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Summary

• In most studies, the distribution of the mycelia of ectomycorrhizal fungi in forest soils has been inferred from areas occupied by fruit bodies. Here, we investigated the below-ground spatio-temporal distribution of *Hebeloma cylindrosporum* by polymerase chain reaction quantification of a specific DNA sequence present in DNA extracted from soil.

• Soil samples were collected in a *Pinus pinaster* stand located in a campsite where fruit bodies of *H. cylindrosporum* had been mapped from 1990 to 2000.

• In samples collected underneath fruit bodies, DNA of *H. cylindrosporum* was always detected in large amounts. However, this DNA was often undetectable in the absence of fruit body even 1 yr after their disappearance. This result was supported by the failure to identify mycorrhizas of this species on seedlings planted in soil samples collected in places where fruit bodies were present 1 yr before sampling.

• This pattern suggests a recolonization of the site each year by *H. cylindrosporum* basidiospores in 'receptive' patches of ground created either by the local elimination of competitors or by local nutrient enrichments, which could frequently occur in a campsite. Our results demonstrate that an ectomycorrhizal species can be completely eliminated from the roots within 1 yr and does not necessarily contribute to the next generation of mycorrhizas.

Key words: below-ground mycelium, soil DNA, DNA quantification, ectomycorrhiza, population structure and dynamic, human disturbance, *Hebeloma cylindrosporum*.

Introduction

In forest soils, ectomycorrhizal (ECM) fungi can contribute up to one-third of the microbial biomass (Högberg & Högberg, 2002) and are associated with almost all assimilative roots of woody plant species in boreal, temperate and some subtropical forests (Smith & Read, 1997). These fungi are key players in biogeochemical cycles and significantly contribute to host plant nutrition. Nutritional exchanges between the two partners of the symbiosis take place in the mycorrhizas but the amount of nutrient transferred could well be related to the biomass of the extraradical fungal mycelium, which increases the volume of soil exploited by the root system.

In natural forest ecosystems, the roots of single trees are almost invariably associated with several different ECM species and different genets of these species (Dahlberg, 2001; Guidot *et al.*, 2001). Although a host plant is permanently associated with ECM fungi, the composition, species richness and diversity of the associated ECM fungal community change during the course of forest ageing (Last *et al.*, 1987) and also as a response to various disturbance regimes such as nitrogen deposition (Lilleskov *et al.*, 2002).

Several studies, usually carried out *in vitro*, have established that different ECM fungal species and different genets within a species display significant quantitative differences for functions related to, for example, nutrient uptake which will ultimately affect host plant nutrition and fitness (Smith & Read, 1997). To appreciate the beneficial effects of the symbiosis on the host plants therefore it appears necessary to identify the factors that affect the establishment, maintenance and turnover rate of the different individual fungal symbionts in the soil.

In this context, several studies have been conducted to unravel the structure and dynamic of ECM fungal populations. In most of these studies, the distribution and size of the fungal individuals (genets) have been inferred from the location and area occupied by genetically identical epigeous fruit bodies. These studies have shown that the spatial and genetic structures of the populations vary considerably between species. Moreover, for a given species these structures can differ across forest habitats and between years (Zhou *et al.*, 2000; Guidot *et al.*, 2002a; Jany *et al.*, 2002). Within a forest stand, the spatial distribution of fruit bodies of an ECM fungal species is often uneven and usually characterized by patches (Ogawa, 1977; Dahlberg, 2001). In some cases, each of these patches can be occupied by a single, large perennial genet which forms several fruit bodies year after year (Dahlberg & Stenlid, 1990, 1994; Dahlberg, 1997; Bonello *et al.*, 1998; Sawyer *et al.*, 1999; Gryta *et al.*, 2000; Fiore-Donno & Martin, 2001; Guidot *et al.*, 2002a). Alternatively, patches can also encompass numerous small, short-lived genets that apparently die soon after (within a year) fruiting (Gherbi *et al.*, 1999; Zhou *et al.*, 1999; Fiore-Donno & Martin, 2001; Redecker *et al.*, 2001; Guidot *et al.*, 2002a). From these results, conclusions have been drawn on the respective roles of long-term survival of the vegetative mycelia in the soil vs permanent recolonization through meiospore dispersal in structuring ECM populations. All of these conclusions are based on the assumption that the sampling and analysis of fruit bodies reflect the spatial distribution and lifespan of the fungal mycelia present in the soil which form the mycorrhizas. However, fruit body formation in most fungal species is known to be limited to a few days or weeks during the year and to be an unpredictable phenomenon which could therefore lead to false or biased estimates of demographic parameters of the populations studied. Therefore, the analysis of the vegetative fungal biomass (mycorrhizas and extraradical mycelia) present in the soil should depict more adequately the distribution, size and persistence of the ECM fungal genets in forest ecosystems (Horton & Bruns, 2001).

Genetic fingerprinting of the mycelia-forming mycorrhizas was performed in populations of *Suillus grevillei* associated with *Larix kaempferi* in Japan (Zhou *et al.*, 2001) and of *Hebeloma cylindrosporum* associated with *Pinus pinaster* in France (Guidot *et al.*, 2001). These studies revealed that, for these two fungal species, fruit body distribution matched the spatial distribution of the mycorrhizas, and that fruit body disappearance was followed by the disappearance of the mycorrhizas. However, from a practical view, the analysis of numerous mycorrhizas is time consuming and therefore the number of soil samples that can reasonably be studied is inevitably low. Moreover, the spatial distribution of mycorrhizas might not exactly match that of the extraradical mycelium, which can extend up to several decimetres away from the roots and may also proliferate in nutrient-rich patches independently of the location of mycorrhizas (Bending & Read, 1996; Lilleskov & Bruns, 2003).

As a substitute to the analysis of mycorrhizas, the study of the spatial and temporal dynamics of ECM populations can be conducted by estimating the distribution of the fungal mycelia in the soil. Soil fungal biomass can be inferred from quantitative polymerase chain reaction (PCR) amplification of a species-specific DNA sequence present in environmental DNA extracted directly from soil samples. This approach was developed for the ECM agaric basidiomycete *Hebeloma cylindrosporum* using the competitive PCR (cPCR) quantification method (Guidot *et al.*, 2002b). In a preliminary study conducted on soil samples collected underneath one patch of fruit bodies, this approach allowed us to show that *H. cylindrosporum* DNA was undetectable at more than 50 cm away from the fruit bodies (Guidot *et al.*, 2002b).

In the present paper, we used the same PCR quantification method to investigate in a forest stand both the below-ground spatial distribution of *H. cylindrosporum* and the life-span of its below-ground mycelia. The study was conducted in a 500 m^2 *P. pinaster* forest stand located in a campsite. This site was established in 1990 as a permanent study site for populations of *H. cylindrosporum*, which were followed till 2000 (Gryta *et al.*, 1997; Guidot *et al.*, 2001, 2002a). In this site, fruit bodies are produced every year within scattered patches of a limited size (often less than 0.25 m^2). These patches always include several small genets, each producing on average 1.6 fruit bodies. The exact locations of these fruit body patches very seldom overlap from one year to the next, thus suggesting that the distribution of the mycelia is patchy and that the genets are annual (Guidot *et al.*, 2002a).

The questions that were addressed in this study were: (1) how patchy is the below-ground distribution of *H. cylindrosporum* in this site?; (2) is it possible to detect DNA of this species outside the patches of fruit bodies?; and (3) is the disappearance of fruit bodies from one year to the next the result of the disappearance of the corresponding vegetative below-ground mycelia? In addition to the quantification of *H. cylindrosporum* DNA by cPCR in soil samples, we also looked for the presence of a potential inoculum of *H. cylindrosporum* capable of forming mycorrhizas on sterile *Pinus pinaster* seedlings planted in soil samples collected in places where fruit bodies of this fungus were absent.

Materials and Methods

Study site

The basidiomycete agaric *H. cylindrosporum* Romagnesi is a pioneer species commonly found in the European Atlantic sand-dune forest ecosystem. In South-west France, this species which is associated with *P. pinaster* trees occurs in two distinct habitats. The first (referred to as the 'dune habitat' in Guidot *et al.*, 2002a) is the extreme western fringe of the forest, which makes the transition with the bare dune. The second (referred to as the 'forest habitat' in Guidot *et al.*, 2002a) is more

Table 1 Soil samples collected in the 'Truc Vert' forest (TVF) site to quantify *Hebeloma cylindrosporum* DNA

Soil samples	Surface area (cm)[a]	Fruiting year	Sampling year	Number of fruit bodies above the collected samples
00/00-1 to -10[b]	10 × 10	2000	2000	5–7
98/99-1[c]	20 × 20	1998	1999	5
98/99-2[c]	30 × 30	1998	1999	6
97/98-1[c,d]	100 × 50	1997	1998	7
97/98-2[c,d]	100 × 50	1997	1998	12
95/98-1	20 × 20	1995	1998	8
95/98-2	20 × 20	1995	1998	13
94/98-1	20 × 30	1994	1998	6
93/98-1	30 × 40	1993	1998	34
90/98-1	50 × 50	1990	1998	17
--/99-1 to -10	20 × 20	–[e]	1999	0

[a]All soil samples were dug to a depth of 10 cm. [b]Samples 1–8 were collected immediately outside the TVF site (at less than 300 m distant). [c]*Hebeloma cylindrosporum* mycorrhizas were looked for in these soil samples (Guidot *et al*., 2001). [d]*Hebeloma cylindrosporum* mycorrhizas were looked for on the roots of pine seedlings planted in these soil samples. [e]Fruit bodies have never been observed on the surface of these soil samples in the 6 yr before sampling.

inland, within the forest, but exclusively in places subjected to human disturbance such as car park borders, recreational areas or campsites. The present study was conducted in the 500 m^2 (20 × 25 m) 'Truc Vert' forest site (TVF site in Guidot *et al*., 2001) located within a campsite within the 'forest habitat'. This site (44°43′ N, 1°15′ W) is approximately 50 km west of Bordeaux and 1300 m away from the Atlantic coastline. The campsite is much frequented in the summer period and almost or completely unoccupied in late autumn.

The area sampled is a monospecific forest stand of planted 50- to 60-yr-old *P. pinaster* trees. Partial litter removal to prevent fire, and human recreational activities prevent the establishment of ground vegetation and the building up of a humus layer. The soil is sandy without any obvious horizon (98–99% noncalcareous sand, 0.5–3% organic matter) with a pH (in water) of about 5.7. Most fine roots with mycorrhizas form a dense network in the top 10 cm of soil. In addition to *H. cylindrosporum*, numerous other ECM fungi fruit in this site including representatives of the genera *Amanita*, *Cortinarius*, *Inocybe*, *Laccaria*, *Lactarius*, *Tricholoma*, *Russula*, Pisolithus and *Suillus*.

Collection of soil samples

Soil samples were collected at the height of the fruiting period in the autumn of 1998, 1999 and 2000. The surface of the soil samples was lightly raked to remove large organic debris (such as pine needles) and the base of the fruit bodies when present and also to minimize the contribution of basidiospore DNA to the total amount of DNA quantified. All soil samples were dug to a depth of 10 cm in order to incorporate most of the fine roots. Different categories of soil samples were collected with respect to the presence or absence of fruit bodies of *H. cylindrosporum*.

In order to estimate the range of below-ground DNA variation underneath existing fruit bodies, 10 soil samples (10 × 10 × 10 cm) were collected in November 2000 underneath five to seven fruit bodies (samples 00/00-1 to 10, Table 1). Because only two patches with more than five fruit bodies were present on the TVF site, the eight others were selected nearby (at less than 300 m away) in the campsite.

In order to estimate the background level of *H. cylindrosporum* DNA in the soil of the TVF site, 10 soil samples (20 × 20 × 10 cm) were collected in places where fruit bodies of *H. cylindrosporum* had never been observed in the previous 6 yr. These samples were collected in 1999 and selected as follows: the 500 m^2 TVF site was divided into 20 5 × 5 m squares and one sample was taken in the centre of each of the 10 squares in which fruit bodies had never been observed between 1993 and 1998 (Fig. 1).

Finally, in order to determine whether or not mycelia of *H. cylindrosporum* genets persisted in the soil after the disappearance of fruit bodies, nine soil samples were collected in 1998 (seven samples) and 1999 (two samples) in places where fruit bodies were absent but where they had been observed 1–8 yr before. The surface area of these latter soil samples matched the surface area of the original patch of fruit bodies (Table 1).

Samples of soil were grossly sieved to remove pieces of long roots and thoroughly homogenized by hand. For each homogenized sample, three 0.5 g subsamples were taken and stored at −70°C until DNA extraction.

Soil DNA extraction and specific quantification of *H. cylindrosporum* DNA

DNA was extracted from the 0.5 g soil samples by using the commercial Fast DNA SPIN Kit for Soil (Bio101, Carlsbad, CA, USA) as described by Guidot *et al*. (2002b). DNA was

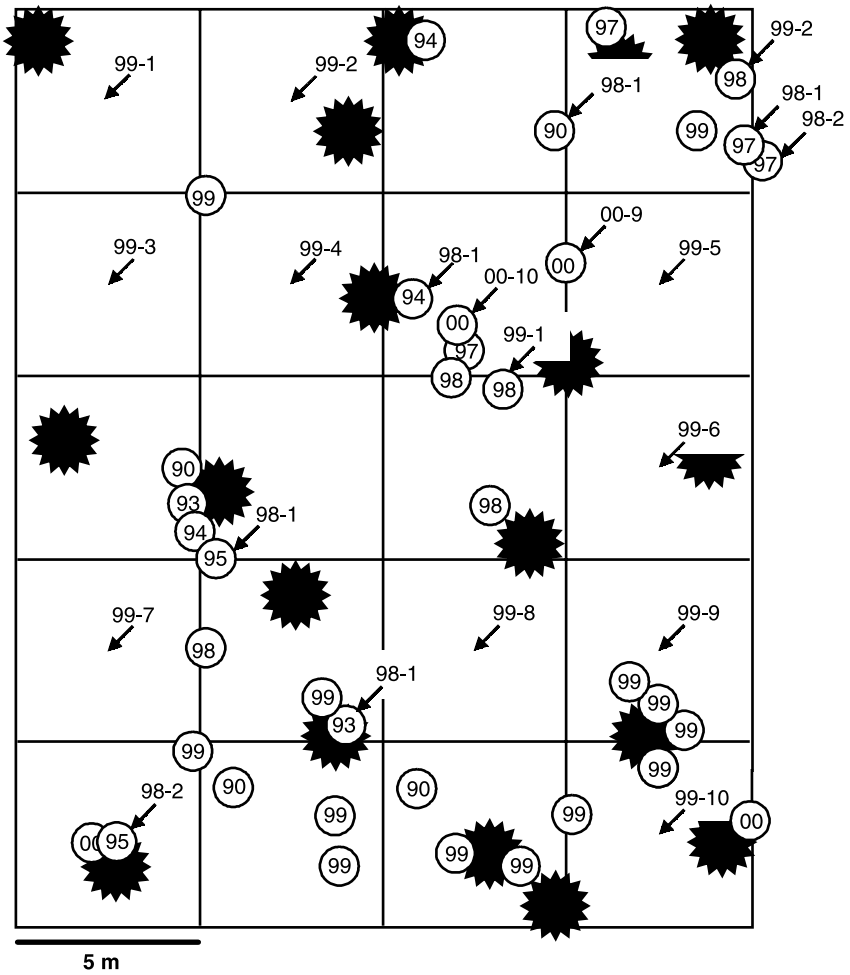

Fig. 1 Spatial distribution of *Hebeloma cylindrosporum* patches of fruit bodies in the 'Truc Vert' forest (TVF) site between 1990 and 2000 and location of the soil samples collected from which the *H. cylindrosporum* biomass was assessed. *Pinus pinaster* trees are represented by stars and each patch of fruit bodies by a circle with its corresponding year of observation, from 1990 (90) to 2000 (00); the site was not visited in 1991 and 1992; 1996 was characterized by a dry autumn, which prevented fruit body formation. Arrows point to the collected soil samples. For each sample, the collection year and a number are given. Only two of the soil samples collected in 2000 beneath fruit bodies appear in the figure; eight others were collected outside, at less than 300 m away, from the TVF site.

eluted using 50 µl H_2O and a final 45 µl solution was routinely obtained and stored at −20°C.

Specific quantification of *H. cylindrosporum* DNA was conducted by cPCR as described by Guidot *et al.* (2002b). The target sequence was a 533-bp long DNA fragment located in the nuclear rDNA untranscribed intergenic spacer 2 (IGS2). The competitor was a 758-bp long fragment constructed by insertion of a 225-bp long fragment into the target sequence (Guidot *et al.*, 2002b). Target and competitor sequences were amplified with the *H. cylindrosporum*-specific primers IGS2A and IGS2B (Guidot *et al.*, 1999, 2002b).

To quantify the target DNA initially present in the cPCR mix before amplification, we used the standard curves constructed in Guidot *et al.* (2002b) with 10 pg and 0.1 pg of competitor and an additional curve constructed with 100 pg of competitor DNA. The detection threshold of this cPCR protocol was 3 pg of *H. cylindrosporum* DNA g^{-1} of soil.

For each soil DNA extract, cPCR amplifications were performed three times. Results are presented as mean ± SE.

To test whether or not DNA extracted from basidiospores could be detected, between one and 10^7 basidiospores were artificially added to 0.5 g of γ-ray sterilized soil devoid of *H. cylindrosporum* DNA before purification by the fast DNA SPIN Kit for soil. Samples of 0.5 g of sterilized soil to which were added 2 mg of *H. cylindrosporum* mycelium or sterile H_2O before DNA extraction were used as positive and negative controls, respectively. The PCR reactions were performed as previously described, both in the absence and in the presence of 0.1 pg of competitor. This test was performed twice.

Bioassay seedlings

In addition to DNA quantification, we also analysed mycorrhizas formed in the laboratory on sterile *P. pinaster* seedlings (bioassay seedlings) planted in soil samples brought back from the field. This experiment was conducted on 50 seedlings planted in soil samples 97/98-1 and 97/98-2 collected in 1998 in places where *H. cylindrosporum* fruit bodies have been observed in 1997. As a positive control, 36 seedlings were planted in two soil samples collected underneath two distinct patches of fruit bodies. Surface-sterilized seeds (Debaud & Gay, 1987) were germinated at 18°C in sterile vermiculite saturated with water. One-month-old seedlings were planted in 400 cm^3 pots filled with soil. Plantlets were grown

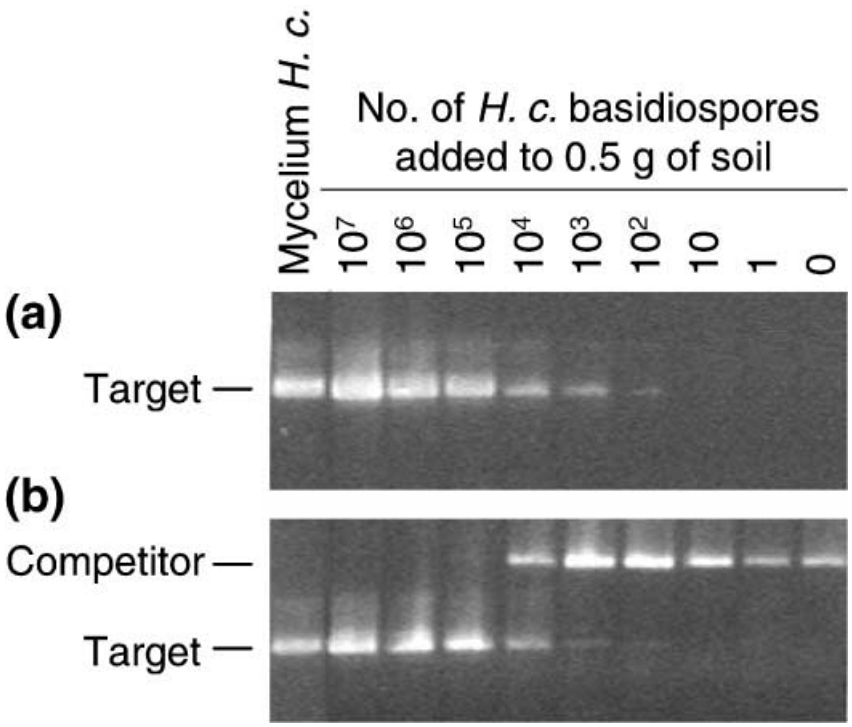

Fig. 2 Detection of *Hebeloma cylindrosporum* basidiospore DNA in soil by polymerase chain reaction (PCR) amplification of a specific target DNA. Between 1 and 10^7 basidiospores were artificially spiked to 0.5 g of γ-ray sterilized soil devoid of *H. cylindrosporum* DNA before DNA extraction. *Hebeloma cylindrosporum* (*H. c.*) mycelium or H_2O added to 0.5 g of sterilized soil before DNA extraction were used as positive and negative controls, respectively. The PCR conditions were similar to those used for the quantification of *H. cylindrosporum* DNA by competitive PCR (cPCR) in soil samples collected in the 'Truc Vert' forest site (Fig. 3). (a) PCR reaction without added competitor; (b) competitive PCR reaction with 0.1 pg of competitor.

3 months in a 22°C culture room under a 16-h light (1.2 W m^{-2} energy fluence rate)/8-h dark regime. Plants were watered once per week. After 3 months, mycorrhizas were collected and sorted by morphotypes. DNA was extracted from each mycorrhiza which matched the '*Hebeloma*' morphotype and the molecular identification of the fungal partner was performed by PCR amplification of the *c.* 1500 bp IGS2.1 segment of the rDNA IGS2 sequence using *H. cylindrosporum*-specific primers as described by Guidot *et al.* (1999, 2001).

Results

All 29 soil samples contained variable amounts of extractable DNA with a mean value of 1.27 μg g^{-1} of soil (range 0.62–2.46 μg g^{-1}, SE = 0.088). The extracted DNA was likely to contain genomic and organelle DNA not only from fungi, but also from bacteria, plant tissues and the microfauna. Mean values were similar whether or not fruit bodies of *H. cylindrosporum* were present on the surface of the samples at the time of collection.

The experiment conducted with *H. cylindrosporum* basidiospores artificially spiked to sterilized soil samples demonstrated that, in addition to mycelial DNA, basidiospore DNA could be extracted and detected. The detection threshold was about 100 basidiospores added to 0.5 g of soil (Fig. 2). As only 1 μl of the 45 μl of soil DNA extract was used for the PCR amplification, this value indicates that our PCR protocol has the potential to amplify the DNA of possibly two basidiospores (i.e. of four haploid nuclei of *H. cylindrosporum*).

The results obtained from specific quantification by cPCR of *H. cylindrosporum* DNA in the soil samples collected in the TVF site were in accordance with the presence or absence of fruit bodies above ground (Fig. 3). In the 10 soil samples collected underneath fruit bodies and used as positive controls, *H. cylindrosporum* DNA was always detected but in variable amounts (between 10 ng DNA and 325 ng DNA g^{-1} of soil; (mean ± SE) 112 ± 37 ng g^{-1} of soil). These values represented 1.5–20.1% of the total DNA in the soil samples. The amount of *H. cylindrosporum* DNA varied significantly even between samples collected underneath identical numbers of fruit bodies. For example, in samples 00/00-1 and 00/00-2, both collected underneath a patch of five fruit bodies, 282 ng and 68 ng of *H. cylindrosporum* DNA g^{-1} of soil were detected, respectively.

In the 10 soil samples collected in places where fruit bodies of *H. cylindrosporum* have never been observed in the previous 6 yr, DNA of *H. cylindrosporum* was not detected except in one sample where only 0.1 ng DNA g^{-1} of soil was quantified (Fig. 3). This value represents only 1% of the lowest value detected in soil samples collected underneath fruit bodies. This soil sample (--/99-10) was one of the closest (at less than 2 m away, Fig. 1) to sporulating fruit bodies at the time of sampling and may have consequently been covered by a higher number of basidiospores compared with other samples. In the other nine soil samples, the target sequence could not be amplified even in the absence of added competitor in the PCR reaction. Successful amplification of 0.1 pg of competitor added to the PCR reactions demonstrated that the lack

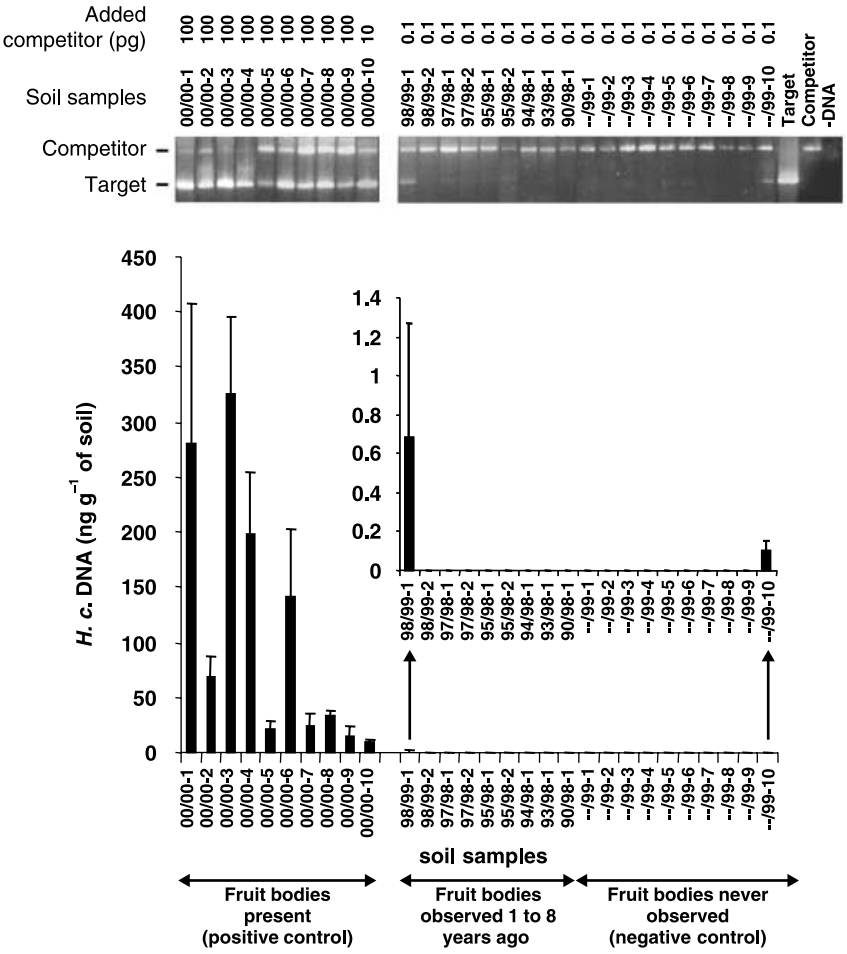

Fig. 3 Quantification of *Hebeloma cylindrosporum* genomic DNA by competitive PCR (cPCR) in soil samples collected in the 'Truc Vert' forest (TVF) site. The characteristics of each soil sample are given in Table 1. The SYBR Gold™ (Molecular Probes, Eugene, OR, USA) stained agarose gel illustrates one of the cPCR reactions carried out on the different soil-DNA extracts with different amounts of competitor DNA. The '-DNA' lane contains a control PCR reaction without added DNA. Mean values ± SE are given in the graphs below (three different 0.5-*g* subsamples were extracted for each soil sample and three cPCRs were performed for each soil extract).

of amplification of *H. cylindrosporum* DNA did not result from the presence of soil inhibitory molecules which would have been co-extracted with soil DNA (Fig. 3).

In the nine soil samples collected in places where fruit bodies had been recorded 1–8 yr before soil sampling, no DNA of *H. cylindrosporum* was detected except in one case where 0.7 ng DNA g^{-1} of soil was quantified (Fig. 3). This value represents 7% of the lowest amount of *H. cylindrosporum* DNA detected in a soil sample collected underneath fruit bodies. This soil sample (98/99-1) was one of the four samples collected in a place where fruit bodies were present 1 yr before sampling.

To confirm the absence of *H. cylindrosporum* infective propagules in soil samples above which fruit bodies were observed 1 yr before soil sampling, 50 sterile bioassay seedlings were planted in two of these samples brought back to the laboratory (samples 97/98-1 and 97/98-2). Three months after planting, almost all root tips were colonized by different ECM fungi. DNA was extracted from 198 mycorrhizas whose morphotype resembled *H. cylindrosporum* mycorrhizas. The *H. cylindrosporum*-specific IGS2.1 sequence (Guidot *et al.*, 1999) could not be amplified from any of these 198 DNA samples. As a positive control, 72 *P. pinaster* seedlings were planted in soil samples collected underneath fruit bodies. DNA was extracted from 96 mycorrhizas and the IGS2.1 sequence was successfully amplified from 88 (92%) of the DNA extracts.

Discussion

Temporal dynamic of the below-ground spatial distribution of *H. cylindrosporum* in a campsite

This study is the first direct analysis which reveals the temporal dynamic of the spatial distribution of below-ground DNA of an ECM species. Until now, seasonal fluctuations in the ECM biomass had been measured without making distinctions between species (Wallander *et al.*, 1997, 2001).

The results were expressed in term of amount of DNA g^{-1} of soil without making distinctions between basidiospore DNA and mycelial DNA. Until now, no reliable method has been developed to separate these two kind of fungal cells which play very different ecological functions. We tried to minimize the contribution of basidiospore DNA to the total

amount of DNA by removing a thin layer of topsoil before collecting the soil samples, but we cannot exclude the possibility that some spores might have migrated in the sandy soil following rainfall. At the time of sampling, sporulating fruit bodies were present in the study site and it can be assumed that the entire soil surface was covered with basidiospores. However, their distribution is not uniform and might be a function of the distance to the nearest fruit bodies.

Our data demonstrate first that within the forest site studied, the mycelia of *H. cylindrosporum* are unevenly distributed, because its DNA could only (except in two cases) be detected in soil samples collected underneath existing fruit bodies. The absence of DNA in soil samples collected away from fruit bodies or in places where fruit bodies had been found in previous years is a clear indication that there are neither basidiospores nor mycelia of the species studied in these samples (at least not in any appreciable quantity). The low amounts of *H. cylindrosporum* DNA found in two patches in the absence of fruit bodies could result from the dilution of a dense network of hyphae colonizing a very small subsample of the soil sample (e.g. a nutrient-rich patch), from a very diffuse network of hyphae colonizing the entire soil sample or from basidiospores present on the soil surface. Earlier studies conducted on a limited number of patches of fruit bodies showed that *H. cylindrosporum* DNA could not be detected in the soil at more than 50 cm away from the nearest fruit body (Guidot *et al.*, 2002b) and also that mycorrhizas of this species were extremely rare or could not be found in the absence of fruit bodies above ground (Guidot *et al.*, 2001). However, even the most exhaustive sampling designs will not guarantee that patches of mycelia which do not form fruit bodies do not exist within the study site. In future studies it would be interesting to test whether the ECM mycelia distribution in a forest ecosystem is random or follows hidden spatial patterns, which could result from tree architecture and tree distribution (Bruckner *et al.*, 1999). Appropriate sampling designs allowing geostatistical analyses can be used to address this question (Ettema & Wardle, 2002). In the case of the TVF campsite, we believe that the locations of the *H. cylindrosporum* patches are most likely the result of a stochastic dynamic resulting from disturbances caused by human recreational activities.

Second, our data clearly demonstrate the near or complete disappearance of *H. cylindrosporum* DNA in the soil in the year following fruit body formation. This result was further supported by the failure to identify *H. cylindrosporum* mycorrhizas on bioassay seedlings planted in soil samples collected in places where fruit bodies were present 1 yr before sampling. This last result also suggests that the basidiospores potentially present in the samples did not act as an active inoculum. Future experiments should focus on the kinetic of below-ground mycelium disappearance during the 12 months after fruit body formation.

Until now, no experimental study has been conducted to identify the causal processes controlling the temporal dynamic and spatial structures of ECM populations. The results obtained on *H. cylindrosporum* populations surveyed over a 10-yr period in the same forest campsite demonstrate that, in this site, numerous new genets appear each year forming mycelia, mycorrhizas and fruit bodies within very small soil areas (< 0.25 m^2) (Gryta *et al.*, 1997; Guidot *et al.*, 2001, 2002a,b; this study). These genets disappear in the year following fruiting while other genets appear in other soil areas. This suggests a major role of the recolonization of the site each year by basidiospores in small 'receptive' patches. Such patches could result from the high level of human-induced disturbances during the summer in this campsite. We may distinguish two types of disturbances: (1) disturbances that affect the site uniformly and permanently, such as soil trampling which prevent the establishment of ground vegetation and the accumulation of litter and humus; and (2) patchy and temporary disturbances that result from local deposit of various detritus or liquid pollutants (e.g. detergents) or soil digging. These two types of disturbances are probably complementary and necessary with respect to the colonization of *H. cylindrosporum*. For example, soil trampling and prevention of litter accumulation have both been reported to affect ectomycorrhizal colonization (Baar & Vries, 1995; Waltert *et al.*, 2002). Pollutant inputs and soil digging are both likely to eliminate locally several soil-dwelling organisms including ECM species thus creating 'empty patches' available for the local recruitment of several genets of *H. cylindrosporum*. An alternative hypothesis to the 'empty patches' one is the localized nutrient enrichment, which may also occur frequently in a campground. Nutrient enrichment leads to shifts in competitive interactions between species because of altered resource levels rather than pioneer colonization of new substrate in the absence of competitors. In support to the 'empty patches' hypothesis, we can say that perennial genets of *H. cylindrosporum* occur in the Dune habitat in which few other ECM species occur (Guidot *et al.*, 2002a). This might suggest that *H. cylindrosporum* is a poor competitor that can only thrive in species-poor communities. In favour of the 'nutrient-enriched patches' hypothesis, several *Hebeloma* species, but not all, have been reported to respond positively to nitrogen inputs (in the form of ammonia or urea) in forest sites and have been qualified as 'ammonia fungi' (Sagara, 1992). However, none of these studies distinguished between two potential effects of nutrient enrichment: simple stimulation of fruit body formation from pre-existing mycelium or *de novo* colonization by means of spores.

New insight on the temporal dynamic of the below-ground ECM community

The vegetative structures of *H. cylindrosporum* disappear within 1 yr after fruit body formation. If it was known that mycorrhizas are short-lived structures, being continuously replaced by new ones on the same roots (Persson, 1979;

Alexander & Fairley, 1983; Santantonio & Santantonio, 1987), it has always been implicitly assumed that the fungal species already present on the root system contributed predominantly to the formation of the new mycorrhizas. Our results demonstrate that this may not always be the case and that one species which can occupy a significant fraction of the root system can be completely eliminated and replaced in the 'next generation' of mycorrhizas. To understand the mechanisms of *H. cylindrosporum* replacement on the root system, it will be necessary to study the entire ECM community to identify the species which possibly outcompete and eliminate *H. cylindrosporum*. It will be of special interest to determine if these species were already present on the root system and if they increase in abundance after *H. cylindrosporum* elimination. These questions can be addressed by community analysis of the DNA extracted from soil samples, as illustrated by the works of Dickie *et al.* (2002) and Landeweert *et al.* (2003).

The forest ecosystem we studied presents some extreme characteristics in term of intensity and frequency of disturbances it is subjected to. Nevertheless, the occurrence of ECM species characterized by genets which form small patches of fruit bodies and only during one season has also been reported in seemingly undisturbed forest ecosystems (Gherbi *et al.*, 1999; Redecker *et al.*, 2001). The respective contribution of perennial vs annual ECM mycelia to the global functioning of the symbiosis remains unknown. Fungal species capable of rapidly colonizing empty niches by means of spores could provide a greater stability to the system by maintaining the entire root system colonized by ECM symbionts.

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