Impact of *Collimonas* bacteria on community composition of soil fungi

Sachie Höppener-Ogawa,1 Johan H. J. Leveau,1,2 Maria P. J. Hundscheid,1 Johannes A. van Veen1,3 and Wietse de Boer1*  
2 Department of Plant Pathology, University of California at Davis, One Shields Avenue, 475 Hutchinson Hall, Davis, CA 95616, USA.  
3 Institute of Biology Leiden, Leiden University, PO Box 95126, 2300 RA Leiden, The Netherlands.

Summary

The genus *Collimonas* consists of soil bacteria that have the potential to grow at the expense of living fungal hyphae. However, the consequences of this mycophagous ability for soil fungi are unknown. Here we report on the development of fungal communities after introduction of collimonads in a soil that had a low abundance of indigenous collimonads. Development of fungal communities was stimulated by addition of cellulose or by introducing plants (*Plantago lanceolata*). Community composition of total fungi in soil and rhizosphere and of arbuscular mycorrhizal fungi in roots was examined by PCR-DGGE. The introduction of collimonads altered the composition of all fungal communities studied but had no effects on fungal biomass increase, cellulose degrading activity or plant performance. The most likely explanation for these results is that differences in sensitivity of fungal species towards mycophagous attack. Indeed, there are indications that such differences between fungal species exist (De Boer *et al.*, 2004; Kamilova *et al.*, 2007).

Introduction

Mycophagy, i.e. the feeding on living fungi, is widely distributed among soil-inhabiting organisms, e.g. fungivorous collembola, nematodes, amoebae and mycoparasitic fungi (cf. Leveau and Preston, 2008). However, little is known on the occurrence of mycophagy among soil bacteria (Leveau and Preston, 2008). This is remarkable as fungi and bacteria coexist in most soils, where they have different interactions based on the acquisition of organic energy sources (De Boer *et al.*, 2005). The availability of degradable carbon compounds is the most common limiting factor for bacterial growth in soils (Demoling *et al.*, 2007). Therefore, it is to be expected that selection has also favoured bacteria exploiting fungal hyphae as organic energy source.

Soil bacteria of the genus *Collimonas* have been shown to grow at the expense of living fungal hyphae in soil-like microcosms and natural soils (De Boer *et al.*, 2004; Höppener-Ogawa *et al.*, 2009; Leveau and Preston, 2008). Höppener-Ogawa and colleagues (2007) showed that collimonads are widely spread in terrestrial environments but that their abundance is rather low (up to $8.7 \times 10^5$ cells g$^{-1}$ dry-weight soil). The low densities of collimonads in soil, as well as the moderate increase (from $10^4$ up to $10^6$ cells g$^{-1}$ dry soil) upon invasion of fungal hyphae in soils or soil-like systems, suggest that their influence on total fungal biomass turnover is of minor importance (De Boer *et al.*, 2001). This is supported by the fact that no significant negative effect of collimonads on fungal biomass production was observed in sand microcosms (Höppener-Ogawa *et al.*, 2009).

A minor effect of collimonads on fungal biomass turnover does, however, not exclude the possibility that collimonads have an effect on soil fungal community composition. As for bacteria, functional redundancy among fungi is high and so competition for the same resources will be severe. This holds true for both saprotrophic and mycorrhizal fungi (Allen *et al.*, 1995; Deacon *et al.*, 2006). Collimonads may have an impact on the outcome of competition between fungi in case there are differences in sensitivity of fungal species towards mycophagous attack. Indeed, there are indications that such differences between fungal species exist (De Boer *et al.*, 2004; Kamilova *et al.*, 2007).
The aim of the current study was to test the effect of the presence of collimonads on the mycelial accumulation and community composition for different functional groups of soil fungi, namely, (i) cellulose- and sugar-degrading fungi after enrichment of a soil with cellulose and (ii) root-exudate consuming fungi and arbuscular mycorrhizal fungi after introducing a plant (Plantago lanceolata). In addition, the effect of the presence of collimonads on composition of fungi invading a sterile sand patch in natural soils was examined.

Results

Effect of collimonads on the community composition of fungi in cellulose-enriched soil

Two weeks after the addition of cellulose to an arable soil, the soil fungal biomass indicator ergosterol had increased from 1.0 ± 0.4 mg kg⁻¹ dry soil to 8.7 ± 1.0 mg kg⁻¹ dry soil in the controls and to 9.6 ± 0.7 mg kg⁻¹ dry soil in the soil inoculated with collimonads respectively. The stronger increase of ergosterol content in the presence of collimonads was nearly significant (P = 0.09). After prolonged incubation (8 weeks after the addition of cellulose) ergosterol contents had decreased to 3.9 ± 0.5 and 4.4 ± 0.2 mg kg⁻¹ dry soil in the controls and in the Collimonas-inoculated soil respectively. The difference between the controls and the Collimonas-inoculated soil was again nearly significant (P = 0.06). After 2 weeks of incubation, cellulase activity in the control samples [89.0 ± 37.6 (nmol RBB release) 24 h⁻¹ g⁻¹ dry soil] did not significantly differ from the Collimonas-inoculated soil [134.6 ± 50.7 (nmol RBB release) 24 h⁻¹ g⁻¹ dry soil].

Two weeks after the inoculation of collimonads, qPCR-based numbers of collimonads had dropped from 1.0 × 10⁶ to 1.6 × 10⁵ ± 2.3 × 10⁴ collimonads g⁻¹ dry soil and the numbers were under the detection limit (1.5 × 10⁴ collimonads g⁻¹ dry soil) after 8 weeks of incubation.

PCR-denaturing gradient gel electrophoresis (DGGE) patterns of fungal 18S rRNA-gene fragments amplified from total DNA differed between the controls and the Collimonas-inoculated soil replicates (Fig. 1, lanes 10–18). These differences were confirmed by distance-based redundancy analysis (db-RDA) (P < 0.001). This indicates a significant effect of the presence of collimonads on the development of the fungal community in the cellulose-enriched soil. No further changes in cellulolytic fungal composition became apparent during prolonged incubation time (from 2 to 8 weeks). Two major bands (E and K, Fig. 1) that were present in the controls were much weaker in the soil inoculated with collimonads. In contrast, five distinct bands (F, G, H, I and J) were

![Fig. 1. DGGE patterns of partial fungal 18S RNA genes from two experiments. Lanes 1–4: Rhizosphere soil of Plantago lanceolata grown in soil without addition of collimonads; lanes 6–9: Rhizosphere soil of Plantago lanceolata grown in soil with addition of collimonads; lanes 10–13: Cellulose-enriched soil without addition of collimonads; lanes 15–18: Cellulose-enriched soil with addition of collimonads. The patterns of the cellulose-enriched soil are from the 8-week incubation. Fungal markers, made of an artificial mix of different fungal species, are presented in lanes 5 and 14. Coding of bands refers to Table 1.](image-url)
detected in the *Collimonas*-inoculated soil. Sequence analysis of the 18S rRNA fragments revealed two high matches (> 98%), namely, one as a taxon within the order Hypocreales (band K, without the addition of collimonads) and one as a taxon within the order Sordariales (band I, with the addition of collimonads).

**Effect of collimonads on the community composition of rhizosphere fungi and arbuscular mycorrhizal fungi associated with roots of *Plantago lanceolata***

The ergosterol content in the rhizosphere soil of *P. lanceolata* was 1.14 ± 0.39 and 0.98 ± 0.43 mg kg⁻¹ dry soil with and without the addition of collimonads respectively. The difference in ergosterol content between the *Collimonas*-inoculated soils and the controls was not significant ($P = 0.48$).

The presence of collimonads had a significant effect on the fungal community structure in the rhizosphere of *P. lanceolata* (Fig. 1, lanes 1–4, 6–9), as shown by db-RDA analysis ($P < 0.004$).

Three distinct bands (A, B and C) were detected in the rhizosphere of the control treatment but were not clearly distinguishable in the presence of collimonads. Sequence analysis revealed that two of these bands (A and B) belonged to the subphylum *Mucoromycotina* (Table 1). One band (D) that was only distinguishable in the soil with collimonads represented an unidentified ascomycete (Table 1).

The presence of collimonads was accompanied by a significant change (db-RDA analysis; $P < 0.004$) in AM fungal community structure inside the root (Fig. 2). Bands AM1 and AM4 were not distinguishable when collimonads were present, whereas bands AM2 and AM3 were only clearly visible in the presence of collimonads. The results of sequence analysis of these bands are shown in Table 1. All sequenced bands, including bands that did not differ between the treatments, had the highest match with the class *Glomeromycota*, confirming the selectivity of the primers specific for arbuscular mycorrhizal fungi.

The addition of collimonads to the soils did not affect the AM fungal colonization of *Plantago* roots (Table 2). There was also no effect of the addition of collimonads on the production of root and shoot biomass by *P. lanceolata*, nor was the nitrogen and phosphorus content of *P. lanceolata* affected by the presence of collimonads (Table 2).

**Field experiment: sand-containing mesh bags**

After the 2-month incubation of sand-containing mesh bags in field soils, qPCR-based numbers of collimonads in the sand had dropped from $1.0 \times 10^6$ to $2.7 \times 10^5 \pm 5.5 \times 10^4$ collimonads g⁻¹ dry soil in the ex-arable site and to $5.3 \times 10^5 \pm 2.6 \times 10^5$ collimonads g⁻¹ dry soil in the forest site.

Quantification of ergosterol contents indicated that the biomass of fungi invading sterile sand in buried mesh bags was low [<0.5 (mg ergosterol) kg⁻¹ sand]. Despite the low fungal biomass present, fungal community structure in the mesh bags could be assessed by PCR-DGGE (Fig. S1). Principal coordinate analysis (PCoA), based on Jaccard similarity, of fungal 18S rDNA-DGGE patterns

---

**Table 1.** Sequence analysis of total fungal and arbuscular mycorrhizal PCR-DGGE bands. Letters indicate the coding of the bands as indicated in Figs 1 and 2.

<table>
<thead>
<tr>
<th>DGGE bands</th>
<th>Order</th>
<th>Phylum affiliations</th>
<th>Accession No., similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1a</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AM2b</td>
<td>Glomerales</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AM3b</td>
<td>Glomerales</td>
<td>Glomeromycota</td>
<td>AY842575, 95%</td>
</tr>
<tr>
<td>AM4a</td>
<td>Glomerales</td>
<td>Glomeromycota</td>
<td>AJ854582, 98%</td>
</tr>
<tr>
<td>A*</td>
<td>Mucorales</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B*</td>
<td>Endogonales</td>
<td>Mucoromycotina*</td>
<td>AF157140, 94%</td>
</tr>
<tr>
<td>C*</td>
<td>Agaricales</td>
<td>Basidiomycota</td>
<td>DQ322638, 93%</td>
</tr>
<tr>
<td>D*</td>
<td>Phylachorales</td>
<td>Ascomycota</td>
<td>AY911691, 92%</td>
</tr>
<tr>
<td>E*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>G*</td>
<td>Lecanorales</td>
<td>Ascomycota</td>
<td>AY584675, 86%</td>
</tr>
<tr>
<td>H*</td>
<td>Maissazales</td>
<td>Basidiomycota</td>
<td>AY083223, 86%</td>
</tr>
<tr>
<td>I*</td>
<td>Sordariales</td>
<td>Ascomycota</td>
<td>EU263613, 99%</td>
</tr>
<tr>
<td>J*</td>
<td>Cantharellales</td>
<td>Basidiomycota</td>
<td>DQ915460, 92%</td>
</tr>
<tr>
<td>K*</td>
<td>Hypocreales</td>
<td>Ascomycota</td>
<td>DQ838790, 100%</td>
</tr>
</tbody>
</table>

a. Band appeared in treatment without collimonads.
b. Band appeared in treatment with collimonads.
c. Band intensity increased in treatment without collimonads.

* Mucoromycotina is a subphylum, whereas the others are phyla (Hibbett et al., 2007) The symbol ‘–’ denotes bands that could not be cloned successfully.
was shown in Fig. 3. Samples clustered according to the soil origin. Statistical analysis (db-RDA) also revealed that the composition of the invading fungi was dependent on the soil origin ($P = 0.001$). The presence of collimonads in the sterile sand had a significant effect on the composition of the invading fungi in the ex-arable site ($P = 0.007$) but not in the forest site ($P = 0.66$).

**Discussion**

Our results clearly indicate that the introduction of collimonads in a soil that was apparently devoid of such bacteria had a significant impact on the composition of fungal communities. The impact on fungal community composition was not accompanied by a reduction in fungal biomass or significant changes in cellulose activity.

The numbers of collimonads that were added to the soil ($10^6 \text{ g}^{-1} \text{ dry soil}$) are similar to the highest densities of collimonads present in natural soils (Höppener-Ogawa et al., 2007). No further increase of collimonads was observed after introduction of cellulose or *P. lanceolata* plants. The actual dynamics of collimonads in these experiments is, however, not clear as the survival after introduction is not known. Yet, it is obvious that the increase of fungal biomass upon introduction of cellulose or plants did not result in densities of collimonads exceeding the initial density of $10^6 \text{ g}^{-1} \text{ dry soil}$. In a previous study we observed that a fungal-induced increase from $10^5$ to $10^6$ collimonads g$^{-1}$ dry soil had no negative effect on fungal biomass production (Höppener-Ogawa et al., 2009).

Therefore, it is not surprising that we did not find a negative effect of collimonads on fungal biomass production in the current study.

Changes in fungal community composition in the presence of collimonads became apparent for all three fungal groups studied (cellulolytic fungi, rhizosphere fungi and AM fungi). Collimonads appeared to affect fungi within all major fungal (sub)phyla that can be found in soils, i.e. *Glomeromycota*, *Mucoromycotina*, *Ascomycota* and *Basidiomycota*. For all these (sub)phyla with exception of the *Mucoromycotina*, both appearing and disappearing amplicons (bands) in the PCR-DGGE analysis were found. The most likely explanation is that differences in

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot dry weight (g/pot)</th>
<th>Root dry weight (g/pot)</th>
<th>AC (%)</th>
<th>VC (%)</th>
<th>HC (%)</th>
<th>N (mg g$^{-1}$)</th>
<th>P (mg g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition of collimonads</td>
<td>0.40$^a$</td>
<td>3.6$^a$</td>
<td>0.47$^a$</td>
<td>0.37$^a$</td>
<td>0.84$^a$</td>
<td>11.6$^a$</td>
<td>4.6$^a$</td>
</tr>
<tr>
<td>Control without adding collimonads</td>
<td>0.58$^a$</td>
<td>3.8$^a$</td>
<td>0.50$^a$</td>
<td>0.31$^a$</td>
<td>0.90$^a$</td>
<td>10.6$^a$</td>
<td>5.0$^a$</td>
</tr>
</tbody>
</table>

Percentage of intersections containing structures of AM fungi: arbuscule-like structures (AC), vesicle-like structures (VC) and hyphal-like structures (HC) were present.

Similar letters denote that there were no significant differences ($P < 0.05$) between the treatments.

Data indicate the means of six replicates. Differences between means were tested for significance using one-way ANOVA.

© 2009 The Authors
sensitivity of fungal species or strains to the presence of collimonads change the competitive relationships between different fungal taxa. Some fungal species that have a selective advantage in the absence of collimonads appear to lose this advantage in their presence and vice versa.

It is tempting to ascribe the changes in fungal community composition to preferential mycophagous growth of collimonads. For instance, the apparent inability of two fungi belonging to the subphylum Mucormycotina to colonize the P. lanceolata rhizosphere in the presence of collimonads is in line with the reported strong mycophagous growth of collimonads at the expense of zygomycetes (De Boer et al., 2001; Höppener-Ogawa et al., 2009). However, selective effects may also have been due to other antifungal activities of collimonads (Kamilova et al., 2007). Small, short-term effects of antibiotic producing biocontrol bacteria on non-target fungi in the rhizosphere have been reported (Glandorf et al., 2001; Scherwinski et al., 2008). Compared with these studies, the effects of collimonads on fungal communities appear to be more consistent. However, this may be due to differences in the experimental set-up (field trials versus containers; seed inoculation versus soil inoculation).

The effects of collimonads on fungal community composition did not appear to have an effect on the cellulose-degrading activity, plant biomass production and plant N and P content. Hence, functionality of fungal communities appeared not to be affected by a change in fungal community composition. This is in line with the expected functional redundancy of soil fungi (Deacon et al., 2006).

Arbuscular mycorrhizal fungi form associations with many plant species and have an important role in the plant’s mineral nutrient acquisition (Rillig, 2004). Earlier studies have indicated that the occurrence of collimonads may be related to the occurrence of AM fungi (Offre et al., 2007). Such a relationship could point at preferential mycophagous growth of collimonads on AM fungi with a possible negative impact on plant performance. The current study showed that the presence of collimonads had a clear effect on the community composition of AM fungi inside the roots. However, there were no apparent effects on colonization of roots of P. lanceolata by arbuscular mycorrhizal fungi or on plant performance. Hence, different sensitivity of AM fungal species/strains to collimonads appears to result in a shift in AM community composition without affecting the plant nutrient acquisition.

In addition to the laboratory and greenhouse experiment, we performed an experiment to see whether collimonads affect fungal community structure under field conditions. Only small amounts of fungi had colonized the sterile sand in the buried mesh bags after 2 months of incubation in the fields. Yet, the effect of the presence of collimonads on the community composition of the invading fungi in the ex-arable soil was apparent. No such effects were seen for the forest soil. Major fungal groups, normally present in ex-arable soils, consist of fast growing saprotrophic fungi and arbuscular mycorrhizal fungi that have less persistent hyphae than lignocellulolytic basidiomycetes and ecto-mycorrhizal fungi, which are typically abundant in forest soils (van der Wal et al., 2006a). Fungi with more persistent hyphae may also be less sensitive to mycophagous attack by collimonads.

As the two field soils differ in acidity, it may be argued that apparent differences in mycophagous performance are due to different pH conditions. However, this is unlikely as an earlier study showed mycophagous growth of collimonads in soils varying in pH from 3.6 to 5.6, i.e. covering the pH values of the soils used in this study (Höppener-Ogawa et al., 2009).

In conclusion, collimonads had a strong effect on the composition of functional groups of soil fungi, whereas the effect on fungal biomass production and functioning of fungi was small. Conceptually, these observations are of great interest as it shows that a minor component of the soil bacterial community can have a strong impact on fungal community composition in the soil ecosystem.
Experimental procedures

Study sites

Two field sites (a deciduous forest and an ex-arable field) used in this study are located in the central part (Veluwe) of the Netherlands. The forest site (major tree species beech and oak), which is located near the village Doorwerth, was only used for the field experiment. The ex-arable field was referred to as Mossel in the study of van der Wal and colleagues (2006b). The soil characteristics of the ex-arable land and forest site (mineral layer) were pH_{water} 5.6 and 3.5, total organic carbon 21 and 35 g kg^{-1}, and C/N ratio 17.1 and 24.3, respectively. More details on the ex-arable site are given by van der Wal and colleagues (2006b). The number of indigenous collimonads in the ex-arable soil was lower than 1.5 \times 10^4 g^{-1} dry-weight soil (detection limit) as assessed by Collimonas-specific real-time PCR (Höppener-Ogawa et al., 2007). At the ex-arable site, soil (0–10 cm layer) was sampled from at least 30 randomly selected points in a 50 \times 50 m plot. Samples were pooled, mixed and sieved (4 mm mesh) before use in microcosm experiments 1 and 2 (see below).

Bacterial strains

The strains used in this study were Collimonas fungivorans Ter 331 (AJ310395) and Ter 6' (LMG 21973), C. arenae Ter 10^7 (LMG 23964) and C. pratensis Ter 91' (LMG 23965) (De Boer et al., 2004; Höppener-Ogawa et al., 2008).

Effect of collimonads on the community composition of fungi in cellulose-enriched soil

The Collimonas strains Ter 6, 10, 91 and 331 were pre-grown on chitin–yeast agar at 20°C for 14 days as described by de Boer and colleagues (2001). Bacteria were suspended in P-buffer (KH_2PO_4, 1 g l^{-1}, pH 6.5), pelleted by centrifugation (16 000 g) and re-suspended in P-buffer. A mixed suspension of Collimonas strains was made by adding equal numbers of cells of each strain (direct microscopic counting) to P-buffer. The bacterial suspension was mixed into autoclaved, acid-purified beach sand to give a moisture content of 14% (w/w). For the control treatment the same amount of P-buffer without collimonads was used. Acid-purified sand with or without collimonads was mixed (1:9) with the homogenized ex-arable soil. The mixing with collimonads-containing sand resulted in a final density of 10^6 cells g^{-1} dry soil. After inoculation, the soil was pre-incubated at 20°C for 1 week to allow the bacteria to adapt to conditions in the ex-arable soil. Next, the soil was enriched with \( \Sigma \) to a final concentration of 2 mg C g^{-1} dry soil. Portions (40 g) of soil were transferred to Petri dishes (diameter, 8.5 cm) and spread evenly. The Petri dishes were sealed with Para-film and incubated at 20°C. Per treatment (i.e. with and without collimonads) and per time interval six replicate samples were incubated. After 2 and 8 weeks of incubation, fungal biomass, fungal community composition and numbers of collimonads were determined. Quantification of ergosterol, via an alkaline extraction method, was used as an estimate of fungal biomass (De Ridder-Duine et al., 2006).

Analysis of cellulase activity was based on the release of remazol Brilliant blue from dyed carboxymethyl cellulose as described by van der Wal and colleagues (2006a).

Fungal community structure was examined using PCR-DGGE (see below). Numbers of collimonads were determined using real-time PCR on extracted soil DNA (see below) (Höppener-Ogawa et al., 2007).

Effect of collimonads on the community composition on rhizosphere fungi and arbuscular mycorrhizal fungi associated with roots of Plantago lanceolata

Seeds of P. lanceolata (Ribwort Plantain) were surface-sterilized by gently shaking them in 0.4% hypochlorite solution for 10 min and rinsed in sterile distilled water for 5 min. Sterile seeds were germinated on 1.5% Bactoagar (#0140-01-0; Difco, Detroit, MI) at 20°C for 7 days. Seedlings free of microbes were used for the experiment.

The ex-arable soil was inoculated with a mixture of collimonads as described in the previous subsection. Non-inoculated controls were also prepared as described above. Portions of 1 kg of soil were put in containers (diameter 11.4 cm; height 14.3 cm). Six replicate pots were prepared for treatments with or without the addition of collimonads. Per container three seedlings were planted. All containers were placed in the greenhouse at a temperature of 25 \pm 1°C (day) and 22 \pm 1°C (night) with a relative humidity of 70% and with a photoperiod of 16:8 h (day : night). Natural daylight was supplemented with 400 W metal halide bulbs (one per 1.5 m²). The weight of the containers was checked daily and, if necessary, water was added to maintain the moisture content of 14% (w/w).

After 1 week, the number of seedlings was reduced to 1 per container. After 8 weeks, plants were harvested. Thirty 1-cm-long sections of thin roots (diameter approximately 2 mm) from each pot were randomly sampled for the determination of colonization by AM fungi, using the magnified intersections method (McGonigle et al., 1990). Rhizosphere soil was collected by brushing soil adhering to roots. Next, roots were washed with water. Half of the roots were used for determination of root dry weight, the other half were used for analysis of AM communities. The shoots were used for determination of dry weight, total C, N and P. Total C and N were determined using a FlashEA 1112 Series NC soil analyser. Total P was determined colorimetrically after acid digestion (van der Wal et al., 2007).

Soil DNA was extracted as described below. The extracted DNA was used for the examination of total fungal and arbuscular mycorrhizal fungal community structure using PCR-DGGE analysis (see below).

Composition of AM fungal community inside the roots was also determined using the freeze dried root tissues. DNA was extracted from 2 g of freeze dried material using bead beating for 3 min to crush the root tissues. DNA extracted from the freeze-dried roots was analysed for AM fungal community structure by PCR-DGGE analysis (see below).
Effect of collimonads on invading fungi  
(Field experiment)

Fungal in-growth bags with an opening on one side were constructed of nylon mesh pieces (mesh size 35 µm; bag size, 10 × 5 × 2 cm) by fusing the edges with a thermo-sealer. The mesh size used, allowed the in-growth of fungal hyphae but not of roots (Wallander et al., 2001). Collimonas strains Ter 6, 10, 91 and 331 were grown and mixed with acid-purified sand as described above, except that the moisture content was kept at 5% (w/w). Portions (120 g) of sand containing collimonads were transferred into the bags. After sealing the opening of the bags, they were placed at 20°C, and pre-incubated for 1 week to allow bacteria to adapt to conditions in sand. For the control treatment, bags with sand without collimonads were prepared. After pre-incubation, the nylon bags were placed horizontally at the interface between the organic horizon (4 cm thick) and the mineral soil in the forest site. In the ex-arable site, the nylon bags were placed at 3 cm below the surface. Bags of the two treatments (with and without collimonads) were placed close to each other. In total, six replicates of both treatments were placed at six randomly selected spots within an area of 100 m².

The start of the field incubation was 11 September 2007, and after 8 weeks of incubation in the field the bags were collected. Per bag, sand was carefully mixed and samples were taken for fungal biomass (ergosterol) measurements (De Ridder-Duine et al., 2006) and determination of fungal community structure by PCR-DGGE analysis (see below). Soil DNA was extracted as described in the next subsection.

PCR-denaturing gradient gel electrophoresis analysis, cloning and sequencing

For all experiments, soil DNA was extracted from an amount of moist soil equivalent to 0.25 g dry soil and an amount of 2 g freeze-dried root tissues using the MOBIO kit (MOBIO laboratories, Solana Beach, CA) and the DNeasy plant mini kit (QIAGEN, the Netherlands) according to the manufacturer’s instruction, except that soil DNA was finally eluted in 50 µl buffer. Table 3 summarizes the primers, thermocycling regimes and electrophoresis conditions used to analyse fungal and AM fungal communities. Partial sequences of 18S rRNA genes and 28S rRNA genes were analysed for total fungi and AM fungi, respectively. All PCR reactions, DGGE and cloning were carried out as described elsewhere (van der Wal et al., 2006a), except that we added 2.5 µl of bovine serum albumin (BSA; 4 mg ml⁻¹) to the PCR reaction mixture to reduce PCR inhibition. The initial denaturation steps were 94°C and 93°C for fungi and AM fungi respectively. Sequencing of DGGE bands was performed by Macrogen (Seoul, Korea). To roughly identify fungi, similarities between obtained sequences were compared with nucleotide sequences available in GenBank by using the nucleotide BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Statistical analyses

The banding patterns of DGGE gels were analysed using the Image Master 1D program (Amersham Bioscience, Roosendaal, the Netherlands). The resulting binary matrices were exported and used in statistical analysis as ‘species’ presence-absence matrices.

Ordination of samples resulting from image analysis of DGGE profiles was carried out using PCoA, based on Jaccard’s similarity. The use of Jaccard’s coefficient is recommended for binary species data like DGGE patterns scored for either presence or absence (Legendre and Anderson, 1999). The effects of the presence of collimonads (all three experiments), the soil type (experiment 3) and interaction of these factors as environmental variables on the microbial structure as analysed by PCR-DGGE were tested by db-RDA (Legendre and Anderson, 1999) in Canoco 4.5 for Windows (Ter Braak and Šmilauer, 2002). To test the effects of each of the two variables (the presence of collimonads and the soil type), the individual variables were recorded, using dummy binary-variables of which one was used in Canoco as the only environmental variable in the model and the other as covariable. To test the interaction, the variable entered in the model was the interaction between the presence of collimonads and the soil type, while both individual factors were included (without interaction) as covariables. The significances of such models were tested with 999 permutations.

All ANOVAs were carried out in Statistica 7.0 (StatSoft, Tulsa, OK). For ANOVA, data normality was tested with Shapiro–Wilks test and variance homogeneity by Levene’s test. When data failed to satisfy one of the tests, an appropriate transformation was applied (log or square root transformation). Turkey’s honestly significant difference (HSD) method modified for unequal sample size (Unequal N HSD in Statistica) was used for post hoc comparison with a 0.05 grouping baseline.

Acknowledgements

We thank Wiecher Smant and Hannes Gamper for technical assistance and advices, Etienne Yergeau for helpful comments about statistical analyses. Financial support for Sachie Höppener-Ogawa was provided by the Netherlands Organization for Scientific Research (NWO). This is publication No. 4416 NIOO-KNAW of the Netherlands Institute of Ecology.
References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DGGE gels of PCR-amplified fragments of 18S-RNA genes (fungi) extracted from nylon mesh bags containing acid-purified sand that had been buried for 8 weeks in an ex-arable field and a forest site. At the start of the field incubation, half of the bags contained sterile sand with addition of collimonads, whereas the other half contained sterile sand only. M: denotes a molecular marker made of an artificial mix of different fungal species.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.