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Testing Potential Effects of Maize Expressing the Bacillus thuringiensis Cry1Ab Endotoxin (Bt Maize) on Mycorrhizal Fungal Communities via DNA- and RNA-Based Pyrosequencing and Molecular Fingerprinting

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1 **Testing potential effects of Bt maize on mycorrhizal fungal communities via DNA- and**
2 **RNA- based pyrosequencing and molecular fingerprinting**
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23 **Abstract**

24 The cultivation of Genetically Modified (GM) crops has increased significantly over the last decades.
25 However, concerns have been raised that some GM traits may negatively affect beneficial soil biota,
26 such as arbuscular mycorrhizal (AM) fungi, potentially leading to alterations in soil functioning. Here,
27 we test two Bt-maize varieties for their effects on soil AM fungal communities. We target both
28 fungal DNA and RNA, which is new for AM fungi, and we use two strategies as an inclusive and
29 robust way of detecting community differences: 1) 454 pyrosequencing using general fungal rRNA
30 gene-directed primers and 2) T-RFLP profiling using AM fungal specific markers. Potential GM-
31 induced effects were compared to the normal natural variation of AM fungal communities across 15
32 different agricultural fields.
33
34 AM fungi were found to be abundant in the experiment, accounting for 8% and 21% of total
35 recovered DNA- and RNA-derived fungal sequences, respectively, after 104 days of plant growth.
36 RNA- and DNA-based sequence analyses yielded most of the same AM fungal lineages. Our research
37 yielded three major conclusions: First, no consistent differences were detected between AM fungal
38 communities associated with GM versus non-GM plants. Second, temporal variation in AMF
39 community composition (between two measured time points) was bigger than GM-trait induced
40 variation. Third, natural variation of AMF communities across 15 agricultural fields in the
41 Netherlands, as well as within-field temporal variation, was much higher compared to GM-induced
42 variation. In conclusion, we found no indication that Bt-maize cultivation poses a risk for AMF.
43
44

45 Key words: fungal RNA-DNA, risk assessment, beneficial soil fungi, GMO, arbuscular mycorrhizal
46 fungi, natural variation, Mon810, DKC3421YG.

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47 **Introduction**

48 In recent decades, significant advances have been made in developing more productive crop
49 varieties. One of these developments has been the generation of Genetically Modified (GM) crops,
50 which has been advocated as a tool to help alleviate some of the major constraints on crop
51 production, such as pest infestations and weed growth (22). However, because genetic modification
52 alters the functioning of crop plants, there is a risk that these changes may have adverse effects on
53 the plant's biotic environment (5, 19), implying that the potential effects of such modifications
54 should be examined (4). This is particularly relevant given the forecasted increases in GM cultivation
55 worldwide (7).
56
57 One important component of a plant's environment is the soil ecosystem, which plays an essential
58 role in nutrient cycling and plant productivity (54). Within this system, arbuscular mycorrhizal (AM)
59 fungi intricately interact with plants; they engage in a symbiosis with over 80% of land plants,
60 including major crops such as maize, wheat, potato and soybean (62), and provide mineral nutrients
61 in exchange for plant carbohydrates (45). Because of these ecological features, they have been
62 identified as an important group of organisms to study for risk assessments of GM crops (25, 31).
63
64 Some studies have shown altered AM fungal development, such as spore production and root
65 colonization, in association with GM versus non-GM plants (52, 60); among which Bt maize; 8,9),
66 suggesting that there is a potential for adverse GM plant-induced effects on AM fungi. However,
67 because these studies involved testing of only a single AM fungal species under otherwise nearly
68 sterile conditions, we do not know whether these results hold for more natural conditions. In nature,
69 soil and plant roots are inhabited by more diverse fungal communities whose members may respond
70 differently to GM plants. In general, studies where roots of GM plants (Bt or other traits) were
71 subjected to a mixture of AM fungal species, either in microcosms or in the field, no effects of GM
72 plants on AM fungal colonisation were reported (12, 24, 28, 38, 49).

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73

74 In addition to root colonization levels, it is important to investigate whether the community
75 composition of AM fungi is altered by the cropping of GM plants. Several studies have shown that
76 plant productivity depends on the identity and diversity of AM fungal species colonizing a plant (33,
77 61). Hence, if GM plants influence AM fungal communities, this can subsequently influence plant
78 productivity. There are only few studies (Hannula *et al.* (21) on GM potato and Tan *et al.* (49) on Bt-
79 maize), reporting whether GM cropping influences AM fungal communities in natural soils.
80 Moreover, no studies to date have targeted RNA to detect changes in AM fungal communities in
81 relation to Bt cropping. Targeting RNA, in addition to DNA, is thought to provide a more
82 comprehensive picture of community dynamics, as RNA has a faster turnover rate and might
83 therefore better capture changes in active fungal communities (3, 23), as compared to DNA-based
84 assays, which may also recover DNA from dead or inactive AM fungal cells or spores (e.g. ref. 29).

85

86 Here, we tested and compared the response of AM fungal communities to two Bt and two non-Bt
87 maize varieties at two different time-points by examining partial 18S rRNA sequences recovered
88 from DNA and RNA, separately. The GM-trait we studied was insect resistance, which was mediated
89 by expression of an insecticidal protein (Bt) that can be present in Bt-maize root exudates (43). This
90 protein does not appear to directly affect AM fungi (15), but some Bt-plants have nevertheless been
91 reported to exhibit an altered interaction with AM fungal symbionts (e.g. ref. 9). The maize plants
92 were grown in a greenhouse in intact soil cores collected from the field to target natural AM fungal
93 communities and to ensure that our results have high ecological relevance.

94

95 We utilized general fungal primers in combination with deep-sequencing technology to minimize
96 bias towards specific AM fungal lineages (17, 26) and to get an estimate of AM fungal abundance
97 compared to other fungi. This approach provided the necessary phylogenetic resolution and
98 sufficient depth of sampling to potentially detect significant community changes. For additional

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99 confirmation of observed trends, and to estimate whether potential Bt-induced effects were
100 comparable to the normal natural variation of AM fungal communities, we also assessed
101 communities by a lower-resolution molecular profiling technique (terminal restriction length
102 polymorphism - T-RFLP) targeting the nuclear 25S rRNA gene with AM fungi-specific primers. In
103 earlier work we used this technique to explore AMF community composition and diversity in a wide
104 range of agricultural fields distributed throughout the Netherlands (58, 59). This enabled us to
105 compare differences between communities in our greenhouse experiment to community variation
106 of AM fungi found in the field. Hence, these comparisons allowed us to assess whether GM crops
107 induce changes that exceed normal temporal community variation in the field, and between fields,
108 and thus whether they represent a potential risk for soil ecological functioning.
109
110 Our aim was to answer the following questions: 1) what are the effects of two GM maize varieties on
111 (active) AM fungal communities? 2) Do these effects exceed normal seasonal and spatial variation of
112 AM fungal communities in the field? And 3) how well do DNA-based and RNA-based measures of
113 communities agree with respect to community trends?
114

115 Materials and Methods

116 Pot experiment and sampling

117 In order to compare AM fungal communities between GM and non-GM plants, seeds were sown into
118 pots that contained soil from a field in which we previously characterized the AM fungal community
119 for multiple years (organic field "Biezenmortel" in ref. 58). Because AM fungal communities have
120 previously been shown to be sensitive to experimental manipulation (47, 57), we collected intact
121 pot-size soil cores from the field and transferred to pots in order to maintain natural stratification
122 and mycelial integrity of AM fungi. Soil cores were collected randomly from within a homogeneous
123 10 x 10 m plot in September 2009. The crop at that time was a grass-clover mixture (*Trifolium*
124 *pratense* L. and *Lolium perenne* L.), which had been sown after maize in fall 2007 and mown twice a

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125 year. Soil chemical properties were as follows: pH ($CaCl_2$-extractable) = 5.8, P ($CaCl_2$-extractable) =
126 5.1 mg kg^{-1}, N = 1.36 g kg^{-1}, OM = 1%.
127
128 In each pot (containing approximately 6 kg of soil, diameter = 20 cm, height = 18 cm), one of four
129 different maize (*Zea Mays* L.) cultivars were grown. These included two GM cultivars; 1) event
130 MON810, cultivar Monumental MON810 and 2) event MON810, cultivar DKC 3421YG. These were
131 matched with two non-GM cultivars (Monumental and DKC3420, respectively). The GM cultivars had
132 both been transformed to express the *Cry1Ab* gene (an insecticidal endotoxin produced by *Bacillus*
133 *thuringiensis* that is active against, among others, the European corn borer *Ostrinia nubilalis* (e.g. ref.
134 34). The non-Bt varieties are non-transformed maize lines with similar background genetics as
135 compared to the Bt counterparts, but do not contain the *cry1Ab* expression cassette. Similar
136 background genetics between Bt and non-Bt varieties is ensured by traditional back-crossing
137 breeding processes. Different cultivars are abbreviated by letters, Monumental = M, DKC = K, and
138 GM cultivars are indicated with "GM" (i.e. M - GM, and K – GM).
139
140 We used three replicates for each cultivar, resulting in 12 pots. Two seeds were sown into each pot
141 on October 1st 2009, which were kept in a greenhouse with a 16/8 hours light/dark cycle. After two
142 weeks, pots were thinned to one seedling each. In pots where no seedlings had emerged after one
143 week (2 pots), new seeds were sown and thinned in the same way after an additional two weeks.
144 Hoagland solution (½ strength P; 250 ml per pot) was applied twice during the first month of plant
145 growth.
146
147 On November 24th, after 47 days of plant growth, soil samples were taken using the following
148 protocol: one core (diameter 1 cm) per pot was taken and the part originating from 5-11 cm depth
149 level was immediately stored on dry ice and transferred to -80°C. Cores were taken 5-6 cm from the
150 edge, which was approximately halfway between the edge of the pot and the stem of the plant. On

151 January 20th, after 104 days of growth, samples were taken as above, but the position of cores was
152 shifted 45° in relationship to the first core to minimize potential disturbing effect of the first
153 sampling event. At the end of the experiment (plant age 130 days; at full maturity of the ears), total
154 above and below ground plant biomass was harvested, dried (7 days at 50°C), and total plant dry-
155 weight and ear (grain plus cob) dry-weight was determined. Of a random subset of roots in each pot,
156 percentage root colonization by AM fungi was determined according to the magnified intersection
157 method (34), based on 50 root intersections per plant.
158
159 **Nucleic acid extraction and cDNA preparation**
160 From each sample, 2 g of soil was used for simultaneous RNA and DNA isolation using the RNA
161 PowerSoil kit and the DNA Elution accessory kit (MO BIO Laboratories inc., Carlsbad, CA, USA). The
162 DNA from total RNA-enriched samples was removed by DNase I (RNase - free DNase set 79254;
163 Qiagen, Hilden, Germany) according to manufacturer recommendations. The total RNA was
164 measured with a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and the
165 quality of the total RNA was checked with Experion (Bio-Rad Laboratories inc., Hercules, CA, USA).
166 The total RNA was cDNA synthesized using random hexamer primers and the superscript double
167 stranded cDNA synthesis kit (Life Technologies Corp., Carlsbad, CA, USA). Resulting DNAs and cDNAs
168 were used as template in parallel analysis using 454 pyrosequencing and T-RFLP as described below.
169
170 **454 pyrosequencing methodology**
171 For 454 pyrosequencing, general fungal primers FR1 and FF390 (53) were used, which amplify a
172 region approximately 350 basepairs in length that includes the V7 and V8 hypervariable region of the
173 SSU rRNA gene. In a recent analysis this primer pair was most suitable for soil fungi based on
174 specificity, coverage and amplicon length (39). We made one small modification to primer FF390
175 (and now refer to it as FF390.1), where at the 5' terminus the third position is now degenerate (see
176 below) to accommodate detection of some "*Glomeraceae*" members as inferred from bioinformatic

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177 analysis of Genbank sequences. Thermocycling conditions were as follows: denaturing at 94°C for
178 30s (after initial denaturation of 4 min. initial annealing temperature was 55°C (1 min.), and every
179 two cycles the annealing temperature was lowered with 2°C until 47°C was reached, which was the
180 annealing temperature used for an additional twenty cycles (thus 29 PCR cycles in total). Extension
181 conditions were 68°C for 2 min. for all cycles. Reactions contained about 25 ng of DNA or RNA
182 template added to a standard PCR mix. The 5' terminus of primers contained an adaptor sequence
183 and a multiplex identifier tag (MID; 12 different 10 bp-long tags), which resulted in the following
184 primer constructs (adaptor in boldface):
185 Forward (FF390.1) 5'- **CTATGCGCCTTGCCAGCCCGCTCAG** - (MID) - CGWTAACGAACGAGACCT - 3'
186 Reverse (FR1) 5' - **CGTATCGCCTCCCTCGCGCCATCAG** - (MID) - AICCATTCAATCGGTAIT - 3'
187
188 Pyrosequencing was performed by Macrogen inc. (Seoul, Korea) using a GS FLX titanium (Roche,
189 Basel, Switzerland). Sequence analysis was done using Qiime 1.2.1 scripts (6) incorporated into the
190 Galaxy interface (18). All reads were checked for containing the right forward and reverse MID-tags
191 and assigned to samples accordingly. Four samples out of 24 were excluded at this stage because
192 they contained the wrong tag – combinations. As a result, "M - GM" and "K" treatments (where "M"
193 and "K" indicate parental cultivars, and "GM" indicate the GM-trait) at first sampling and "M" and
194 "K" treatments at second sampling are represented by two, instead of three replicates (see also
195 Table 1 for an overview of replicate number per treatment). Of all other samples, barcodes and tags
196 were removed and sequences were denoised using Denoiser 0.91 (42) and clustered at 97%
197 similarity using the UCLUST 1.2.21 algorithm (14). The resulting "operational taxonomic units" (OTU),
198 represented by the most abundant sequence within each OTU, were assigned to eukaryote families
199 through BLAST searches against the QIIME-compatible version of the Silva 104 release (41). After this
200 we sorted this dataset into "non-fungal", "fungal" and "putative AMF", of which the latter two sets
201 were used for subsequent analysis.
202

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203 **Analysis of putative Glomeromycota sequences**

204 Assignment to eukaryote families yielded 185 OTUs having highest BLAST hits with families within
205 the phylum of Glomeromycota (clustered at 97% similarity and after excluding singletons according
206 to recommendations by Tedersoo *et al.* (50)). These were additionally blasted against the NCBI
207 database for confirmation, and were aligned with all Glomeromycota sequences (excluding those
208 classified as "environmental") within the SILVA database and, as outgroup, the protist
209 *Corallochytrium limacisporum* (acc. nr: ENAL42528; which has been proposed to be an appropriate
210 outgroup for fungal phylogenetic analysis; (56)). These analyses gave low confidence of membership
211 of Glomeromycota for the majority (94%) of OTU's for the following reasons: 1) inclusion within the
212 Glomeromycota had low bootstrap support and many distinct clades were formed containing only
213 sequences from this study, and thus separated from all database sequences of "known" AM fungi.
214 And 2), similarity of many of the obtained sequences to known AM fungal sequences was low, which
215 averaged 93.9%, and often did not strongly exceed similarity to the highest non-AM fungal BLAST
216 hits in the NCBI database (average 92.4%; see table S1 for all putative AM fungal OTU's and BLAST
217 scores). Therefore, as an operational definition it was decided to consider OTU's to be conclusive
218 members of the phylum of Glomeromycota only if similarity to known members exceeded 97%.
219 Alignment, Neighbour Joining (NJ) tree construction and subsequent bootstrapping analysis (1000
220 replicates) were performed using the program MEGA 4.1 (48). Nucleotide sequences were deposited
221 in GenBank under the Accession No. HE970441-HE970631.

222

223 **AM fungal community analysis by T-RFLP**

224 AM fungal communities were also analyzed by T-RFLP. This was done to obtain additional
225 information about the effects of Bt maize on AM fungal communities using a different molecular
226 profiling technique. Moreover, the use of T-RFLP made it also possible to compare potential "GM
227 induced" effects to the normal natural variation of AM fungal communities across 15 different
228 agricultural fields, which we determined in earlier work (see Verbruggen *et al.* (58)). DNA and RNA

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229 derived from each of the pot soil samples at each of time points were analyzed by T-RFLP after a
230 nested PCR with primers LR1-FLR2 and FLR3-FLR4 (50, 54) with a standard PCR mix, respectively, and
231 subsequent restriction digestion with *Taq*I. Cycling consisted of denaturing at 95 °C for 5 minutes, 30
232 cycles of 15 s at 95°C, 30s at 58°C, and 60s at 72°C, with a final extension of 10 min. The product of
233 the first PCR was diluted 500 x prior to second PCR. Reactions and electrophoresis conditions were
234 as in (58)). The resulting electropherograms were analyzed using the program T-Rex (12) with a
235 clustering threshold of 2.5 and exclusion of T-RFs less than 45 bp in length, or contributing less that
236 0.5% of peak area per sample. As a reference for "normal" community variation, we analyzed
237 samples from the same field from which soil was taken for the pot experiment (a maize field on
238 sandy soil). These samples were taken in July '07, September '07, July '08, September '08 and at time
239 of the collection of experimental soil in September '09. Freeze-dried roots (±100 mg) of six plants
240 from each sampling date were homogenized and subjected to DNA isolation using the DNeasy Plant
241 Mini Kit (Qiagen, Hilden, Germany). T-RFLP was performed using the same methods as the pot
242 experiment to assess the AM fungal community present at each sampling time. Results were
243 compared with AM fungal communities from 15 maize fields on sandy soil from earlier published
244 work (58). However, because not from all samples soil DNA was available, we analyzed communities
245 found in plant roots for these samples. Even though these might differ from those in soil, this
246 procedure should still allow distinguishing main trends, especially since we previously found low
247 systematic variation between root and soil AM fungal communities (59).
248
249 **Data analysis and statistics**
250 T-RFLP and pyrosequencing derived community data were analyzed in a complementary manner. T-
251 RFLP was specifically used to compare AM fungal communities among pot treatments and to field
252 communities. Pyrosequencing was used to compare communities between pot treatments, increase
253 taxonomic information, and to get an estimate of AM fungal abundances as related to other fungi.
254 DNA and RNA derived communities were compared between GM and non-GM plants (factor GM),

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255 their parental cultivars (factor cultivar), and the interaction between these two factors in a crossed
256 analysis using two-way NPMANOVA (permutation with 10,000 replicates) with Bray-Curtis similarity
257 indices. The Bray-Curtis similarity index is a commonly used similarity estimate in ecology (10) and
258 previously used for comparing AM fungal communities (44). We also estimated AM fungal richness
259 through a two-way ANOVA with the same factors. Here we used the average of forward and reverse
260 T-RF's for T-RFLP (as each unique sequence produces both a forward and a reverse peak), and by
261 individual rarefaction analysis for pyrosequencing based on 100 reads (as for the majority of samples
262 more than 100 reads were obtained), to account for variation in read numbers among samples. To
263 assess whether the communities derived by analysis of DNA and RNA were correlated, we compared
264 Bray-Curtis similarities between RNA and DNA derived from the same sample versus DNA derived
265 communities from all other samples with a non-parametric Mann-Whitney U test. Non-metric
266 multidimensional scaling; NMDS (27) was performed to assess similarity of communities of pots at
267 different plant stages for pyrosequencing and T-RFLP derived community data. We analyzed the
268 correlation between these two methods through a Mantel test of Bray-Curtis similarities
269 (permutation with 10,000 replicates). These analyses were performed in the program PAST (20).
270
271 Usage of general fungal primers for pyrosequencing also enabled us to compare number of
272 sequencing reads of AM fungi relative to those of all fungi, and thus give an indication of AM fungal
273 relative abundance, and its development through time. Relative abundance of each OTU at each
274 time-point, for DNA as well as RNA, was tested for significant differences between GM and non-GM
275 plant – associated communities using a non-parametric Kruskal-Wallis test. Relative abundance of
276 AM fungal reads to total fungi (pyrosequencing), AM fungal richness (T-RFLP and pyrosequencing),
277 percentage root colonization, total plant dry weight, and ear dry weight were assessed using a one-
278 way ANOVA comparing GM versus non-GM plants. These analyses were performed using SPSS
279 version 17.0.
280

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281 **Results**

282 **Plant colonization and growth**

283 Roots of all maize plants were colonized by AM fungi, with percentage root length colonization
284 varying from 12% to 64% (not shown). This variation was, however, not attributable to the GM trait,
285 as colonization percentages did not differ significantly between plant varieties ($F_{3,8}$ = 2.40; P = 0.14).
286 Total plant dry-weight (average: 74.9 g ± 2.3 SE) and ear dry weight (average: 25.5 g ± 3.9 SE) also
287 did not differ between varieties (total dry weight: $F_{3,8}$ = 0.22, P = 0.88, N =12; ear dry weight: $F_{3,8}$ =
288 0.17, P = 0.91, N =12). Ear dry weight was significantly positively correlated with AM fungal root
289 colonization (R^2 = 0.46, P = 0.016, N = 12), but total dry weight was not (R^2 = 0.07; P = 0.40, N = 12).
290

291 **AM fungal taxa assessed by pyrosequencing**

292 The pyrosequencing approach yielded 222,401 reads after denoising with a mean length of 351 bp
293 (SD = 7.7), and a minimum length of 284 bp. Of these, 155,206 reads belonged to OTU´s assigned to
294 fungal families, and 20,465 (~ 9.2% of total, or 13.2% of fungi) were assigned to AM fungal families
295 (excluding eight singleton OTU's). However, as the majority of these could not be confidently
296 assigned to the phylum of Glomeromycota (see Materials and Methods) we have defined an OTU as
297 belonging to AM fungi on the basis of having at least 97% similarity to sequences classified as AM
298 fungi in the NCBI database. This conservative approach yielded 11 OTUs that could be unequivocally
299 assigned to the Glomeromycota and showed good phylogenetic support of topology of the
300 containing clades (Figure 1). These OTUs represented a considerable portion of the putative AM
301 fungal reads: 15,518 (75.8%) out of the 20,465 putative AM fungal sequencing reads. They represent
302 most of the major AM fungal lineages, including *Glomus* group A (family *Glomeraceae*; six OTU's,
303 comprising 58% of reads), *Glomus* group B (or *Claroideoglomus*; one OTU/15%), *Paraglomus* (one
304 OTU/15%), *Diversispora* (one OTU/11%), *Scutellospora* (one OTU/0.2%) and *Archaeospora* (one
305 OTU/0.05%). The 11 AM fungal OTU's include all of the abundant putative AM fungal taxa, as the

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306 most abundant putative AM fungus not included in these is only responsible for 3% of all putative
307 AM fungal reads.
308
309 **AM fungal communities associated with GM and non-GM plants are not different**
310 Comparing the number of AM fungal reads to total fungal reads revealed a high presence of AM
311 fungi, which increased from an average of 11% to 21% for RNA over the course of the experiment
312 (Figure 2a). AM fungi were represented at higher levels in RNA compared to DNA, where they were
313 very low at first sampling (Figure 2a). There were no differences in AM fungal communities between
314 GM and non-GM plants at either growth stage, as assessed by pyrosequencing (Table 2). Also, when
315 analyzed separately, none of the AM fungal taxa occurred at significantly different relative
316 abundances in GM or non-GM plant – associated soils (Figure 2b). However, communities did differ
317 between the two plant growth stages (Table 2, see also Figure 2b), and for RNA derived communities
318 there was a significant "cultivar" effect at late growth stage (Table 2). Non-metric multidimensional
319 scaling of Bray-Curtis similarities of pyrosequencing data (Figure 3a) indicated that AM fungal
320 communities changed during the course of plant growth in a fairly similar direction for all
321 treatments. In Table 1, estimated AM fungal taxa richness is presented for each plant variety. Also
322 here, there was no significant effect of the GM trait, based upon RNA-derived sequences at the late
323 plant stage. For RNA at early growth stage and for DNA-derived sequences, these statistics were not
324 performed because less than 100 AM fungal reads were obtained for some samples.
325
326 **T-RFLP also does not detect differences between GM and non-GM plants**
327 A total of 34 unique T-RF signals were found in the experimental pots, 17 of which contributed more
328 than 1% to the total signal. This translates into an estimated total of 17 different "taxa" present
329 across treatments (see Materials and Methods). There were no significant differences in AM fungal
330 richness between GM and non-GM plants at either growth stage using either DNA or RNA (Table 1).
331 Overall, richness estimates derived from DNA- and RNA-based community fingerprints were similar,

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332 and tended to increase slightly with plant age. Using this method, there were also no significant
333 differences found in community composition between GM and non-GM plant associated AM fungal
334 communities (Table 2). As with the pyrosequencing analysis, there was a significant effect of time on
335 AM fungal communities assessed by RNA-based analysis, as well as "cultivar" (referring to parental
336 variety, but not GM vs. non-GM; see above) at second sampling for RNA (Table 2). Here, time was
337 also found to have a significant effect for DNA-based analysis, as well as "cultivar" for AM DNA-
338 based analysis at first sampling and "GM vs non-GM" for RNA at first sampling. This was caused by
339 two replicates of the "K" variety, which were very different at the first sampling. Because these were
340 the two pots where initially no seedling emerged, and communities converged afterward, this result
341 may not be a true cultivar effect. An NMDS plot including these two samples is presented in Figure
342 S1, but these are excluded in Figure 3b and 3c to show overall community trends. These two
343 replicates did not strongly contribute to the measured "time" effect on RNA-based analysis (Table 1),
344 because when these were excluded there was still a significant difference between communities
345 based on sampling time (not shown).
346
347 **High similarity between present and active AM fungal communities**
348 T-RFLP community profiles derived from DNA and RNA were significantly related (Bray-Curtis
349 similarity of RNA communities and DNA communities in the same sample versus DNA communities
350 in other samples are 0.72 versus 0.47, respectively, $Z = -5.72$; $P < 0.0001$), indicating that the AM
351 fungal community found in a sample through RNA-targeted analysis was relatively similar to DNA-
352 targeted analysis of the same sample. For the pyrosequencing data, the relationship was less strong,
353 but also significant (0.56 versus 0.43, $Z = -3.14$ $P < 0.001$). However, when only communities at the
354 late plant stage were analyzed, thus excluding the effect of the low number of DNA reads at
355 sampling one (Figure 2a; Table 1), similarity between communities represented by DNA and RNA as
356 recovered by pyrosequencing increased (0.69 versus 0.53; $Z = -3.30$; $P < 0.001$). Also, community
357 trends as derived from T-RFLP and pyrosequencing were significantly correlated ($R = 0.21$; $P = 0.009$),

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358 indicating that they partially detect similar AM fungal community compositional variation. For a
359 more detailed account of community trends using T-RFLP and pyrosequencing see Notes S1.
360
361 **Discussion**
362 In this study, we compared AM fungal communities associated with two GM and two non-GM maize
363 varieties using complementary approaches. Our analysis of RNA and DNA aimed to provide a
364 sensitive and comprehensive assessment of community composition. DNA is much more stable than
365 RNA, and thus, it has been argued to have a strong "historical component" (2). In contrast, owing to
366 its fast degradation, RNA is potentially more suitable for analysis of active communities at a given
367 time-point (40). Moreover, we analysed DNA and RNA by pyrosequencing with general fungal
368 primers and by T-RFLP using AM fungal specific primers. Using this combined and sensitive approach,
369 we did not find significant differences between AM fungal communities associated with GM and
370 non-GM maize plants. Moreover, variation including GM induced variation was much lower than the
371 natural variation of AMF communities across a wide range of fields.
372
373 Our pyrosequencing - based assessment of total soil fungal communities provided us with novel
374 information on AM fungal abundance and activity compared to other fungi (Figure 2a). In most
375 instances, relative abundance of AM fungal reads was very high, in particular in the RNA derived
376 assessments. This indicates that RNA based analysis can provide a good estimate of AM fungal
377 communities, which was in our case even superior to DNA based analysis regarding contribution to
378 total fungal reads. In our experiment, this contribution was lowest for DNA derived assessment of
379 young plants, where AM fungi only accounted for less than 1% of total fungal reads. A possible
380 explanation is that the experimental procedure represented a strong disturbance, causing increased
381 growth of fungi other than AMF, or a decrease in the latter.
382

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383 At plant maturity, however, AM fungal reads constituted an average of > 20% of all fungal RNA
384 reads, and >10% of all fungal DNA reads. A similarly high contribution was observed recently by
385 Jumpponen (23) in a North-American tallgrass prairie system, where AM fungi were abundant in the
386 rRNA pool (assessed on RNA), with a relative contribution ranging from about 15% to 35% of total
387 fungal reads. In another study in North-American prairie, Miller and Kling (36) estimated AM fungi to
388 contribute up to 23% of total microbial biomass. This may indicate that activity and abundance of
389 AM fungi in our system is in the same order of magnitude as in these grassland systems. However, as
390 rRNA is constitutively transcribed in living cells it may not be a precise measure of taxa activity.
391 Potentially transcription of other genes which are indicative for specific physiological processes (e.g.
392 phosphate transporters) can be assessed in future research as an approximate for specific activities
393 (e.g. see Gamper *et al.* (16) for a discussion).
394
395 The high relative abundance of AM fungi observed here has important implications for studies on
396 environmental risk of transgenic crops. In other studies on fungal community responses to GM
397 plants (e.g. ref. 30, 63), the primers used have been shown to display a bias towards other fungal
398 groups over Glomeromycota, and in some cases may not detect AM fungi at all. This means that
399 these studies potentially underestimate a numerically significant and important group of soil fungi.
400
401 AM fungal community assessments by pyrosequencing and T-RFLP were significantly correlated,
402 confirming that both methods are able to detect the same community trends. However, these
403 methods did not always agree with respect to the relative abundances of individual taxa. These
404 discrepancies are partially explained by the fact that our T-RFLP method highly underestimated the
405 abundance of *Paraglomus* and *Diversispora*, and thus relative abundance of other taxa is
406 consequently overestimated. Another factor that is likely to have contributed is the usage of two
407 different rRNA regions and methods, SSU for pyrosequencing versus LSU for T-RFLP. These regions
408 differ in their phylogenetic resolution (17), and the methods of T-RFLP versus full amplicon

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409 sequencing differ as well in this respect. For economic and practical reasons, the number of
410 replicates per treatment was low in this study (2 or 3 replicates per treatment). An alternative would
411 have been to include more replicates thereby reducing the number of reads obtained per sample.
412 However, because some AMF OTU's had very low abundance (e.g. Archaeospora represent 0.05% of
413 AMF reads) this is likely to result in missing OTU's. Despite low replication, effects of time (see Table
414 2) on AM fungal communities were stronger compared to cultivar or GM- induced differences,
415 indicating that our experiment had sufficient statistical power to measure community changes.
416
417 Additionally, we tested how community variation in our experiment relates to that in 1) the field
418 where soil was derived from five samplings over the course of three years, and 2) 15 other
419 agricultural maize fields (Figure 4). As expected, we found that the variation of AM fungal
420 communities was much larger among 15 different agricultural fields, and communities for each of
421 the experimental varieties clustered within the seasonal variation of the donor-field at the late
422 sampling stage (Figure 4). However, AM fungal communities at 47 days showed lower similarity to
423 those obtained from the field, but did not cluster outside the natural range found in other
424 agricultural fields, nor did these show any systematic change in response to the GM-trait. It should
425 be noted that a variety of maize cultivars was cropped in the agricultural fields, and thus our
426 assessment of natural variation includes cultivar-associated variation. Because our goal has been to
427 sample a representative set of "normal" agricultural conditions, we think it is appropriate to include
428 different cultivars in estimating baseline community variation. Therefore, our study suggests that the
429 GM-trait we assessed does not cause AM fungal communities to change outside "baseline" variation.
430 This provides additional support to the conclusion that Bt maize poses no risk for the investigated
431 soil fungi. For other GM-traits, agricultural systems, or non-target organism classes, such changes
432 may occur. We therefore suggest that future studies on effects of GM plants on soil (microbial)
433 communities address how effects, if any, scale against natural variation. This will provide important
434 insight in the extent of GM-imposed disturbance and potential effects on soil ecological quality (25,

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435 46). Moreover, it would be of great interest to extend this approach towards other soil microbes
436 such as bacteria and decomposing and pathogenic fungi.
437
438 In our study, the majority of putative AM fungal OTU's (but not reads) had a low similarity to known
439 AM fungal species. This is in contrast to previous studies where AM fungal communities were
440 examined by pyrosequencing of rRNA genes. For these studies, recovered sequences typically had
441 highest BLAST hits with known AM fungal sequences (and thus putative AM fungal sequences) that
442 exceeded 97% (13, 32, 37). One reason for this discrepancy may be that our primers target a
443 different portion of the SSU rRNA gene, i.e. the V3-V4 and V7-V8 hypervariable regions respectively,
444 and that these portions differ in variability and/or Phylum discrimination potential. Because other
445 studies using the same primer set as used here rarely report the recovery of AM fungal sequences, it
446 remains to be tested whether this is a common problem. We observed that affiliation of low-
447 similarity OTU's with the Glomeromycota was not strongly supported through alignment and
448 phylogenetic analysis, and therefore we have used very stringent similarity criteria to identify AMF.
449 We acknowledge that this may have caused a significant underestimation of AMF diversity, although
450 the OTU's included do represent the majority of putative AM fungal reads and should thus be
451 sufficient to analyze major community trends. Because Phylum assignment through BLAST of
452 sequences obtained from soil material through 454 pyrosequencing can be problematic, at least for
453 this SSU rRNA gene region, we recommend performing additional phylogenetic analysis to confirm
454 affiliation of OTU's.
455
456 **Conclusions**
457 In this study, we thoroughly analysed community composition of important and beneficial soil fungi
458 in association with transgenic and non-transgenic crops, targeting DNA as well as RNA at two plant
459 growth stages. To our knowledge, assessing both DNA and RNA simultaneously has not previously
460 been done for these fungi. Our assessment revealed that AM fungi represent a major portion of soil

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461 fungal communities, especially at the RNA (activity) level. We have not detected consistent changes
462 in AM fungal community composition in response to the two GM maize cultivars compared to non-
463 GM maize cultivars. Given the large contribution of AM fungi to the estimated total fungal activity
464 we observed, and the key ecological functions they perform, AM fungi should not be overlooked in
465 risk-assessment of GM crops. Our approach of comparing GM-related changes to within and among
466 field community variation can provide a suitable framework for scaling potential GM-imposed
467 effects to natural community variation.
468
469 **Acknowledgements**

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473 maize seeds used in the experiment.
474
475 **Supporting information**

476 See the appendix section for the following Supporting Information:
477
478 **Table S1** List of OTU's assigned to Glomeromycota
479 **Figure S1** NMDS biplots of T-RFLP of DNA and RNA including all samples
480 **Notes S1** descriptive comparison of taxa detected by T-RFLP and pyrosequencing
481 **Table S2** Bray-Curtis similarities of samples and obtained T-RFLP profiles
482

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Table 1: Average AM fungal richness for each plant variety at first (47 days) and second sampling (104 days). Assessment by pyrosequencing and by T-RFLP (average of forward and reverse T-RF) are presented separately, assessed by DNA and RNA analysis. Different plant varieties are abbreviated by letters, Monumental = M, DKC = K. GM varieties are indicated with an additional identifier “GM” (i.e. M - GM, and K – GM). For pyrosequencing, to account for differences in total AM fungal read numbers when more than 100 reads were present, AM fungal richness of samples was estimated by rarefaction analysis estimate of 100 individual sequences (e.g. ref. 1). SE = standard error in the mean. N = number of observations. “reads” represents the mean number of AM fungal reads obtained. In none of the instances there was a significant effect of the GM-trait (GM versus non-GM), the cultivar (M versus K) or their interaction on AM fungal richness.

		Pyrosequencing								*T-RFLP*					
		47 days				104 days				47 days			104 days		
	variety	Mean	SE	N	reads	Mean	SE	N	reads	Mean	SE	N	Mean	SE	N
DNA	M	***2.7†***	0.9	3	9	***6.0***	0.0	2	137	**6.2**	0.2	3	**6.5**	0.5	3
	M-GM	***3.5***	0.5	2	35	***5.3***	0.4	3	67	**4.2**	1.0	3	**5.5**	0.6	3
	K	***2.0***	2.0	2	3	***6.5***	0.5	2	37	**4.3**	1.3	3	**6.3**	1.5	3
	K-GM	***2.3***	0.9	3	8	***5.3***	0.3	3	146	**5.0**	1.0	3	**5.2**	0.8	3
RNA	M	***5.0***	1.0	3	459	**5.8**	0.0	2	1159	**4.0**	1.0	3	**6.0**	1.2	3
	M-GM	***3.8***	0.4	2	1109	**5.9**	0.0	3	718	**4.5**	0.8	3	**6.3**	0.4	3
	K	***4.6***	1.4	2	190	**5.2**	0.4	2	815	**5.3**	0.7	3	**5.3**	1.4	3
	K-GM	***4.7***	0.7	3	651	**5.7**	1.0	3	792	**5.0**	0.5	3	**5.5**	0.8	3

† for values in italics (Pyrosequencing of DNA at both times and RNA at 47 days) not all replicates were represented by at least 100 AM fungal reads and thus no statistics are performed on richness estimates comparing GM with non-GM.

657 Table 2: Two-way NPMANOVA of Bray-Curtis similarities of plant-associated AM fungal communities,
658 represented by DNA or RNA, sampled at either a plant age of 47 days or 104 days. The crossed
659 factors are "GM versus non-GM", and "cultivar" (K versus M parental cultivar; independent of the
660 GM-trait). There were no significant interactions between "cultivar" and "GM vs. non-GM" in any of
661 the analyses. The effect of the factor "time" is also presented, where a one-way NPMANOVA was
662 performed contrasting communities at "104 days" versus "47 days". F = NPMANOVA statistic, P =
663 chance to obtain result if null-hypothesis is true, N = number of observations. No analyses were
664 performed on pyrosequencing data for DNA at 47 days because of the low number of reads.
665 Similarities between separate samples are presented in Table S2.

			pyrosequencing			*T-RFLP*		
			F	P	N	F	P	N
DNA	GM vs non-GM	47 days				2.73	NS	24
	cultivar					**3.68**	**0.01**	
	GM vs non-GM	104 days	0.83	NS	20	0.72	NS	24
	cultivar		1.69	NS		0.81	NS	
	time					**2.76**	**0.02**	48
RNA	GM vs non-GM	47 days	0.82	NS	20	**2.86**	**0.04**	24
	cultivar		0.13	NS		2.75	NS	
	GM vs non-GM	104 days	0.49	NS	20	1.34	NS	24
	cultivar		**3.00**	**0.02**		**5.01**	**0.01**	
	time		**4.38**	**0.003**	40	**3.30**	**0.006**	48

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668 **Figure legends**

669

670 Figure 1: Neighbour Joining (NJ) tree showing topology of OTU's having at least 97% similarity to
671 known AM fungal taxa (sequences obtained in this study are in bold and are preceded with a
672 number; an identifier for storage purpose, as in Table S1). OTU's are named according to placement
673 within the phylogenetic tree. Bootstrap values are given at the branch points.

674

675 Figure 2: The total number of sequences derived from AM fungi is shown as a percentage of total
676 fungal reads (a; mean ± SE). At both samplings, AM fungi were highly overrepresented in RNA
677 compared to DNA, and both increased from the first to the second sampling date. The relative
678 abundance of AM fungi to total fungi was similar between GM and non-GM. The mean relative
679 abundance of each OTU (b; left and right column of each panel representing non-GM and GM plants,
680 respectively) also did not differ between GM and non-GM plants at any of the two time points or
681 nucleic acid type (RNA or DNA; $P > 0.05$ for each OTU). For both figures N = 5, except for GM at 104
682 days (both DNA and RNA): N = 6, and non-GM at 104 days (both DNA and RNA): N = 4.

683

684 Figure 3: NMDS biplots of Bray-Curtis similarities among communities found in the experimental
685 treatments using pyrosequencing for RNA (a; stress = 0.14; N = 20) and T-RFLP (b; RNA, stress = 0.16,
686 N = 22, c; DNA; stress = 0.14; N = 22; note that for T-RFLP two samples have been excluded, see
687 Supplementary material for plots with these samples included).

688

689 Figure 4: Detrended Correspondence Analysis (DCA; eigenvalue of first axis = 0.53, second axis =
690 0.21) of the soil AM fungal communities obtained in this study represented by DNA, together with
691 "season"; seasonal variation in AM fungal communities at one field site (site where soil was derived
692 for this study) obtained during five field sampling events over three years, and "15 fields" ; variation
693 of maize root AM fungal communities across 15 different agricultural fields sampled in September

694 2007 (data from (57)). AM fungal community composition was assessed using T-RFLP for all data
695 points. Note that the different Maize varieties of the greenhouse study represent average
696 communities of soil samples of three replicate plants (to accommodate comparison to pooled
697 samples); “season” and “15 fields” are pooled root samples of six plants. Three to four months- old
698 maize roots were collected for “15 fields” while maize roots for season had variable age (from 2 to 4
699 months).

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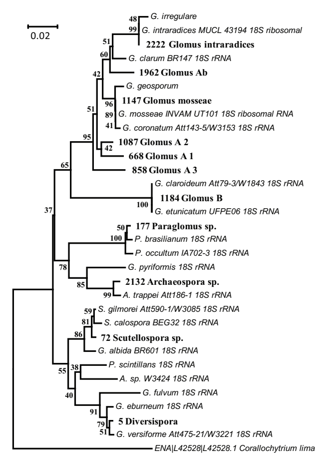

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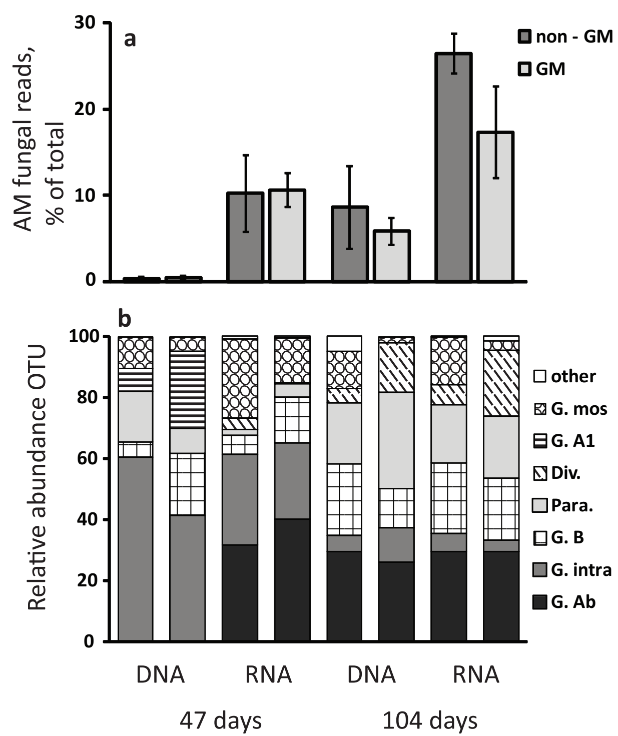

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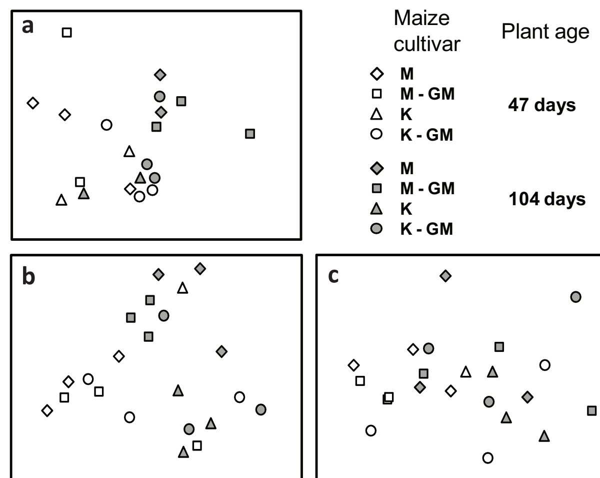

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