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

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30 **Abstract**

31

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

48

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

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51

52 **Introduction**

53 Although conventional breeding has been successful in developing plants with desired traits, transgenic  
54 techniques have extended these possibilities by enabling the introduction of interesting genes from other  
55 organisms (Jones 2011). The introduction of new genes into crop-species may increase consistent food  
56 production for the growing world population as crop losses due to pests are reduced and optimal crop yields  
57 can be obtained. However, there are ethical concerns about the use of transgenic crops as have been  
58 discussed in many public forums and have spurred numerous discussions regarding their safety (Jones  
59 2011). Despite these concerns, the number of fields allocated to transgenic crop production has increased  
60 each year worldwide (James 2012). However there are strong differences between continents; while in  
61 Europe companies are pulling out of the market due to the negative public opinion towards genetically  
62 modified (GM)-crops and more strict EU-legislation, growing of GM crops, such as herbicide resistant  
63 soybean, is a common practice in USA. The main concerns regarding the use of GM crops in agro-  
64 ecosystems are related to the possibility of unintended transgene flow to indigenous plants, development of  
65 super weeds, and the effects of transgenic plants on non-target organisms, including soil microbial  
66 communities (Wolfenbarger and Phifer 2000).

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

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

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

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

88

### 89 **Fungal diversity and functioning in agro-ecosystems**

90 Before considering the potential effects of genetically modified plants on soil fungi, it is necessary to  
91 consider the general effects of agriculture on fungi. Besides the major effects that soil physical and  
92 chemical factors such as pH, moisture, soil texture, vegetation have on the composition and functioning of  
93 soil microbial communities, many studies have reported a reduction of fungal biomass under agriculture  
94 compared to more natural systems, and bacteria are thought to dominate in agricultural ecosystems  
95 (Kennedy 1999; Berg and Smalla 2009). This has been attributed both to the constant removal of crop  
96 plants, thereby reducing the input of litter (Berg and Smalla 2009) and to mechanical actions such as  
97 plowing which can potentially break extensive hyphal networks (Wang et al. 2010). Different agronomic  
98 practices such as tillage and fertilization also have profound impacts on the fungal communities (Oehl et al.  
99 2010). Studies have found that organic farming had a significant positive effect on soil AMF richness  
100 compared to conventional farming (Verbruggen et al. 2010) and that type of fertilizer applied significantly  
101 affects fungal biomass (Heinze et al. 2010). Contradicting the idea of agro-ecosystems being bacterial  
102 dominated, recent evidence gained with stable isotope methods show that fungi might be more important  
103 organisms in the rhizosphere of crop-species than earlier thought (Gschwendtner et al. 2011; Hannula et al.  
104 2012a). Further evidence suggests that the importance of fungi might be larger later in the season when the  
105 plant is more mature and fungi have had more time to establish their hyphal networks (Hannula et al. 2010)  
106 while bacteria are more abundant in the rhizosphere of early stages of plant growth and in the bulk soils  
107 (Inceoglu et al. 2010).

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

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

123

#### 124 **Mechanisms by which GM-plants can affect soil fungi**

125 GM crops can influence soil ecosystems positively, negatively or neutrally, (Oger et al. 1997). Birch et al.  
126 (2007) pointed out that the potential impacts of GM crops on soil ecosystem can be (1) direct (e.g. toxicity  
127 of an expressed new protein on key non-target species), (2) indirect (e.g. effects via trophic interactions),  
128 (3) caused by unintended changes in the metabolism of the plant and thus altering rhizodeposition and/or  
129 (4) caused by changes in the management regime used to cultivate GM crops. The effects of GM-trait can  
130 potentially be on the fungal biomass, community function or community composition. Harmful effects on  
131 fungal community function may cause a decrease in fertility and nutrient cycling in the soil which  
132 subsequently affects the following crops (de Vries et al. 2011), and changes in biomass and community  
133 structure via elimination of beneficial fungi such as AMF may affect plant growth as well and may result  
134 in the increased sensitivity of the plant to pathogens (van der Heijden et al. 2008). GM-caused shifts in

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

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

139

140

#### 141 *Root exudation and soil fungal communities*

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

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

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

176         Despite the importance of arbuscular mycorrhizal fungi (AMF) in plant-soil systems, only few  
177 studies evaluated the non-target effects of GM-crops on AMF colonization and community structure. (Liu  
178 2010) (Fig 1). As plants vary naturally in their AMF-hosting ability, the GM trait in plants might, in some  
179 cases, alter their relationship with AMF. Because AMF are obligate symbionts and thus require the plant  
180 host for nutrition and reproduction, they may be more sensitive to changes in the physiology of the host  
181 plant than free-living soil fungi (Liu 2010; Cheeke et al. 2011). Earlier, it was shown that AMF are  
182 sensitive to different agronomic practices such as tillage and fertilization (Oehl et al. 2010). AMF are  
183 thought to be especially important in low input agro-ecosystems and are, therefore, an important  
184 component of sustainable agriculture (Verbruggen and Kiers 2010) and it is therefore crucial to understand  
185 the impacts of GM-traits on functioning and diversity of AMF. In two studies a reduction in arbuscular  
186 mycorrhizal colonization of the roots of a Bt-cultivar of maize was reported (Castaldini et al. 2005; Cheeke  
187 et al. 2012). Another study found no effect on colonization of the Bt-maize roots but an effect on the AM-  
188 fungal community structure assessed by DGGE (Tan et al. 2011). Transient effects of GM-crops on soil  
189 AMF community structure or AMF colonization of roots have been reported in 4 other studies on Bt-maize,  
190 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

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

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

238

### 239 **Normal variation versus GM-induced variation**

240 A common issue in the debate and a possible explanation for the lack of coherence in the results obtained in  
241 the previous studies on possible, harmful, side-effects of GM-crops is the difficulty to discern the effects of  
242 the modification from all the other abiotic and biotic factors (Fig.2). Usually 'normal variation' is defined  
243 as the variation in the responses of the fungal/microbial community to the non-GM crops under the  
244 prevailing conditions of the common agricultural practices (Kowalchuk et al. 2003). Factors such as  
245 weather, agricultural management and plant developmental stage can affect the outcome of the experiments  
246 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

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

315

#### 316 **New methods and new possibilities in GM-research**

317 Traditionally most of the studies on effects of GM-crops on fungi have used cultivation based methods and  
318 root colonization counts of AMF (Table 1) to assess the effects of GM crops on the size and the diversity of  
319 the fungal community. However, the inherent limitations of the studying of culturable microbes have also  
320 here prevented a full scale assessment of the effects of GM crops on fungal diversity and functioning. In  
321 few cases DNA based fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE)  
322 (Milling et al. 2004; Götz et al. 2006) or terminal restriction length polymorphism (T-RFLP) (Hart et al.  
323 2009) were applied. However, in order to answer fundamental questions still open in this field such as on  
324 the totality of the effects of GM-crops on soil fungal diversity and in particular the rare and non-culturable  
325 fractions of the community as well as the functioning of the community through the impact of changes in  
326 rhizodeposition patterns, new methodology is ready to be adopted. In this section we will discuss  
327 methodologies that could help answering these key questions.

328

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

330 Many studies have reported on the differences in the community composition of rhizosphere fungi between  
331 cultivars and have hypothesized that this would have been the result of changes in rhizodepositions.  
332 However, only few studies have actually measured rhizodepositions or monitored carbon flow from the plant  
333 into the rhizosphere, the subsequent utilization of the rhizodepositions by the microbial community, and how  
334 this influence both the structure and the functioning of the rhizosphere community . An elegant approach to  
335 do such measurements is stable isotope probing (SIP,) where the whole plant is (pulse) labeled with  $^{13}\text{C}\text{O}_2$   
336 and the incorporation of  $^{13}\text{C}$  in microbes is followed in the endosphere and rhizosphere. Alternatively, plant  
337 residues containing  $^{13}\text{C}$  can be used in a decomposition study to monitor the soil fungi involved in break-  
338 down of the (GM)-plant material. The measurement of the isotope in the DNA/RNA or fatty acids  
339 (PLFAs) extracted from soil allows for the detection and identification of the microbes actively involved in  
340 the assimilation of the labeled compound i.e.  $^{13}\text{C}$  from the plant roots or dead plant material (Radajewski et  
341 al. 2000). SIP can be used to follow the fate of carbon in any system and has been used to study for  
342 example effects of soil management (Rangel-Castro et al. 2005; Lu et al. 2007) and climate change (Drigo  
343 et al. 2010) on soil microbial community structure and is proven to be a robust technique which can provide  
344 a quantitative insight in the rhizodeposit metabolizing community. In combination with phospholipid fatty  
345 acid (PLFA-SIP) analyses it has been used to evaluate the effects of GM-plants on carbon partitioning to  
346 different groups of soil organisms (Wu et al. 2009; Gschwendtner et al. 2011; Hannula et al. 2012a). All of  
347 these studies have shown the great importance of both saprotrophic fungi and especially AMF in the  
348 rhizosphere assimilating the ( $^{13}\text{C}$ ) from the plant. The first two studies did not find significant differences  
349 between the GM-and its parental cultivar, although Wu et al. (2009) found significant differences between  
350 the Bt and parental rice in the amount of  $^{13}\text{C}$  distribution at the seedling, booting and heading stages. The  
351 last study (Hannula et al. 2012a) found that a starch modified GM-potato line affected soil fungal  
352 communities slightly differently than its parental isolate did, but these effects were deemed transient. A  
353 study done with DNA-SIP revealed cultivar dependent distinctions in  $^{13}\text{C}$ -label flow to endophytic bacteria  
354 of potato (Rasche et al. 2009). However, in these studies the baseline of environmental variation was not  
355 investigated and thus it is not clear whether these differences are ecologically relevant. SIP methodology  
356 will, nevertheless, offer a great opportunity to study the effects of GM-varieties on active members of  
357 rhizosphere communities.

358

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

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

385

386 *Final remarks*

387 One issue, that has been addressed only marginally in GM crop studies, but in our opinion highly relevant  
388 to evaluate the real risk associated with the use of GM crops, is the effects of GM crops after harvest and/or  
389 in the following growing season(s). Few examples of such studies are a study by Castaldini *et al.* (2005),  
390 who reported a lower AMF colonization of *Medicago sativa* (alfalfa) roots grown in pots that had  
391 previously been cultivated with Bt maize and contained extra Bt plant residues. Contradictory, Cheeke *et al.*  
392 2012 did not find any residual effect of Bt maize cultivation on AMF colonization of subsequently  
393 planted *Glycine max* (soybean). Besides, other studies in field and greenhouse conditions could not detect  
394 an effect of GM-crops on soil fungi after their harvest and/or in the following growing season (Oliveira *et al.*  
395 2008; Powell *et al.* 2009; Cheeke *et al.* 2012; Hannula *et al.* 2012b). We strongly argue that such  
396 measurements should be considered in future risk assessment studies on the use of GM crops especially in  
397 cases when GM-crops are grown consecutively in the same soils for years as continuous growing of GM-  
398 crops can cause additive effect may strengthen the (negative) effects of the transgene.

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

408

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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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**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 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 phyllosphere		
	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 (gbs)	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 lysozyme	Microscopic analyses and DGGE	Field study	Differences in relative abundances between treatments		
	Wei et al. 2006	Papaya	mutant gene of papaya rinpot 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 ̢-1,3-glucanase 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 (gbs)	qPCR	Field and greenhouse studies	No effect on fungi		
	Hannula et al. 2010	Potato	granule bound starch synthase gene (gbs)	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 (gbs)	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 (gbs)	SIF- TRFLP	Greenhouse study	Transient effect on fungi and AMF		
	Hannula et al. 2012b	Potato	granule bound starch synthase gene (gbs)	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	Maize / 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 Vaulleury et al. 2007	Maize	Bt	Infectivity and colonization	Artificial greenhouse system (microcosm)	No effect on AMF	
		Powell et al. 2007	Soybean	Herbicide resistant	Colonization counts	Greenhouse study	Transient effect	
		Knox et al. 2008	Cotton	Bt and Glyphosate resistant	Colonization counts	Field study	No effect on AMF	
		Hannula et al. 2010	Potato	granule bound starch synthase gene (gbs)	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	Structural change	Colonization counts	Greenhouse study	No effect on AMF	
		Hiebbach 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	
Seppinen 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 hyphal 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 (gbs)	TRFLP, qPCR	Greenhouse study	Transient effect		