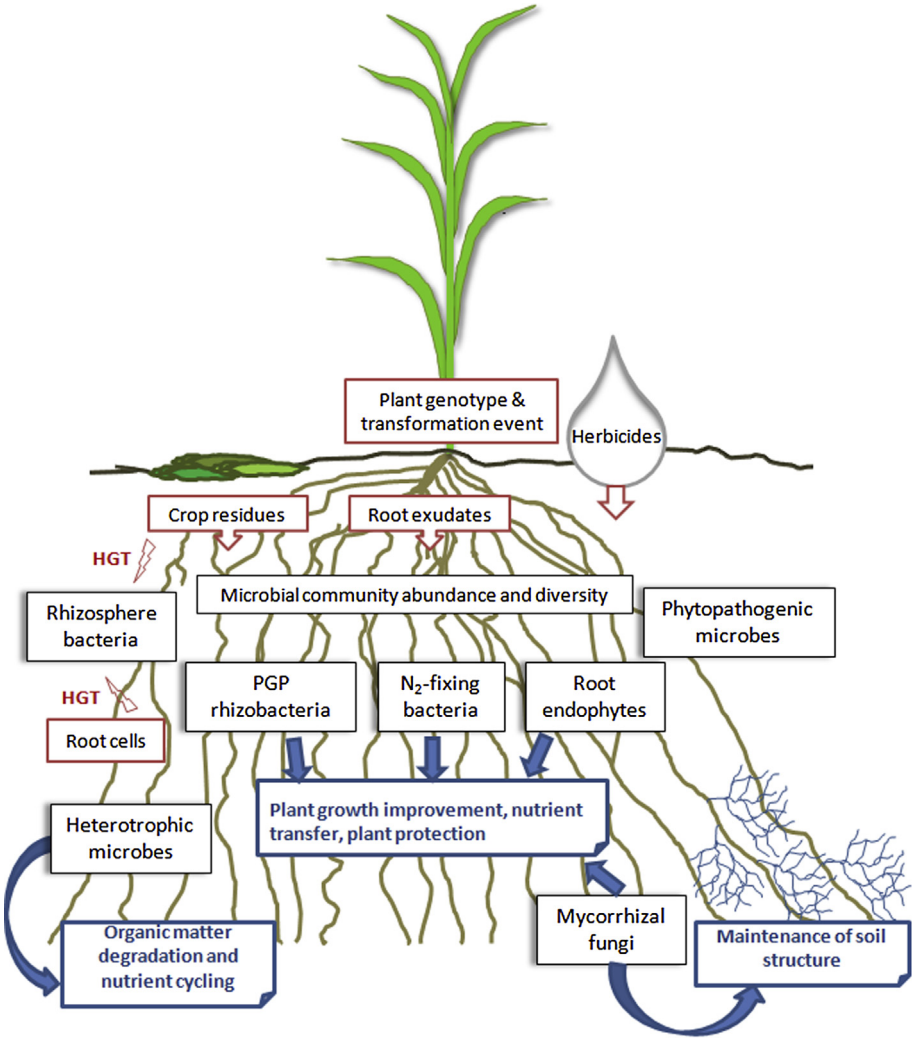

Fig. 1. Schematic drawing representing direct and indirect impact of transgenic crops on soil microbial communities and microbe-mediated processes and functions. Red: sources of potential impact; blue: microbe-mediated processes; black: soil microbial functional groups. PGP, Plant Growth Promoters; HGT, Horizontal Gene Transfer.

no effects on the decomposition rate were detected by Hopkins and Gregorich [31], either in microcosm experiments or when residues of some *Bt* hybrids were ploughed into field soil.

A distinctive group of beneficial soil microorganisms, arbuscular mycorrhizal (AM) symbionts, have been extensively investigated as potential key non-target organisms in studies on the environmental impact of GMPs, given their strong responsiveness to agricultural practices and environmental changes. A reduction in AM colonization was shown in *Bt*11, *Bt*176 and MON810 maize lines expressing the Cry1Ab toxin compared with non-*Bt* isogenic lines (Fig. 2), whereas no effects were detected in other trials with different maize and cotton lines expressing Cry1Ab, Cry1Ac and Cry2Ab (Table 1). A recent study on 14 *Bt* and non-*Bt* maize lines expressing different numbers and types of engineered traits revealed that all the various transgenic lines reduced mycorrhizal colonization by indigenous AM fungi occurring in the greenhouse [32], while no changes in AM fungal colonization were found in the field [33,34], but AMF spore abundance was lower in field plots with a Bt maize cultivation history than in control plots [35]. Such contrasting results might be explained by the different nutrient status of soil or by the differential nutrient uptake of *Bt* and non-*Bt* plants [32,36].

In general, great attention should be paid when discussing data obtained in short-term experiments or single-point assessments, because microbial communities living in the soil and in the rhizosphere are subject to seasonal shifts, which represent further factors affecting the complex network of interactions characterizing natural and agricultural ecosystems. Time-course investigations based on large spatial and long temporal scales are needed to assess putative long-term modifications occurring in microbial community structure and composition during and after GMP cultivation. Soil environment should continue to be monitored after Bt crop use, as there are data showing that repeated cultivation of *Bt* corn over many years resulted in greater microbial biomass, enzyme activity and functional diversity than conventional corn grown in rotation [37].

Table 1
Pleiotropic (P) and undetermined (pleiotropic or direct, U) effects of transgenes expressed in different plant species on soil microorganims, as revealed by culture-dependent and independent methods. Consistent (+), transient (+/−) and no effects (−) are reported.

Protein or *gene*	Plant	Impact occurrence	Methods	Organism	References
Expression of *Bt* toxins					
Cry1Ab	Corn	+ (U)	ARISA[a]	Rhizosphere bacterial community	[24].
		+ (U)	DGGE	Rhizosphere bacterial community	[25].
		+ (U)	AMF colonization	Glomeromycota	[25,32], [1s[b], 2s]
		+ (U)	AMF spore count	Glomeromycota	[35].
		+ (U)	Soil respiration	Soil and rhizosphere bacterial communities	[24], [3s]
		+ (U)	Quantitative PCR	Ammonia oxidizing archaea and bacteria, nitrogen fixing bacteria	[23].
		+ (U)	Enzymes activities, microbial biomass, CLPP	Soil microbial communities	[37].
		+ (U)	N e C cycle activities, DGGE	Rhizosphere microbial communities	[26].
		± (U)	CLPP, plate counts, soil respiration	Soil bacterial communities	[4s]
		± (P)	Fungal growth measurement	*Fusarium graminearum* and *Trichoderma atroviride*	[29].
		± (U)	Plate counts	Rhizosphere and soil culturable bacterial and fungal communities or specific functional bacterial groups	[15,21].
		± (U)	DGGE	Soil bacterial community	[15].
		−	Plate counts	Rhizosphere bacterial and fungal community	[19,24,27], [5s]
		−	CLCP, CLPP	Rhizosphere bacterial and fungal community	[24], [6s, 7s]
		−	DGGE	Rhizosphere and soil bacterial and AMF communities	[6s, 8s, 9s]
		−	DGGE	Bacterial communities	[23], [10s]
		−	PFLA	Rhizosphere and soil bacterial communities	[7s, 11s]
		−	Microarrays	*Bacillus subtilis* and *Streptomyces coelicolor*-related bacteria	[12s]
		−	SSCP	Rhizosphere bacterial community	[13].
		−	454 pyrosequencing, T-RFLP	Fungi	[13s]
		−	T-RFLP, cloning and sequencing	Glomeromycota	[34].
		−	454 pyrosequencing	Rhizobacterial communities	[20].
		−	Soil respiration	Soil microbial communities	[14s, 15s]
		−	Soil enzymatic activities	Rhizosphere microbial communities	[15,21].
		−	Mineralizable C, total soil C and N, lignin	Rhizosphere microbial communities	[14s, 16s, 17s]
		−	AMF colonization	Glomeromycota	[33], [18s]
Cry1Ab	Cotton	+ (U)	Soil enzymatic activities	Soil bacterial communities	[19s]
		± (P)	PCR-RFLP	Rhizosphere bacterial and fungal communities	[16].
		± (P)	Plate counts	Soil bacterial and fungal communities	[16].
		−	Soil enzymatic activities	Soil bacterial communities	[20s]
		−	CLPP	Soil microbial community, specific functional bacterial groups	[27], [20s, 21s]
		−	Plate counts	Soil microbial communities	[22s]
Cry1Ab	Rice	+ (U)	T-RFLP	Fungal communities	[23s]
		+ (U)	RNA-SIP, clone libraries	Rhizosphere methanogenic archeal communities	[24s]
		± (U)	Plate counts	Aerobic bacteria, actinomycetes and fungi	[25s]
		± (U)	Soil enzymatic activities	Soil bacterial communities	[25s, 26s]
		−	TGGE	Soil bacterial communities	[26s]
		−	T-RFLP	Soil bacterial and fungal communities	[27s]
		−	PLFA	Rhizosphere bacterial communities	[28s]
Cry1Ab/CrylAc	Rice	+ (U)	DGGE, quantitative PCR	Methanogenic archeal and methanotrophic bacterial communities	[29s]

Table 1 (*continued*)

Protein or *gene*	Plant	Impact occurrence	Methods	Organism	References
Cry1A.105	Corn	–	T-RFLP	Bacterial endophytes	[30s]
Cry1Ac	Brinjal	±	Quantitative PCR, biomass, RFLP	Microbial communities	[31s]
CrylAc	Cotton	+ (U)	Soil enzymatic activities	Soil bacterial communities	[19s, 32s]
		± (P)	PCR-RFLP	Soil bacterial and fungal communities	[16].
		± (P)	Plate counts	Soil bacterial and fungal communities	[16].
		–	AMF colonization	Glomeromycota	[33s]
	Rice	± (U)	CLPP, DGGE	Rhizosphere microbial communities	[34s]
		–	Enzymatic activities	Rhizosphere microbial communities	[34s]
Cry1Ac	Turnip	–	DGGE	Rhizosphere bacterial community	[35s]
Cry1F	Corn	+ (U)	AMF colonization	Glomeromycota	[32].
		+ (U)	AMF spore count	Glomeromycota	[35].
		+ (U)	Quantitative PCR	Ammonia oxidizing archaea and bacteria, nitrogen fixing bacteria	[23].
		–	CLPP, plate counts	Rhizosphere bacterial community	[11s]
		–	DGGE	Bacterial communities	[23], [10s]
		–	AMF colonization	Glomeromycota	[33].
Cry2Ab2	Corn	–	T-RFLP	Bacterial endophytes	[30s]
Cry2Ab	Cotton	–	AMF colonization	Glomeromycota	[33s]
Cry3Bb1	Corn	+ (U)	AMF colonization	Glomeromycota	[32].
		+ (U)	AMF spore count	Glomeromycota	[35].
		± (U)	Plate counts	Rhizosphere and soil culturable bacterial and fungal communities or specific functional bacterial groups	[15,21].
		–	T-RFLP	Soil decomposer community	[22].
		–	ARISA, DGGE, microarrays, SSCP, T-RFLP	Rhizosphere and soil bacterial community or specific functional bacterial groups or bacterial endophytes	[15], [30s, 36s, 37s]
		–	AMF colonization	Glomeromycota	[33].
		–	N-mineralization	Soil microbial communities	[36s]
		–	Soil enzymatic activities, soil respiration	Soil microbial communities	[15].
		–	Soil enzymatic activities, lignin content	Rhizosphere microbial communities	[38s]
Cry34/35Ab1	Corn	+ (U)	AMF colonization	Glomeromycota	[32].
		+ (U)	AMF spore count	Glomeromycota	[35].
		–	AMF colonization	Glomeromycota	[33].
Resistance to pathogens other than insects					
Agglutinin	Potato	± (P)	Soil enzymatic activities	Rhizosphere microbial communities	[38].
		–	CLPP	Rhizosphere microbial communities	[38].
Cecropin B	Potato	± (U)	PCR-RFLP	rhizosphere *Bacillus* spp.	[43].
Cecropin B/attacin		± (U)	T-RFLP, clone libraries, soil enzymatic activities	Rhizosphere bacterial communities	[41].
Chicken egg white cystatin	Potato	+ (U)	PLFA	Rhizosphere bacterial communities	[39].
Chitinase	Rice	+ (U)	AMF colonization	Glomeromycota	[45].
Chitinase	*Nicotiana sylvestris*	–	AMF colonization	Glomeromycota	[48].
Concanavalin A	Potato	± (P)	CLPP	Soil bacterial communities	[38].
CSA synthesis genes	Tobacco	+ (U)	AMF colonization	Glomeromycota	[49].
Defensin Dm-AMP1	Aubergine	–	AMF colonization	Glomeromycota	[5].
ESF39A	Elm	–	AMF colonization	Glomeromycota	[39s]
Lactonase AttM	Tobacco	–	Plate count	Specific microbial groups	[44].
		–	DGGE	Rhizosphere bacterial communities	[44].
NahG gene	Tobacco	+ (U)	AMF colonization	Glomeromycota	[49].
pm3b gene	Wheat	± (P)	DGGE	Pseudomonads	[51].
		± (P)	AMF colonization	Glomeromycota	[51].
PR-2	Tobacco	+ (U)	AMF colonization	Glomeromycota	[47].
T4 lysozyme	Potato	+	T-RFLP	Bacterial communities	[42].
		± (U)	T-RFLP, clone libraries, enzymatic activities	Rhizosphere bacterial communities	[41].

(*Continued on next page*)

Table 1 (*continued*)

Protein or *gene*	Plant	Impact occurrence	Methods	Organism	References
		−	Plate counts, CLCP, PCR-RFLP, DGGE	Rhizosphere bacteria	[40s] [42].
		−	Plate counts, FAME	Beneficial plant-associated bacteria	[40s–42s]
Resistance to herbicides					
Ahas gene	Soybean	−	DGGE	Soil microbial community	[43s]
Cp4-epsps gene	Canola	+ (U)	CLPP	Rhizosphere bacterial communities	[44s]
		+ (U)	FAME	Rhizosphere bacterial communities	[68]; [44s]
		± (U)	CLPP, T-ARDRA, FAME	Rhizosphere bacterial communities	[67].
		± (U)	Microbial biomass, CLPP, soil enzymatic activities	Soil bacteria	[45s]
Cp4-epsps gene	Cotton	−	AMF colonization	Glomeromycota	[33s]
Cp4-epsps gene	Corn	−	T-RFLP, quantitative PCR	Denitrifying and fungal communities	[70].
Cp4-epsps gene	Soybean	+ (U)	FAME	Microbial community	[46s]
		± (U)	Microbial biomass, soil enzymatic activities	Microbial community	[72].
		+ (P)	Plate count and specific test	Specific microbial groups	[71].
		−	AMF colonization	Glomeromycota	[47s]
Cp4-epsps gene	Wheat	± (U)	Microbial biomass, CLPP, soil enzymatic activities	Soil bacteria	[45s]
pat gene	Canola	+ (U)	DGGE	Rhizosphere bacterial community	[69].
		+ (U)	Soil enzymatic activities	Rhizosphere bacterial communities	[48s]
		−	DGGE	Rhizosphere bacterial community	[48s]
pat gene	Corn	−	SSCP	Rhizosphere bacterial communities	[66], [49s]
		± (U)	PFLA	Microbial community	[50s]
		−	ELFA, CLPP, basal respiration, decomposition	Rhizosphere bacterial communities	[51s]
ppo gene	Rice	−	T-RFLP	Rhizosphere bacterial and fungal communities	[52s]
Traits for the benefit of the food industry and consumers					
Alpha-amylase	Alfalfa	+ (U)	ERIC-PCR	Rhizosphere bacterial community	[53s]
		+ (U)	plate counts	Rhizosphere and soil bacterial communities	[53s, 54s]
		+ (U)	CLPP, soil respiration	Soil bacterial communities	[54s]
		−	PCR-RFLP	Soil bacterial and fungal communities	[54s]
RNAi of *gbss* gene	Potato	+ (U)	T-RFLP, PFLA-SIP	Rhizosphere fungal communities	[77].
		+ (U)	PFLA-SIP, clone libraries	Rhizosphere bacterial communities	[76].
		+ (U)	DGGE	Rhizosphere bacterial communities	[74,76].
		± (U)	T-RFLP, soil enzymatic activities	Rhizosphere fungal communities	[55s, 56s]
		± (U)	DGGE	Rhizosphere bacterial communities	[75].
		−	PFLA	Rhizosphere bacterial communities	[78].
		−	T-RFLP	Fungal communities; Actinomycetales, α and β Proteobacteria	[55s]
Lignin metabolism (suppressed cynnomyl alcohol dehydrogenase activity)	Poplar	−	454 pyrosequencing, DGGE	Fungal communities	[57s, 58s]
lignin peroxidase	Alfalfa	+ (U)	plate counts, CLPP, soil respiration	Soil bacterial communities	[54s]
		−	PCR-RFLP	Soil bacterial communities	[54s]
Mn-dep. lignin peroxidase	Alfalfa	+ (U)	CLPP, ERIC-PCR	Rhizosphere bacterial communities	[53s]
malate dehydrogenase	Alfalfa	+ (U)	CLPP, PCR-RFLP	Rhizosphere bacterial communities	[59s]
Lhcb1-2 genes	Eucalyptus	± (U)	Plate counts, ARDRA, DGGE	Rhizosphere bacterial community	[60s]
Phytase	Tobacco	−	T-RFLP	Surface and endophytic bacteria; Glomeromycota	[61s]

[a] ARISA (automated ribosomal spacer analysis), CLCP/CLPP (community-level catabolic/physiological profiling), DGGE (denaturing gradient gel electrophoresis), ERIC-PCR(enterobacterial repetitive intergenic consensus sequence-PCR), ELFA (ester linked fatty acid analysis), FAME (fatty acid methyl ester profiles), MPN (most probable number), PCR-RFLP (PCR-restriction fragment length polimorphisms), PLFA(phospholipid fatty acid analysis), PFLA-SIP (phospholipid fatty acid analysis-stable isotope probing), SSCP (single strand conformational polymorphisms), T-ARDRA (terminal-amplified ribosomal DNA restriction analysis), T-RFLP (terminal-restriction fragment length polimorphisms).

[b] s (References in Supplementary Material).

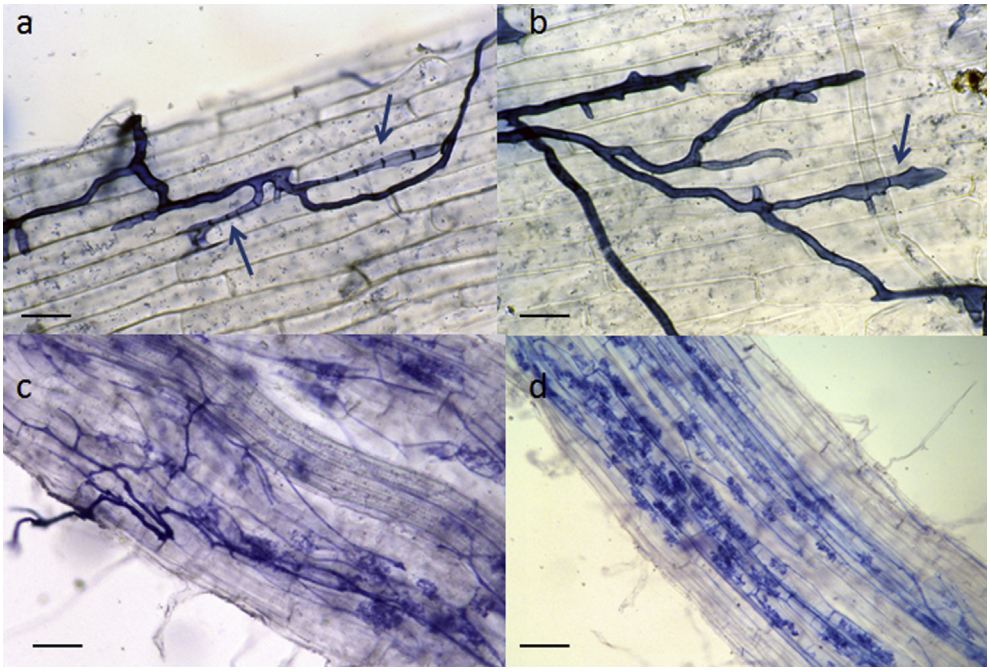

Fig. 2. Light micrographs showing fungal development in transgenic and non-transgenic maize plants (*Zea mays*) during the different stages of mycorrhizal symbiosis establishment. (a, b) Appressoria developed by *Funneliformis mosseae* on *Bt*176 maize roots producing infection pegs which become septate and devoid of protoplasm (arrows). (a) Scale bar = 35 μm; (b) Scale bar = 25 μm. (c) *F. mosseae* appressorium and entry point successfully colonizing a non-transgenic maize root. Scale bar = 50 μm. (d) Mycorrhizal colonization of a non-transgenic maize root by *F. mosseae*. Scale bar = 80 μm.

2.2. Transgenic plants resistant to phytopathogenic bacteria and fungi and to phytoparasites other than insects

Alongside GMPs expressing *Bt* toxin, other transgenic plants have been developed to control either invertebrate pests - by expressing lectin or proteinase inhibitors - or phytopathogenic fungi - by expressing plant-derived defensins, lysozymes, cecropins, pathogenesis-related proteins and systemic acquired resistance (Table 1).

Engineered potatoes producing *Galanthus nivalis* agglutinin, conferring resistance to nematodes, showed a reduction in microbial activity and different physiological profiles of rhizosphere microbial communities [38]. Transgenic potato plants expressing the cysteine proteinase inhibitors able to control potato-cyst nematode, tested in the field for two growing seasons, showed a reduction in bacterial and fungal abundance after one year's growth [39]. On the other hand, the impact of transgenic potato plants expressing a phage T4 lysozyme gene on bacterial communities was comparable to the effects of plant genotype, vegetation stage, soil type and pathogen infection [40–42]. Accordingly, other transgenic events expressing lytic peptides able to control phytopathogenic bacteria, like cecropin B- and quorum quenching lactonase AttM-expressing plants, did not cause significant changes in soil bacterial communities [43,44] (Table 1).

Several transgenic plants resistant to pathogenic fungi were obtained by inserting genes encoding pathogenesis-related proteins such as chitinases. A chitinase-expressing transgenic rice showed a reduction in root colonization by endophytic and mycorrhizal fungi and an increase in intraradical bacteria [45]. Symbiotic fungi and bacteria represent a very important group of non-target microbes to be monitored in impact studies of this particular kind of GMP, since they can be affected by plant antimicrobials. The establishment of mycorrhizal symbiosis by *Funneliformis mosseae* was not affected in the roots of *Nicotiana* spp. expressing chitinases and pathogenesis-related proteins, although a delay in mycorrhizal colonization was observed in plants expressing the PR-2 protein [46–48]. Other reports confirmed such results, showing delayed root colonization by AMF in tobacco plants modified for the expression of enhanced systemic acquired resistance, compared with non-transgenic lines [49]. The low sensitivity of AMF symbionts to antifungal enzymes may be ascribed to their differential expression in root tissues: thus in transgenic tobacco roots, chitinase levels were only 2–4 times higher than in controls, whereas their content increased 23–44 times in the leaves [50]. Differences in AMF root colonization and *Pseudomonas* population dynamics were observed among wheat plants expressing the *pm3b* mildew resistance transgene and parental lines. Conversely, no differences were detected between GM and non-GM sister lines, obtained through the same tissue culture and regeneration process, demonstrating that the differences in root colonization may be ascribed to the transformation technology [51].

Defensins are antimicrobial proteins able to inhibit the growth of phytopathogenic fungi by reducing hyphal elongation through specific binding to sphingolipid sites in hyphal

membranes. Defensin-engineered *Solanum melongena* (aubergine) plants expressing the gene for the Dm-AMP1 protein in all tissues were not affected in their ability to establish mycorrhizal symbiosis by *F. mosseae*. Interestingly, the antimicrobial protein was exuded from the roots into the surrounding environment, where it maintained the ability to control phytopathogenic fungal growth [4]. Unfortunately, no information is available on the persistence of the Dm-AMP1 protein released from transgenic roots in the rhizosphere and in the nearby soil.

Some of the antimicrobial compounds produced by transgenic plants do not accumulate in the rhizosphere, since they are degraded by proteases, as in the case of cecropin B [52], or are bound to plant residues, as in the case of lysozyme [53]. However, only limited information about the persistence of other, protease-resistant compounds, such as chitinases and vacuolar PR proteins, is available [54]. In vitro assays on transgenic tobacco plants expressing hen egg lysozyme detected release of the active enzyme through roots [53], whereas the enzymes β-1,3-glucanase and chitinase remained bound on the root surface of transgenic barley [55]. In addition, biological and chemical degradation of antimicrobial proteins may be hindered by adsorption of enzymes to soil inorganic or organic colloids, as reported above for Cry proteins: retention of root-bound β-1,3-glucanase and chitinase activity in silty loam soil was detected, even in the presence of rhizosphere microorganisms [56].

Although the available studies provided some data on the impact of transgenic plants on soil microbial communities and beneficial symbionts, the experiments performed failed to discriminate between pleiotropic effects and effects due to the transgene products. Further research should devote particular attention to other groups of non-target beneficial microorganisms and to the development of highly specific systems for phytopathogenic control.

2.3. Transgenic plants expressing antibiotic resistance genes

The production of transgenic plant varieties is generally obtained by engineering a genetic construct which includes not only the gene of interest and the relevant promoter for protein constitutive expression, but also an antibiotic-resistant gene. Such a gene is introduced exclusively for technical reasons, as it represents an optimal selectable marker, allowing easy detection of transformed cells incorporating the transgenes. One of the most widely used antibiotic resistance genes is *nptII*, deriving from a bacterial transposon (Tn5 from *Escherichia coli*) whose product inactivates aminoglycoside antibiotics such as kanamycin and neomycin. Other antibiotic markers have been utilized, often in combination: as an example, a cassette containing *nptII*, *Gent* and *Tet* genes, conferring resistance to neomycin, gentamicin and tetracycline antibiotics, has been used to develop papaya plants resistant to Papaya ringspot virus (PRSV) infection.

The release of antibiotic resistance genes in the field and in the soil by transgenic crops raised concerns about the possibility of their uptake by native soil bacteria through horizontal gene transfer (HGT), a fundamental mechanism of genetic recombination and evolution in many bacterial species. In particular, the rhizosphere represents a “hot spot” for bacterial recombination, given its high nutrient content and water/exudate fluxes, as compared with bulk soil. For example, a high transfer frequency of plasmids with antibiotic resistance has been reported to occur in wheat rhizosphere [57]. In such a peculiar ecological niche, various events of HGT occurring by conjugation, transformation and transduction have been described, including the acquisition of plant-derived genes by rhizosphere bacteria [58,59]. Transformation was found to be active in the transfer of kanamycin resistant genes from transgenic plant DNA to the rhizosphere bacterium *Acinetobacter* sp. in soil microcosms [60,61], confirming previous data on the possibility of bacterial natural transformation [62].

The possible spread of antibiotic resistance genes in agricultural and natural ecosystems through competent soil bacteria carrying homologous sequences and their HGT represents a potential risk to be taken into account when assessing the environmental impact of GMPs. For this reason the World Health Organization recommended the development of new technologies to obtain GMPs while avoiding the use of antibiotic resistance genes [9]. Indeed, some transgenic events have been developed using, as a selection marker, the phosphinothricin N-acetyltransferase (PAT) enzyme that confers resistance to the herbicidal activity of glufosinate. GMPs could also be produced by removing antibiotic resistance selection markers, using either co-transformation followed by the segregation of relevant genes or site-specific recombination with excision of marker genes [63]. Other promising strategies involve the use of positive selection markers based on hormone, saccharide and amino acid metabolism [64] or of modified tubulin genes [65].

2.4. Herbicide tolerant transgenic plants

Many crop species, including widely cultivated beet (*Beta vulgaris* L.), maize (*Zea mays* L.) and oilseed rape (canola) (*Brassica napus* L.), have been genetically engineered to express bacterial genes that confer herbicide tolerance. The aim of this technology is to improve pre-emergence and post-emergence control of many different weeds by using herbicides (e.g., glyphosate, glufosinate-ammonium) without harming the cultivated crops. Thus far, contrasting effects have been described on the composition and diversity of soil and microbial communities living in the rhizosphere of herbicide-tolerant (HT) *B. napus*, maize and soybean [66–72] (Table 1). Interestingly, pleiotropic effects of genetic transformation were reported for glyphosate-resistant (GR) soybean, which exuded higher amounts of carbohydrates and amino acids at a rate higher than non-transgenic plants. Such exudates could significantly enhance the growth of *Fusarium* regardless of glyphosate treatment [73]. Moreover, significant effects of GR crops and the relevant herbicides were observed for some important functional groups of microbes, such as nitrogen-fixing bacteria, pseudomonads, and rhizobacteria [71].

As cultivation of such plants entails the use of herbicides, such as glyphosate, which inhibits key enzymes occurring not only in plants, but also in fungi and bacteria [71], further work should focus on the effects of HT plant cultivation on microbial groups (e.g., mycorrhizal fungi, antagonists, nitrogen-fixing bacteria), either associated with the rate and time of herbicide application or linked to the relevant management practices, e.g. altered rotations, land use or the tillage system.

2.5. Transgenic plants modified to express different traits for the benefit of the food industry and consumers

Certain plant species have been engineered with the aim of modifying a range of properties in order to reduce processing costs and offer greater benefit for consumers. Examples include potatoes, which have been genetically modified to contain more starch and accumulate less sugar, thus reducing processing costs, or tomatoes, which have been modified to increase their shelf life by delaying ripening; the content and type of sugars and starch has been modified in a number of target crops, to obtain more uniform starches, or starches with altered branching or degree of polymerization. Only a few studies investigated the impact of such GMPs on soil microbes.

Transgenic potato plants with altered starch content affected ammonia oxidizer communities [74], rhizosphere bacteria [75,76] and mycorrhizal fungi [77]. As other studies carried out at earlier growth development did not find any differences in fungal biomass and plant exudation [78], further works should consider plant growth stages when assessing GMP impact, by using time-course assessments. In addition, since soil showed a short-term ability to restore the original rhizosphere and rhizoplane communities in transgenic tobacco [79], revealing a high potentiality to act as a strong buffering agent, different soil types should be included in the analysis of GMP effects in the years to come.

3. Concluding remarks

The available experimental data investigating the effects of transgenic plants on the different components of the soil microbiota have generally overlooked the natural variability which may occur among different varieties of crop plants, as they evaluated the potential impact on soil microbial communities comparing transgenic with parental lines. This approach is scientifically sound, but it fails to answer the interesting question of whether the effects of a specific transgenic crop are clearly beyond the differences that would be found between a range of conventional cultivars. Interestingly, a recent quantitative assessment of soil functioning was devised to detect the natural variation (or normal operating range, NOR) in soil function, allowing discrimination of soil critical parameters. Such a method could be used to understand the relevance of changes induced by GMPs [80]. On the other hand, scarce information is available on microbiological changes resulting from agricultural practices inherent to transgenic crops, such as broad-spectrum herbicide application rates and timing, altered rotations and production schemes, land-use forms and tillage systems, which may affect below-ground microbial biodiversity and food webs. Accordingly, such questions should be tackled in future assessments in order to achieve a better understanding of the realistic effects of transgenic crops on soil microbes.

The development of effective and integrated methodologies for the assessment of the impact of transgenic crops on soil microorganisms remains a major scientific challenge. New data should be produced using adequately designed and standardized tests, sampling methods and statistical analyses, not only in short-term small-scale laboratory or glasshouse experiments, but also in long-term systematic and continued field trials, during and after crop removal. Special attention should be paid to the monitoring of key sensitive microbial functional groups fundamental for soil fertility and plant nutrition and governing the most important soil ecological functions, e.g. nitrification, nitrogen fixation, phosphate mobilization, organic carbon cycling and sink. Finally, further studies should be focused not only on punctiform effects on single organisms, but also on all other possible outcomes, such as combinatorial and cumulative effects which characterize the complex network of interactions ruling soil life.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.02.006.

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