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Title: **Do genetic modifications in crops affect soil fungi? ; a review**

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Abstract

The use of genetically modified (GM) plants in agriculture has been a topic in public debate for over a decade. Despite their potential to increase yields, there may be unintended negative side-effects of GM-plants on soil micro-organisms that are essential for functioning of agro-ecosystems. Fungi are important soil organisms and can have beneficial or harmful effects on plants. Their benefits to agro-ecosystems come from their activities as free-living saprobes breaking down soil organic matter thereby releasing nutrients to the crops, as well as from mutualistic interactions. On the other hand soil-borne plant pathogenic fungi can cause severe damage in crops. Understanding of the impact of GM plants on the dynamics and functioning of soil fungi is essential to evaluate the possible risks of introduction of GM plants for ecosystem functioning. In recent years, over 50 studies have addressed the effects of various GM-traits in crops on soil fungal community structure and function. These studies showed that GM-crops can have positive, negative or neutral effects on both free-living and plant-associated soil fungi. The observed discrepancy in results of these studies is discussed. This is done by highlighting a number of case studies. New methods developed in recent years have enabled microbial ecologists to get a better picture on the functioning and assembly of soil fungal communities. This review presents and discusses two of the most promising methods which are also readily usable in risk assessment of GM-plants on soil fungi and that could help answer remaining key questions in the field.

Keywords: Genetically modified (GM) plants – soil fungi – risk assessment – 'normal operating range'

Introduction

Although conventional breeding has been successful in developing plants with desired traits, transgenic techniques have extended these possibilities by enabling the introduction of interesting genes from other organisms (Jones 2011). The introduction of new genes into crop-species may increase consistent food production for the growing world population as crop losses due to pests are reduced and optimal crop yields can be obtained. However, there are ethical concerns about the use of transgenic crops as have been discussed in many public forums and have spurred numerous discussions regarding their safety (Jones 2011). Despite these concerns, the number of fields allocated to transgenic crop production has increased each year worldwide (James 2012). However there are strong differences between continents; while in Europe companies are pulling out of the market due to the negative public opinion towards genetically modified (GM)-crops and more strict EU-legislation, growing of GM crops, such as herbicide resistant soybean, is a common practice in USA. The main concerns regarding the use of GM crops in agro-ecosystems are related to the possibility of unintended transgene flow to indigenous plants, development of super weeds, and the effects of transgenic plants on non-target organisms, including soil microbial communities (Wolfenbarger and Phifer 2000).

Effects on composition and activity of soil biota could occur via changes in the chemical composition and quantity of crop residues and rhizodeposits (compounds released by roots) as a result of the modification of the crop. However, growing of different non-GM crop species in crop rotations is nearly always coinciding with changes in the soil microbial communities making the interpretation of the implication of differences in microbial community composition between GM-crops and parental crops complicated (Bruinsma et al. 2003; Liu et al. 2005). The majority of the studies on GM-crop effects on soil microbes have focused on bacteria investigating numbers, activities and community composition whereas only relatively few studies have addressed the impacts on fungi in similar detail despite the importance of fungi for the functioning of soil ecosystems (Carlile et al. 2001).

In the 2003 review by Bruinsma et al (2003) , it was thought that the remaining gaps regarding the evaluation of impact of GM-crops on soil microbes were (1) incomplete knowledge of the functioning of soil microbial communities, (2) poor understanding of the range of responses of the microbial community to “normal” variation in soil systems (such as due to changes in season, weather, and

agricultural management practices including fertilizer use, crop rotation, pesticide use, etc.), and (3) inability to convert complex laboratory procedures to practical assays that are easy to perform and interpret. These knowledge gaps, in particular in the area of effects on fungi, have not yet been filled.

Here, we summarize the information on the effect of GM-plants on activity, biomass and community composition of non-target soil fungi and discuss possibilities to fill the remaining knowledge gaps. We pay special attention to recently developed methods such as next-generation sequencing and stable isotope probing which have the potential, both in their own way, to facilitate the evaluation of the response of soil fungi (and other microbes) to GM-crops.

Fungal diversity and functioning in agro-ecosystems

Before considering the potential effects of genetically modified plants on soil fungi, it is necessary to consider the general effects of agriculture on fungi. Besides the major effects that soil physical and chemical factors such as pH, moisture, soil texture, vegetation have on the composition and functioning of soil microbial communities, many studies have reported a reduction of fungal biomass under agriculture compared to more natural systems, and bacteria are thought to dominate in agricultural ecosystems (Kennedy 1999; Berg and Smalla 2009). This has been attributed both to the constant removal of crop plants, thereby reducing the input of litter (Berg and Smalla 2009) and to mechanical actions such as plowing which can potentially break extensive hyphal networks (Wang et al. 2010). Different agronomic practices such as tillage and fertilization also have profound impacts on the fungal communities (Oehl et al. 2010). Studies have found that organic farming had a significant positive effect on soil AMF richness compared to conventional farming (Verbruggen et al. 2010) and that type of fertilizer applied significantly affects fungal biomass (Heinze et al. 2010). Contradicting the idea of agro-ecosystems being bacterial dominated, recent evidence gained with stable isotope methods show that fungi might be more important organisms in the rhizosphere of crop-species than earlier thought (Gschwendtner et al. 2011; Hannula et al. 2012a). Further evidence suggests that the importance of fungi might be larger later in the season when the plant is more mature and fungi have had more time to establish their hyphal networks (Hannula et al. 2010) while bacteria are more abundant in the rhizosphere of early stages of plant growth and in the bulk soils (Inceoglu et al. 2010).

Fungi perform a wide range of ecosystem functions in agricultural soils and their importance in agro-ecosystems come from their activities as saprobes breaking down soil organic matter thereby releasing nutrients to the crop species as well as from their mutualistic and pathogenic interactions with the plant (Raaijmakers et al. 2009). Thus, a reduced fungal biomass in a field can potentially cause changes in both carbon and nutrient cycling and therefore change the functioning of the system (de Vries et al. 2011). Furthermore, as fungi are in the base of the soil foodweb, a change in either biomass or community composition of fungi caused by a GM-plant can have unforeseen cumulative effects on the higher trophic levels (de Vries et al. 2013).

According to the insurance hypothesis, the loss of biodiversity would cause a reduction in the ecosystem stability due to diminished probability to find species best able adapt to changing conditions (Loreau et al. 2002). The positive effect of increasing fungal biodiversity on stability of the soil ecosystems have been shown for simple communities (Setälä and McLean 2004) but the effect of biodiversity is less evident in natural systems (Nielsen et al. 2011). In this review the effects of GM-crops both on fungal community structure and diversity (and via that to resilience and resistance (Griffiths and Philippot 2013)), and on biomass and function are discussed.

Mechanisms by which GM-plants can affect soil fungi

GM crops can influence soil ecosystems positively, negatively or neutrally, (Oger et al. 1997). Birch et al. (2007) pointed out that the potential impacts of GM crops on soil ecosystem can be (1) direct (e.g. toxicity of an expressed new protein on key non-target species), (2) indirect (e.g. effects via trophic interactions), (3) caused by unintended changes in the metabolism of the plant and thus altering rhizodeposition and/or (4) caused by changes in the management regime used to cultivate GM crops. The effects of GM-trait can potentially be on the fungal biomass, community function or community composition. Harmful effects on fungal community function may cause a decrease in fertility and nutrient cycling in the soil which subsequently affects the following crops (de Vries et al. 2011), and changes in biomass and community structure via elimination of beneficial fungi such as AMF may affect plant growth as well and may result in the increased sensitivity of the plant to pathogens (van der Heijden et al. 2008). GM-caused shifts in

fungal community and biomass may also affect higher soil organisms through a cascade of effects in the soil food web in which fungi are a major channel of energy and nutrients.

The possible effects discussed in this review are related to GM-induced changes in the chemical composition of living and dead stages of crops that may affect non-target fungi.

Root exudation and soil fungal communities

Rhizodeposition has been identified as an important factor for the development of rhizosphere microbial communities (Lynch and Whipps 1990; Berg and Smalla 2009). A substantial amount of photosynthetically fixed carbon is released into the rhizosphere by roots and the composition and quantity of these exudates differs among plant species and plant growth conditions (Berg and Smalla 2009). Therefore, the first mechanism by which GM-crops can affect soil fungal communities is via intentional or unintentional changes in rhizodeposition quantity and quality. The latter does not only include changes in composition of well-known root-exudates (sugars, organic acids and amino acids) but also the presence of toxins, introduced into the soil from the root. It has been shown that the presence of novel compounds in root derived materials of a transgenic plant may confer a selective advantage to a specific group of soil bacteria which are able to utilize this compound (Savka and Farrand 1997). However such a specific process has not yet been demonstrated for fungi. The effects of toxin releases from roots of Bt-crops and its persistence in the soils has been discussed in detail in an earlier review (Icoz and Stotzky 2008).

Several studies have compared GM-crops and parental isoline and other varieties of the same crop species in field trials and greenhouse experiments. Most of these studies have shown that GM-crops do not affect the composition of the free-living soil fungi nor the fungal biomass differently than their parental isolines. Only in five studies significant differences in the soil fungal biomass or community structure between the GM-variety and its parental isoline were observed (Fig. 1). The reason why these studies, and not others have found differences between GM- and their parental isolines remains unclear as there is little in common in these studies; different GM-traits were introduced and different methods were used. Two of the studies were carried out with GM potatoes (increased resistance against nematode and pathogenic bacteria) (Cowgill et al. 2002; Götz et al. 2006) , one with both GM maize and GM potato (Bt and viral

resistance) (Xue et al. 2005), one with GM soybean and GM maize (herbicide tolerance) (Kremer and Means 2009) and the last one with viral resistant GM papaya (Wei et al. 2006). Remarkably, other studies carried out with the same crops and same modifications showed no effect on fungi (Table 1). For example, the study by Kremer and Means (2009) found that frequency of potential plant pathogenic fungi (Fusaria) colonizing glyphosate resistant maize roots was higher than in the roots of the parental cultivar, whereas in the same year Hart et al. (2009) reported no differences in abundance or community structure of rhizosphere fungi between the same parental and GM-varieties. This lack of coherence in results is probably due to the large variety in interactions between crops and biotic and abiotic factors such as soil type, plant growth stage studied, climate and interaction with other soil organisms. All these factors are known to strongly influence dynamics of soil fungi. Furthermore, in seven studies differences in fungal biomass or community structure were found between GM- and parental varieties, but due to the large variation in time and space, these effects were deemed transient (Fig 1.). These 'transient' effects and lack of coherence in results are discussed later in this review.

Despite the importance of arbuscular mycorrhizal fungi (AMF) in plant-soil systems, only few studies evaluated the non-target effects of GM-crops on AMF colonization and community structure. (Liu 2010) (Fig 1). As plants vary naturally in their AMF-hosting ability, the GM trait in plants might, in some cases, alter their relationship with AMF. Because AMF are obligate symbionts and thus require the plant host for nutrition and reproduction, they may be more sensitive to changes in the physiology of the host plant than free-living soil fungi (Liu 2010; Cheeke et al. 2011). Earlier, it was shown that AMF are sensitive to different agronomic practices such as tillage and fertilization (Oehl et al. 2010). AMF are thought to be especially important in low input agro-ecosystems and are, therefore, an important component of sustainable agriculture (Verbruggen and Kiers 2010) and it is therefore crucial to understand the impacts of GM-traits on functioning and diversity of AMF. In two studies a reduction in arbuscular mycorrhizal colonization of the roots of a Bt-cultivar of maize was reported (Castaldini et al. 2005; Cheeke et al. 2012). Another study found no effect on colonization of the Bt-maize roots but an effect on the AM-fungal community structure assessed by DGGE (Tan et al. 2011). Transient effects of GM-crops on soil AMF community structure or AMF colonization of roots have been reported in 4 other studies on Bt-maize, starch modified potatoes and herbicide tolerant soybean (Turrini et al. 2004; Powell et al. 2007; Cheeke et

191 al. 2011; Hannula et al. 2012a). In the study by Powell et al. (2007) different levels of rhizobial and
192 mycorrhizal colonization were observed between conventional and GM- soybeans. However, these
193 differences could be attributed to variation found between the three different non-modified and six different
194 modified cultivars and not the GM status of the plant. Other studies did not find effects of the GM-
195 modification on any aspect of AM biology studied. For instance, four different modifications introducing
196 insect resistance or herbicide tolerance in cotton had no effect on AMF colonization (Knox et al. 2008). In
197 addition, de Vaufleury *et al.* (2007) did not find any significant effect of Cry1Ab (Bt-) modification of
198 maize on AMF colonization. However, the total number of studies about effects of GM-crops on AMF is
199 rather low and certain traits such as the herbicide tolerance and resistance to pathogens have been only
200 studied in two studies (Table 1) which makes it difficult to come to a definite conclusion about the effects
201 of GM-crops on the AMF community.
202
203 *Effect of GM plants on residue decomposition and decomposer fungi*
204 Decomposition of litter is a key function in the cycling of elements and, consequently, in mineral nutrient
205 supply to plants thus any change in plant litter composition may potentially significantly affect soil
206 functioning (Deacon et al. 2006; Berg and McClaugherty 2008; van der Wal et al. 2013). In general, fungi
207 are more significant as litter-decaying agents than bacteria (Deacon et al. 2006). However, the large bulk of
208 the relevant studies have addressed litter decomposition as a functional response to GM-traits without
209 referring explicitly to the fungal communities involved.
210 The Bt-varieties of corn, cotton and rice have been the most studied modifications in litter
211 decomposition due to the observed unintended effect of Cry1Ab on the lignin content of the plants (Saxena
212 and Stotzky 2001b). Slower decomposition resulting from this altered lignin concentration has been
213 reported in few studies (Castaldini et al. 2005; Flores et al. 2005) while a greater number of studies did not
214 find a difference in decomposition between Bt and non-Bt corn (Jung and Sheaffer 2004; Fang et al. 2007;
215 Zwahlen et al. 2007; Daudu et al. 2009; Zurbrügg et al. 2010). An early study on Cry1A expression in
216 cotton found more species of fungi based on colony counting in the soils incubated with transgenic leaves
217 than in the soil incubated with leaves from the parental variety (Donegan et al. 1995). However, this study
218 seems to be an exception as it is the only one in which significant differences between the GM crop and the

parental isoline could not be explained by other factors than the genetic modification. The majority of studies on fungi in decomposing plant material did not show any significant effect or only a transient effect of genetic modifications on certain aspects of the fungal community (Fig.1). One of these studies on Cry3Bb expressing Bt-corn in a field experiment revealed no difference in the decomposition rate of roots, stalks, cobs or leaves between the Bt- and its parental variety at different locations but did detect a significant difference in fungal community composition as determined by T-RLFP in one of the soils tested and in one year which points to the transient nature of the observed effect (Xue et al. 2011). Other studies detected effects at one or more time points during decomposition but not for the overall decomposer community dynamics or for the total amount of plant material that was decomposed (Wu et al. 2004; Castaldini et al. 2005; Lu et al. 2010a).

As for AMF, it should be noted that effects of genetic modifications on decomposer fungi have only been addressed for a limited number of modifications and majority of the studies have investigated the effects of Bt-modifications (Table 1). Since modifications of pathogen resistance and structural changes of plant parts would be the most obvious GM-traits to affect the non-target decomposer fungal communities, it is surprising that no decomposition studies have addressed pathogen-resistance related modifications and only three dealt with the effect of structural changes of GM plants (lignin synthesis in tobacco, chitinase in birch and starch in potatoes) (Henault et al. 2006; Seppänen et al. 2007; Hannula et al. 2013). In the case of plants with genetic modifications to structural parts such as lignin synthesis or starch quality, risk assessment studies taking into account the effects on soil microbes and processes are essential.

Normal variation versus GM-induced variation

A common issue in the debate and a possible explanation for the lack of coherence in the results obtained in the previous studies on possible, harmful, side-effects of GM-crops is the difficulty to discern the effects of the modification from all the other abiotic and biotic factors (Fig.2). Usually ‘normal variation’ is defined as the variation in the responses of the fungal/microbial community to the non-GM crops under the prevailing conditions of the common agricultural practices (Kowalchuk et al. 2003). Factors such as weather, agricultural management and plant developmental stage can affect the outcome of the experiments more than the genetic modification (Griffiths et al. 2000; Lukow et al. 2000; Dunfield and Germida 2001).

247 For example, Hannula et al. (2012b) investigated the impact of different potato cultivars, including a GM
248 amylopectin-accumulating potato line, on rhizosphere fungal communities over a period of three years
249 under field conditions using molecular microbiological methods; they revealed occasional differences
250 between the transgenic line and its parental variety, indicating that differences, if realistic at all, were
251 mainly transient in nature and could only be detected either in one soil, at one growth stage or over a one-
252 year period. Furthermore, decomposition of plant material is also affected by the soil type and burying
253 depth of the tested plant material (Holland and Coleman 1987; Burgess et al. 2002; Powell et al. 2009).

254 The first variables to consider are site related variables. In general, soil type and field conditions,
255 including the history of the site are considered to be among the most influential factors governing soil
256 fungal community structure and function directly and indirectly via better plant growth (Costa et al. 2006;
257 Singh et al. 2007; Wang et al. 2009; Chaparro et al. 2012). In case of Bt-modifications it is known that the
258 physicochemical and biological characteristics of soils may influence the persistence of Cry class proteins
259 (Icoz and Stotzky 2008) in the environment thus influencing the outcome of the studies. Unfortunately,
260 only relatively few studies addressing possible effects of GM crops on soil fungal communities have
261 included more than one soil type (Fig. 2). For instance, Blackwood and Buyer (2004) investigated the
262 effects of Bt-modified maize on soil fungi in three soils and found that the soil type, but not the
263 modification, had a significant effect on the fungal biomass. Furthermore, the decomposition of litter is
264 found to vary between sites. Indeed, studies on Bt maize and rice have shown that both the site and the
265 burying depth are very important factors governing the decomposer processes and the structure of
266 associated fungal communities (Cortet et al. 2006; Lu et al. 2010b; Xue et al. 2011).

267 Few studies have compared effects of agricultural management practices in combination with GM-
268 crops on soil fungi (Fig. 2). Cheeke et al. (2011) inoculated AMF *Glomus mosseae* in Bt- maize and
269 parental roots and found that there was a significant interaction effect of cultivar and fertilizer level. The
270 effect of the GM-trait could only be seen in the low or no fertilizer treatments but not in the high fertilizer
271 treatment. Yet, this is an important aspect to consider as AMF are thought to be more beneficial to the plant
272 in low-input agro-ecosystems.

273 The growth stage of the plant is a second factor determining the activity and community structure
274 of fungi in soil. Jones et al. (2004) indicated that the amount and composition of rhizodeposition changes

275 during plant development with important consequences for the microbial activity and community
276 composition in the rhizosphere. Indeed, this seems to be valid for saprophytic fungi as well (although their
277 role in the rhizosphere is still a matter of debate) as plant growth stage and sampling time were found to
278 have the largest effect on activity and composition of both fungi in general and AMF in many experiments
279 (Fig. 2). The effect of growth stage was not seen in the bulk soil (Milling et al. 2004) or in the AMF
280 community under a tree (aspen) (Kaldorf et al. 2002) but was apparent in all other studies in which the
281 stage was evaluated. For example studies on genetically modified potatoes (Donegan et al. 1995; Cowgill et
282 al. 2002; Weinert et al. 2009; Gschwendtner et al. 2010; Hannula et al. 2012b) have shown that growth
283 stage is the single most important factor affecting the fungal biomass and community structure in the
284 rhizosphere. In field trials effects of growth stage can be affected by coinciding changes in temperature and
285 water availability, which are both important determinants of microbial growth. However, greenhouse
286 experiments have shown that in controlled conditions there is an effect, although smaller than in the field,
287 of plant growth stage on soil fungal communities (Girlanda et al. 2008; Wu et al. 2009; Gschwendtner et al.
288 2011). Finally, there is emerging evidence that plant parts collected at different stages of growth, also
289 decompose differently (Zurbrügg et al. 2010) and might, thus, also have different effects on fungal
290 communities.

291 Annual variation, including climatic factors such as precipitation and temperature often explains
292 large part of the variation observed in decomposer experiments (Fig. 2). From 11 studies dealing with the
293 effects of GM-crops on soil fungi in which annual variation was accounted for, 9 observed differences in
294 fungal community composition or abundance between years whereas in 2 studies no annual variation was
295 apparent (Milling et al. 2004; Li et al. 2011) . An elegant field study during 3 years revealed that ‘year’ was
296 the strongest explaining factor for changes in decomposition rate and structure of the associated fungal
297 communities and far more important than the Bt-trait of maize (Xue et al. 2011). In a four year field study
298 of Bt-corn, ‘year’ was shown to be a highly significant explanatory factor while the Bt-and its parental
299 variety differed only in one of the years and thus had a transient effect on numbers of culturable soil fungi
300 (Icoz et al. 2008).

301 The last important factor to consider when evaluating the effects of GM–traits on soil fungi is the
302 variation in traits that affect the soil microbial community among cultivars that exists due to their long

history of breeding. This may explain some of the transient effects observed in those studies that have compared multiple GM-varieties or multiple 'normal' varieties against the GM (Fig. 2). In most cases it was found that the normal variation among cultivars and thus the variable impact of conventionally bred varieties on environment was larger than the difference between GM-variety and its parental cultivar. Icoz et al. (2008) compared 4 Bt-varieties and their corresponding parental isolines and observed that the Bt-modification did not have an effect on numbers of fungi while crop variety had a significant albeit transient effect on the soil fungal community. Cheeke et al. (2012) investigated the colonization percentages of AMF on 16 maize lines (9 Bt lines and 7 parental varieties) and were the first to find a significant relationship between Bt-trait and the (lowered) colonization of AMF in a multiple cultivar study. However, this pattern seemed to hold only for greenhouse conditions as in the field study with a subset of these cultivars no effect on AMF colonization was found (Cheeke et al. 2013). This further highlights the importance of including environmental factors when evaluating GM effects on AMF.

New methods and new possibilities in GM-research

Traditionally most of the studies on effects of GM-crops on fungi have used cultivation based methods and root colonization counts of AMF (Table 1) to assess the effects of GM crops on the size and the diversity of the fungal community. However, the inherent limitations of the studying of culturable microbes have also here prevented a full scale assessment of the effects of GM crops on fungal diversity and functioning. In few cases DNA based fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) (Milling et al. 2004; Götz et al. 2006) or terminal restriction length polymorphism (T-RFLP) (Hart et al. 2009) were applied. However, in order to answer fundamental questions still open in this field such as on the totality of the effects of GM-crops on soil fungal diversity and in particular the rare and non-culturable fractions of the community as well as the functioning of the community through the impact of changes in rhizodeposition patterns, new methodology is ready to be adopted. In this section we will discuss methodologies that could help answering these key questions.

Monitoring differences in root exudation patterns with stable isotope probing (SIP)

Many studies have reported on the differences in the community composition of rhizosphere fungi between cultivars and have hypothesized that this would have been the result of changes in rhizodepositions. However, only few studies have actually measured rhizodeposits or monitored carbon flow from the plant into the rhizosphere, the subsequent utilization of the rhizodeposits by the microbial community, and how this influence both the structure and the functioning of the rhizosphere community . An elegant approach to do such measurements is stable isotope probing (SIP,) where the whole plant is (pulse) labeled with $^{13}CO_2$ and the incorporation of ^{13}C in microbes is followed in the endosphere and rhizosphere. Alternatively, plant residues containing ^{13}C can be used in a decomposition study to monitor the soil fungi involved in break-down of the (GM)-plant material. The measurement of the isotope in the DNA/RNA or fatty acids (PLFAs) extracted from soil allows for the detection and identification of the microbes actively involved in the assimilation of the labeled compound i.e. ^{13}C from the plant roots or dead plant material (Radajewski et al. 2000). SIP can be used to follow the faith of carbon in any system and has been used to study for example effects of soil management (Rangel-Castro et al. 2005; Lu et al. 2007) and climate change (Drigo et al. 2010) on soil microbial community structure and is proven to be a robust technique which can provide a quantitative insight in the rhizodeposit metabolizing community. In combination with phospholipid fatty acid (PLFA-SIP) analyses it has been used to evaluate the effects of GM-plants on carbon partitioning to different groups of soil organisms (Wu et al. 2009; Gschwendtner et al. 2011; Hannula et al. 2012a). All of these studies have shown the great importance of both saprotrophic fungi and especially AMF in the rhizosphere assimilating the (^{13})C from the plant. The first two studies did not find significant differences between the GM-and its parental cultivar, although Wu et al. (2009) found significant differences between the Bt and parental rice in the amount of ^{13}C distribution at the seedling, booting and heading stages. The last study (Hannula et al. 2012a) found that a starch modified GM-potato line affected soil fungal communities slightly differently than its parental isoline did, but these effects were deemed transient. A study done with DNA-SIP revealed cultivar dependent distinctions in ^{13}C-label flow to endophytic bacteria of potato (Rasche et al. 2009). However, in these studies the baseline of environmental variation was not investigated and thus it is not clear whether these differences are ecologically relevant. SIP methodology will, nevertheless, offer a great opportunity to study the effects of GM-varieties on active members of rhizosphere communities.

Possibilities of high-throughput sequencing to reveal fine scale differences between GM- and parental variety

It has been recognized that in addition to using broad scale keystone indicators such as fungal biomass and community composition, there is a need to improve the sensitivity of detection methods for detailed analyses of the impacts of GM-crops on soil microbial communities (Lilley et al. 2006). This should further target relevant species and functions for each combination of modification and species. Earlier, microbial biodiversity was thought to be a very sensitive parameter to perturbation and a good indicator for soil functioning (van der Heijden et al. 1998; Kennedy 1999; Garbeva et al. 2004) but this has recently been debated (Prosser 2012). In earlier studies in which differences in fungal community structure have been found, often these changes have been small without clear influence on the functioning of the system. However, it is not known how rare microbes affect the functioning of the system and how a change in community structure and diversity affects its function (Nielsen et al. 2011). Modern molecular methods such as 454-sequencing are useful in evaluating the effects of GM-crops on soil diversity replacing the DGGE and T-RFLP methods (Lindahl et al. 2013). A recent study on the effects of Bt maize varieties on AMF communities done using 454-sequencing combined with T-RFLP analyses revealed a significant correlation between the two methods even though some of the relative abundances of individual taxa differed (Verbruggen et al. 2012). Thus, there is no need to repeat existing evaluations of various GM-crops with this new methodology but the method itself is a powerful tool for future studies on the effects of GM-crops on soil fungal community structure and diversity. However, changes in diversity and community structure might not always lead to changes in ecosystem function as the relationship between diversity of soil micro-organisms and soil ecosystem functioning remain unclear (Nielsen et al. 2011). Thus, functional parameters or indicators of community functioning (i.e. production of extracellular enzymes) should be measured parallel to the diversity estimates. These functional measurements are discussed in an earlier review in more detail (Bruinsma et al. 2003). In future, function based sequencing and meta-transcriptomics can be used to when evaluating the effects of the GM-crops on soil fungi and will give a more detailed insight into the functional consequences of the modifications.

Final remarks

One issue, that has been addressed only marginally in GM crop studies, but in our opinion highly relevant to evaluate the real risk associated with the use of GM crops, is the effects of GM crops after harvest and/or in the following growing season(s). Few examples of such studies are a study by Castaldini *et al.* (2005), who reported a lower AMF colonization of *Medicago sativa* (alfalfa) roots grown in pots that had previously been cultivated with Bt maize and contained extra Bt plant residues. Contradictory, Cheeke et al. 2012 did not find any residual effect of Bt maize cultivation on AMF colonization of subsequently planted *Glycine max* (soybean). Besides, other studies in field and greenhouse conditions could not detect an effect of GM-crops on soil fungi after their harvest and/or in the following growing season (Oliveira et al. 2008; Powell et al. 2009; Cheeke et al. 2012; Hannula et al. 2012b). We strongly argue that such measurements should be considered in future risk assessment studies on the use of GM crops especially in cases when GM-crops are grown consecutively in the same soils for years as continuous growing of GM-crops can cause additive effect may strengthen the (negative) effects of the transgene.

The results available on the impact of GM plants on natural and agricultural ecosystems show that specific effects of single transformation events should be tested on a case-by-case basis in a natural setting where the baseline factors are all taken into the consideration, including biochemical, physiological, and molecular parameters. As fungi are important to soil functioning and plant growth, an evaluation of these organisms should be performed when evaluating the effects on soil biota. Furthermore, there is a need for statistical methods which can evaluate the effects of GM-trait in relation to the baseline 'noise' in the system. The new techniques such as SIP-experiments and high throughput sequencing and metatranscriptomics should be used in parallel with carefully designed field experiments considering all the 'baseline' factors including effects on the subsequent crop species.

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Legends for figures:

Figure 1 Studies on the effect of GM-crops on general fungal communities arbuscular mycorrhizal fungal (AMF) communities and decomposer fungal communities. This classification was done for convenience and because of the large differences between the types of studies and organisms. The observed effect of GM crop was categorized as i) a (lasting) effect, ii) a transient effect or iii) no effect on fungi. In the mycorrhizal studies, only effects on AMF were included; the few studies addressing effects of lignin-modified trees on ectomycorrhiza were not included. Despite the presumed role of fungi in decomposition processes, only studies actually measuring fungal activity were included. Further, studies based on fatty acid analysis (FAME, PLFA) were not included in AMF studies. For more details, see text.

Figure 2. Published studies on GM that have included the effect of other parameters than GM-trait on fungal community composition. List of studies which investigated each parameter and either found an effect (darker color) or did not detect an effect (lighter color). In the first row the studies detecting a significant effect of GM are marked with black, no effect with light gray and 'transient' effect with dark gray. Next rows are the effects of plant growth stage, field site and soil related parameters, season and climate and cultivar. For details on these categories, see text. Darker color marks that this factor was a significant explanatory factor in the study while lighter color marks that factor was studied but no effect was found. The totals are total number of studies looking at the factor (and studies in which an effect of the factor in question was detected). Some studies are featured many times in the table as they have looked at multiple aspects.

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776 **Table 1.** Studies on the effects of GM-crops on soil fungi, AMF and decomposer fungi. The primary crop species and modification studied, the method used, the
777 set-up of the experiment and the outcome are listed in the columns.

Organisms	Research	Crop	Modification	Method	Environment studied	Outcome
Fungi	**Donegan et al. 1996**	Potato	Bt	CFU	Field study	Minor effect of GM trait in phyllopshere
	Donegan et al. 1999	Alfalfa	Lignin peroxidase and alpha amylase	CFU	Field study	No effect on fungi
	Saxena & Stotzky 2001	Maize	Btk (Cry1Ab)	CFU	Greenhouse study	No effect on fungi
	Cowgill et al. 2002	Potato	Nematode resistance	FAME	Field study	Decrease in fungi in GM treatment
	Dunfield & Germida 2003	Canola	Glyphosate resistant	FAME	Field study	Transient effect
	Blackwood & Buyer 2004	Maize	Bt	PLFA	Greenhouse study	No effect on fungi
	Milling et al. 2004	Potato	granule bound starch synthase gene (gbss)	DGGE	Field study	No effect on fungi
	Xue et al. 2005	Maize and Potato	Bt and PVY (potato virus Y)	FAME	Field study	Decrease in fungi in GM treatment
	Gotz et al. 2006	Potato	T4 lyzozyme	Microscopic analyses and DGGE	Field study	Differences in relative abundances between treatments
	Wei et al. 2006	Papaya	mutant gene of papaya rinspot virus	CFU	Greenhouse study	Increase in fungi in the soils grown with GM-papaya
	Weaver et al. 2007	Soybean	Glyphosate resistant (RoundUP)	FAME	Field study	No effect
	Girlanda et al. 2008	Tomato	Expression of tobacco b-1,3-glutanase and chitinase	CFU and AMF colonization	Greenhouse study	No effect on AMF or fungi
	Icoz et al.2008	Corn	Bt	CFU	Field study	Transient effect
	Liu et al. 2008	Rice	Bt	TRFLP	Field study	No effect on fungi
	O'Callaghan et al. 2008	Potato	The antimicrobial peptide magainin II (against bacteria)	CFU	Field study	More fungi in roots of GM- potato
	Oliveira et al. 2008	Corn	Bt	CFU	Field study	Transient effect
	Hart et al. 2009	Corn	Glyphosate resistant (RoundUP)	qPCR - TRFLP	Field study	No effect on fungi
	Kremer & Means 2009	Soybean & corn	Glyphosate resistant	CFU of endophytes	Field study	Increase in Fusaria colonizing the GM-roots
	Weinert et al. 2009	Potato	carotenoid zeaxanthin accumulation	DGGE	Field study	No effect on fungi
	Wu et al. 2009	Rice	Bt	FAME	Greenhouse study	No effect on fungi
	Gschwendtner et al. 2010	Potato	granule bound starch synthase gene (gbss)	qPCR	Field and greenhouse studies	No effect on fungi
	Hannula et al. 2010	Potato	granule bound starch synthase gene (gbss)	TRFLP - enzymatic measurements, fungal biomass	Field study	No effect on AMF or fungi
	Tan et al. 2010	Corn	Bt	DGGE	Greenhouse study	No effect on fungi
	Gschwendtner et al. 2011	Potato	granule bound starch synthase gene (gbss)	FAME	Greenhouse study	No effect on fungi
	Lee et al. 2011	Rice	Fusion of trehalose-6-phosphate synthase	TRFLP and qPCR	Field study	No effect on fungi
	Li et al. 2011	Cotton	Bt	CFU- diversity	Field study	No effect on fungi
	Hannula et al. 2012a	Potato	granule bound starch synthase gene (gbss)	SIP- TRFLP	Greenhouse study	Transient effect on fungi and AMF
	Hannula et al. 2012b	Potato	granule bound starch synthase gene (gbss)	TRFLP, enzymatic measurements and fungal biomass	Field study	No effect on AMF or fungi
	Chun et al. 2012	Rice	Herbicide resistant	TRFLP	Field study	No effect on fungi
	Tilston et al. 2013	Tobacco	Phenylpropanoid metabolism	PLFA	Greenhouse study	Decrease in fungi in GM treatment
	Kuramae et al. 2013	Corn	Bt	454-Sequencing of DNA and RNA	Greenhouse study	No effect on fungi
AMF	**Kaldorf et al. 2001**	Aspen	Phytohormone balance	Colonization counts	Field study	No effect on AMF
	Turrini et al. 2004	Maize & Aubergine	Bt / antifungal	Colonization counts	Artificial greenhouse system (microcosm)	Transient effect
	Castaldini et al. 2005	Maize	Bt	Colonization counts	Artificial greenhouse system (microcosm)	Decreased colonization in one of the non-GM varieties
	de Vaufleury et al. 2007	Maize	Bt	Infectivity and colonization	Artificial greenhouse system (microcosm)	No effect on AMF
	Powell et al. 2007	Soybean	Herbiside restistant	Colonization counts	Greenhouse study	Transient effect
	Knox et al. 2008	Cotton	Bt and Gluphosate resistant	Colonization counts	Field study	No effect on AMF
	Hannula et al. 2010	Potato	granule bound starch synthase gene (gbss)	Colonization counts, TRFLP	Field study	No effect on AMF
	Cheeke et al. 2011	Maize	Bt	Colonization counts	Artificial greenhouse system (microcosm)	Transient effect
	Tan et al. 2011	Maize	Bt	Colonization counts and DGGE	Greenhouse study	Effect on the community structure but not on colonization
	Verbruggen et al. 2012	Maize	Bt	Microscopy, 454-seq RNA and DNA / T-RFLP	Greenhouse study	No effect
	Cheeke et al. 2012	Maize	Bt	Colonization counts	Greenhouse study	Decreased colonization in one of the GM varieties
	Wrobel-Kwiatkowska et al. 2012	Flax	Sturucturural change	Colonization counts	Greenhouse study	No effect on AMF
	Fließbach et al. 2012	Maize	Bt	Colonization counts	Greenhouse study	Transient effect
	Cheeke et al. 2013	Maize	Bt	Colonization counts and spore diversity estimate	Field study	No effect on AMF
Decomposer Fungi	**Donegan et al. 1995**	Cotton	Bt	CFU	Greenhouse study	Transient effect
	Wu et al. 2004	Rice	Bt	CFU	Greenhouse study	Transient effect
	Castaldini et al. 2005	Maize	Bt	Colonization counts	Greenhouse study	Transient effect
	Flores et al. 2005	Maize, canola, potatoes, cotton, rice, tobacco	Bt	CFU	Greenhouse study	No effect
	Henault et al. 2006	Tobacco	Lignin-synthesis	FAMEs	Greenhouse study	Twice as much fungal marker in the GM soil
	Naef & Defago 2006	Corn	Bt	Multiplex PCR - micro assay	Field study	Transient effect
	Seppänen et al. 2007	Birch	Lignin-synthesis	Ergosterol	Field study	No effect
	Lawhorn et al. 2009	Corn	Bt	Extracellular enzymes	Field study	No effect
	Powell et al. 2009	Soybean - maize	Glyphosate-tolerant	DFS and hyphael length	Greenhouse study	No effect
	Lu et al. 2010a	Rice	Bt	TRFLP	Field study	Transient effect
	Lu et al. 2010b	Rice	Bt	TRFLP	Field study	No effect
	Tan et al. 2010	Corn	Bt	DGGE	Greenhouse study	Transient effect
	Xue et al. 2011	Corn	Bt	TRFLP	Field study	Transient effect
	Hannula et al. 2013	Potato	granule bound starch synthase gene (gbss)	TRFLP, qPCR	Greenhouse study	Transient effect

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