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Competitive Interaction between Two Aquatic Hyphomycete Species and Increase in Leaf Litter Breakdown

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Abstract

Aquatic hyphomycete species produce large numbers of conidia which rapidly colonize the leaf litter that falls into rivers during autumn. Our objective was to understand how a species which produces many fewer conidia than another in laboratory conditions can nevertheless be codominant in a natural setting. In microcosm studies with two pioneer dominant species, *Flagellospora curvula* and *Tetrachaetum elegans*, inoculated on alder leaves, we first verified that the ratio of the conidium production of both species (6 to 7:1) was inverse to that of individual conidial masses (1:7) as previously described. Calculating the percentage of leaf mass loss that corresponds to 1 mg of conidial mass produced, the combination of the two species produced 2.9-fold more loss than the mean of each species. By contrast, the reproductive biomasses of *F. curvula* and *T. elegans* were 5.2- and 2.6-fold lower, respectively. As a result, the conidium production of *F. curvula* in the combination was only 3.2-fold that of *T. elegans* instead of 6- to 7-fold in pure culture. In a mixed culture of the two species, *T. elegans* conidia had a high germination potential (>90%) whereas the proportion of germinated *F. curvula* conidia was only 50%. Moreover, *T. elegans* reduced the area on which *F. curvula* could grow on poor and rich solid media. These results indicate that the dominance of *F. curvula* conidia in the river may be partly controlled by *T. elegans* and suggest that a negative interaction between microfungi may have a positive effect on the ecosystem functioning.

Introduction

Aquatic hyphomycetes are a key group in the breakdown of terrestrial leaf litter submerged in streams [4]. High estimates of fungal biomass associated with submerged leaf litter [2, 5, 10, 11, 23] and correlation between leaf breakdown coefficients and fungal growth parameters [11, 16, 26] provide two lines of evidence that litter breakdown is primarily mediated by these fungi.

Aquatic hyphomycetes are very early colonizers on fresh fallen leaves and twigs whose input can represent several tons of allochthonous organic matter per hectare of river bed [29]. To colonize multiple substrate units, aquatic hyphomycete species allocate 50% or more of their production to the formation of large numbers of conidia [12, 22] of various shapes and sizes [13, 14]. This results in the development of several colonies of the same species on a single decomposing leaf [6, 7, 19] that is typically colonized by 4–12 species through much of the leaf's decay [19, 20, 27]. This suggests that intra- and interspecific interactions can frequently occur among these fungi. *In vitro* testing of intraspecific hyphal interactions during growth of aquatic hyphomycetes revealed that nonaggressive behaviour between individuals is a general rule [3]. By contrast, almost all interspecific interactions resulted in growth inhibition of one or both of the members of an interacting pair [21].

Interaction between aquatic hyphomycete species may occur at the various steps of the leaf litter colonization [4]: conidial attachment and germination, mucilage secretion, appressorium formation, and hyphal growth within the substrate. Conidium production varies >10-fold between the different species, and the number of conidia produced is inversely proportional to the individual conidial mass [9]. Germination rates and development of attachment structures by conidia on artificial substrates change considerably among aquatic hyphomycete species [1, 18]. Mycelial growth at a given temperature along with the optimal temperature of growth and conidium production also vary with the species [9]. Large differences in particular exoenzyme activities produced by aquatic hyphomycetes and in-

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volved in the degradation of natural polymers have been reported [25].

Interspecific interactions are expected to partly control aquatic hyphomycete community structure and dynamics. Since the strategy of these fungi is to produce large numbers of conidia, our objective was to understand how a species which produces many fewer conidia than others in laboratory conditions can nevertheless be codominant in a natural setting. We selected two pioneer dominant species from southwestern France, *Flagellospora curvula* Ingold and *Tetrachaetum elegans* Ingold, whose individual conidial masses are in a 1:7 ratio, respectively. We first confirmed that the number of conidia produced was inversely proportional to the individual conidial mass. We then found that both reproductive output and leaf biomass loss were modified when the two species were in combination. Hence, we investigated fungal germination and growth rates to explain the interactions between the two species.

Methods

Leaf Litter Processing and Fungal Isolates. Freshly fallen alder (*Alnus glutinosa* (L.) Gaertn.) leaves were collected in autumn. Intact leaves with no visible fungal colonization were rinsed with deionized water and frozen in small packs. Upon thawing, 1-cm diameter disks were cut from all the leaves of a pack avoiding principal and secondary veins. After drying at room temperature the leaf disks were autoclaved [12].

Two monosporic isolates, *Flagellospora curvula* Ingold 129–680 and *Tetrachaetum elegans* Ingold 29–675, were used. Conidia were isolated from streams of the Montagne Noire (southwestern France). These strains were maintained on solid 2% (w/v) malt extract 2% (w/v) agar (MEA) medium. Individual conidial masses of 0.19 and 1.42 ng for *F. curvula* and *T. elegans*, respectively, were determined by weighing as reported by Chauvet and Suberkropp [9]. These values were used in calculations of reproductive output.

Conidium Production. Conidium production was determined in stream-simulating microcosms as described by Suberkropp [22] and Chauvet and Suberkropp [9]. Twenty leaf disks were incubated in 40 mL sterile medium (10 mg of KNO_3, 0.55 mg of K_2HPO_4, 10 mg of $MgSO_4$ $7H_2O$, 0.1 g of $CaCl_2$ $2H_2O$, and 0.5 g of 3-morpholinopropanesulfonic acid (MOPS) per liter of water, pH 7.0) under constant aeration. The medium was replaced every 2 days and the number of conidia released for each 2-day period was determined. The number of conidia inoculated per microcosm varied from 100 to 10,000. Leaf disks were submerged overnight in the medium under aeration before the experiment. After inoculation, the aeration was maintained for 15 min, stopped for 2 h, and then restarted for the duration of the experiment. The temperature of incubation was 16°C. The end of the experiments for the different inocula corresponds to the day of the last conidial count. For each inoculum size, three microcosms were inoculated.

Leaf Litter Mass Loss. Dry mass of leaf disks was determined at the end of conidium production by filtration on preweighed 47-mm-diameter Whatman GF/C filters. Drying of filters before preweighing and leaf disks on filters was performed at 105°C for 3 days. The determinations were carried out in triplicate. The percentage of leaf mass loss was determined as $(DM_L - DM_F)/DM_L \times 100\%$ where DM_L is the dry mass of 20 autoclaved (three replicates), noninoculated leaf disks after leaching for 40 d in the medium (changed every 2 d) and DM_F the final dry mass of inoculated leaf disks at the end of the experiment.

In Vitro Germination. To determine germ tube production by single species, conidial suspension was taken from a microcosm 20 or 30 days after inoculation with 3000 conidia of *Flagellospora curvula* or *Tetrachaetum elegans*, respectively. One mL of the conidial suspension (containing 150–300 conidia of each species) was spread over a 90-mm-diameter Petri dish containing 2% (w/v) agar (Agar) media. Petri dishes were incubated at 16°C, and conidia producing at least two germ tubes were recorded as germinated from 100 conidia examined at different times from 0 to 24 h. Three 50-mm-diameter Petri dishes were prepared for each determination.

To study interactions between the species, two experiments were performed. One experiment was designed to test the effect of interaction between conidia and colonized leaf disks: five disks taken from a microcosm 20 days after inoculation with *F. curvula* together with five disks taken from a microcosm 30 days after inoculation with *T. elegans* were incubated for 3 days for conidial production in a new microcosm with 20 mL of fresh medium. The other experiment was designed to test whether the inhibition of germ tube production is mediated by a soluble compound: after 3 days incubation, 10 mL conidial suspension from each microcosm used to determine germ tube production by single species (see above) was mixed under aeration in a new microcosm without leaf disks for 1 day. After incubation, 1 mL of the conidial suspension was processed at each time as described above.

Mycelial Growth and Growth Interaction. Growth of single species and growth interaction between *Flagellospora curvula* and *Tetrachaetum elegans* were measured on MEA and Agar media at 16°C as described in Chauvet and Suberkropp [9] with modifications. When a single

species was studied, a cylindrical plug (1 cm diameter) was cut from the leading edge of a colony grown on MEA and inoculated in the center of a 50-mm-diameter Petri dish containing either MEA or Agar medium.

To measure growth interaction, two sets of experiments were carried out as described by Bärlorcher [3]: (1) a plug of *F. curvula* was placed at a distance of ~1 cm from a plug of *T. elegans* and (2) one half-plug of each species was placed side by side in the center of the Petri dish. The radius of each colony (two measurements per plate) was measured at intervals of 1 to 4 days. Three 50-mm-diameter Petri dishes were used for each determination.

Statistical Analysis. Percentages of leaf mass loss were arcsine-transformed before ANOVA. Differences were considered significant when $P < 0.05$. Statistical analyses and calculations were performed with the Systat 5.2.1. computer package [30].

Results

Sporulation as a Function of Inoculum Size. The sigmoidal pattern of cumulative conidium production described by Chauvet and Suberkropp [9] was observed whatever the inoculum size for both *Flagellospora curvula* (Fig. 1A) and *Tetrachaetum elegans* (Fig. 1B). The total conidium production on alder leaf disks increased as a function of inoculum size for each species up to 3000 conidia inoculated. Comparable cumulated numbers of conidia were produced at the two highest inocula, namely 3000 and 10,000 conidia, indicating that a saturation of conidium production occurred at these inoculum sizes. Depending on the inoculum size, *F. curvula* produced 4- to 6.8-fold more conidia than *T. elegans* (Fig. 1). The lag time before production of conidia for both species was inversely related to the inoculum size from 100 to 3000 conidia (> 54–70 days, 30–52 days, 26–44 days, and 12–16 days for 100, 300, 1000, and 3000 conidia, respectively). The length of the period of highest production rate (corresponding to the peak of daily production and the near-linear part of the sigmoidal curve) varied slightly between *F. curvula* and *T. elegans* for inocula 1000 (10 and 12 days) and 300 (16 and 18 days). By contrast, for inocula 10,000 and 3000 the length of this period for *T. elegans* (28 and 32 days) was 2- and 2.9-fold that observed for *F. curvula* (14 and 11 days).

Leaf Biomass Loss and Fungal Biomass Allocated to Conidia. Percentage of leaf biomass loss and fungal biomass allocated to conidia significantly decreased as the inoculum size of either *Flagellospora curvula* or *Tetrachaetum elegans* decreased from 10,000 to 100 (Table 1; ANOVA, $P < 0.05$). However, the percentages

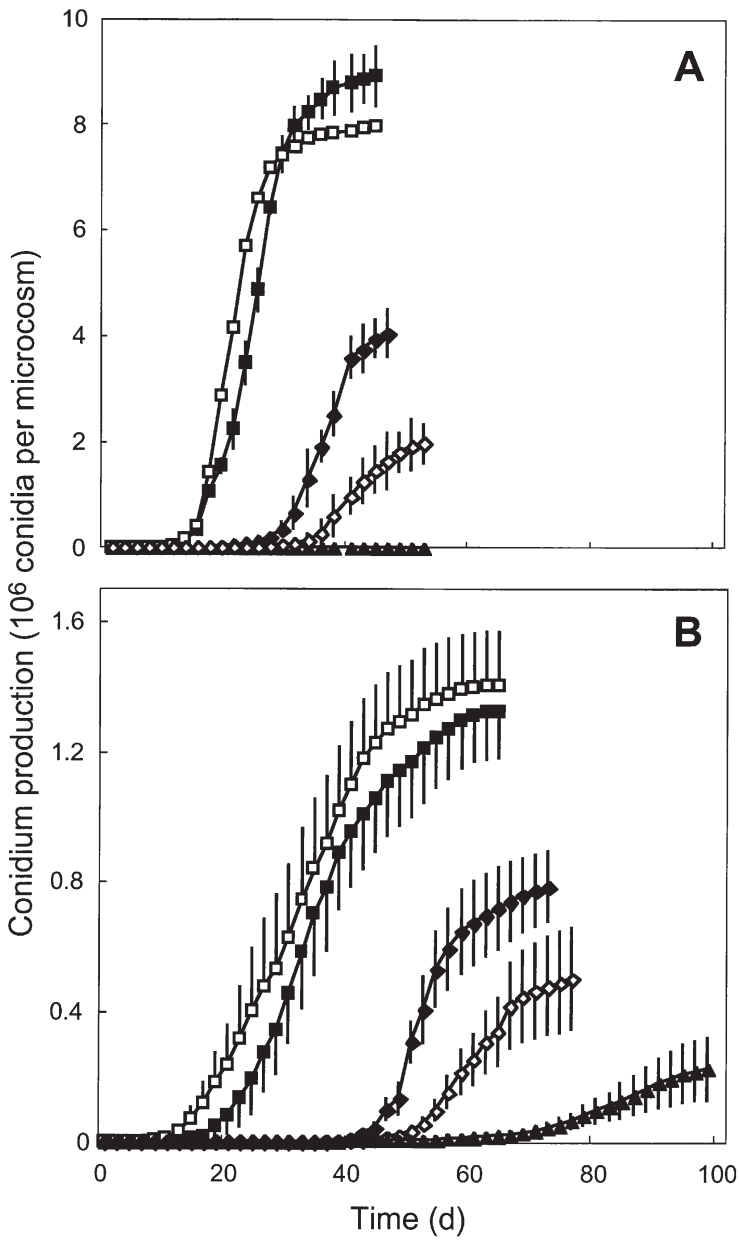

Figure 1. *Flagellospora curvula* (A) and *Tetrachaetum elegans* (B). Sporulation on alder leaf disks as a function of the number of conidia inoculated, ■: 10,000; □: 3000; ♦: 1000; ◇: 300; ▲: 100 conidia per microcosm. The experiment with the microcosms inoculated with 100 *F. curvula* conidia had to be stopped for technical reasons. Vertical bars indicate ± 1 SE ($n = 3$).

did not differ significantly between the two inoculum sizes of 3000 and 10,000 conidia or between the two species (Bonferroni's adjustments). Hence, the mean of the four values of leaf mass loss (30.5, 32.5, 34.7, and 36.8%; Table 1) was calculated as 33.6 ± 2.7(SD)%. As no significant change in the percentage of leaf mass loss was observed between inoculum sizes of 3000 and 10,000 conidia for both species, an inoculum size within this range was expected to produce a percentage close to the calculated mean. The effect of species assemblage was then tested using an inoculum of 5000 conidia of each species (corresponding to a total of 10,000 conidia). In the case of an additive effect, the

Table 1. Percentage of leaf mass loss and fungal biomass allocated to conidia on alder leaf disks incubated with various inocula of *Flagellospora curvula*, *Tetrachaetum elegans*, or a combination of the two species[a]

	Inoculum (conidia per microcosm)				
	10,000	*3,000*	*1,000*	*300*	*100*
F. curvula					
Mass loss	30.5% (3.8)	32.5% (n.a.)	29.9% (11. 2)	17.1% (3.8)	n.a. (n.a.)
Conidia mass	1.71 mg (0.19)	1.52 mg (n.a.)	0.76 mg (0.15)	0.38 mg (0.12)	n.a. (n.a.)
T. elegans					
Mass loss	34.7% (2.6)	36.8% (4.7)	25.7% (7.0)	13.0% (7.1)	5.8% (5.3)
Conidia mass	1.85 mg (0.21)	1.91 mg (0.21)	0.99 mg (0.12)	0.71 mg (0.24)	0.28 mg (0.13)
F. curvula (5,000) + *T. elegans* (5,000)					
Mass loss	56.3% (1.0)				
Conidia mass	1.02 mg (0.13) [*F. curvula*: 0.31 mg (0.05); *T. elegans*: 0.71 mg (0.18)]				

[a] The initial dry mass of 20 leaf disks was 98.4 ± 2.1 mg (n = 3) and DM_L = 73.4 ± 1.6 mg. The mass of reproductive output is the product of the individual conidium mass by the total number of conidia released in the results shown in Fig. 1. Mixed inoculum: the total reproductive biomass along with the contribution of each species in the combination are given. Values: mean (SE). (No. of replicates = 3; n.a.: not available.)

leaf mass loss would still be within the range of the calculated mean. In combination, the time course of conidial production, including the lag period and the duration of conidia release for each species, was similar to those observed for single species for inocula 3000 and 10,000 (data not shown). This combination of species resulted in a 67% significant increase in the percentage of leaf mass loss (−33.6 to −56.3%; t-test, $P < 0.00001$). Calculating the percentage of leaf mass loss that corresponds to 1 mg of conidial mass produced from the data presented in Table 1, the combination produced 2.9-fold more loss (−55.2%) than the mean of each species for inocula 3000 and 10,000 (−19.3%). On the contrary, the reproductive biomass of *F. curvula* and *T. elegans* decreased 5.2- (from 1.62 to 0.31 mg) and 2.6-fold (from 1.88 to 0.71 mg), respectively. In the combination, the number of conidia produced by *F. curvula* was only 3.2-fold that produced by *T. elegans* (1.63 and 0.51 × 10^6 conidia, respectively).

Germination Potential. When each species was incubated alone in a microcosm, germ tube production by *Flagellospora curvula* conidia was faster than that observed with *Tetrachaetum elegans*: after 5 h, 63% *F. curvula* conidia had germinated as compared to <1% for *T. elegans* (Fig. 2). Both species reached a plateau at 9–11 h corresponding to 97–98% germination. In a mixed culture of the two species *T. elegans* conidia still had a high germination potential (>90%) whereas the proportion of germinated *F. curvula* conidia was only 50%. Germ tube production by *F. curvula* and *T. elegans* conidia was delayed for about 5 and 2 h, respectively, as compared to the germination of single species. Similar results were obtained when conidia containing medium from single cultures of each species were mixed for one day (Fig. 2) indicating that *T. elegans* conidia and/or conditioned medium can inhibit germ tube production by conidia of *F. curvula* in suspension.

In Vitro Mycelial Growth. As shown in Fig. 3 growth rates of *Flagellospora curvula* and *Tetrachaetum elegans* were similar on MEA medium with 0.70 and 0.71 mm day^{-1}, respectively. On Agar medium, the growth rate of *T. elegans* was significantly higher than

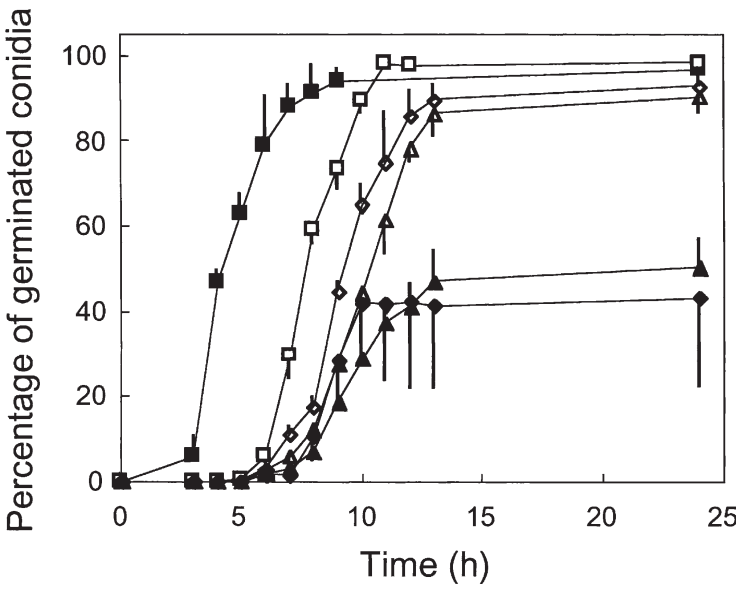

Figure 2. *Flagellospora curvula* and *Tetrachaetum elegans*. Kinetics of germ tube production (see Methods for details). Germination of *F. curvula* (closed symbols) and *T. elegans* (open symbols) conidia produced from (1) (■ and □) single cultures on alder leaf disks inoculated with either *F. curvula* or *T. elegans*, (2) (▲ and △) a mixed culture of two samples of leaf disks, each sample inoculated with either *F. curvula* or *T. elegans*, and (3) (♦ and ◇) a mix of conidia containing media from single cultures of the two species. Vertical bars indicate ± 1 SD (n = 3).

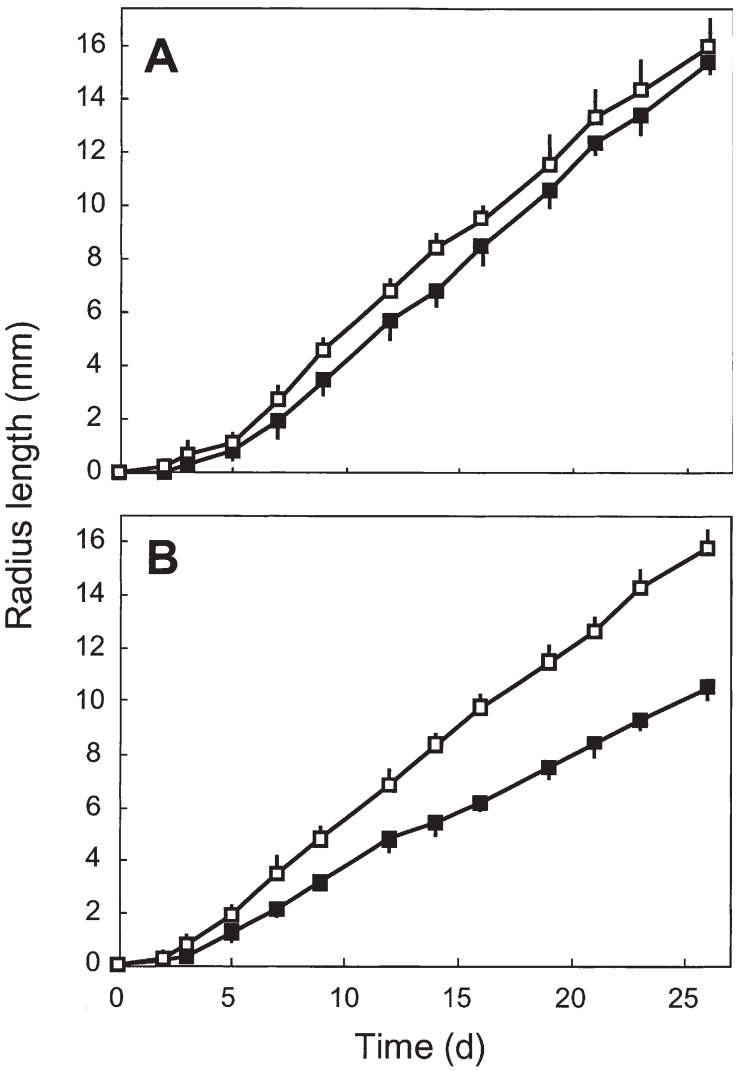

Figure 3. *Flagellospora curvula* and *Tetrachaetum elegans.* Growth kinetics from single plain plugs placed in the center of a Petri dish containing MEA (A) and Agar (B) media. ■: *F. curvula* and □: *T. elegans*. Vertical bars indicate ± 1 SD (n = 3).

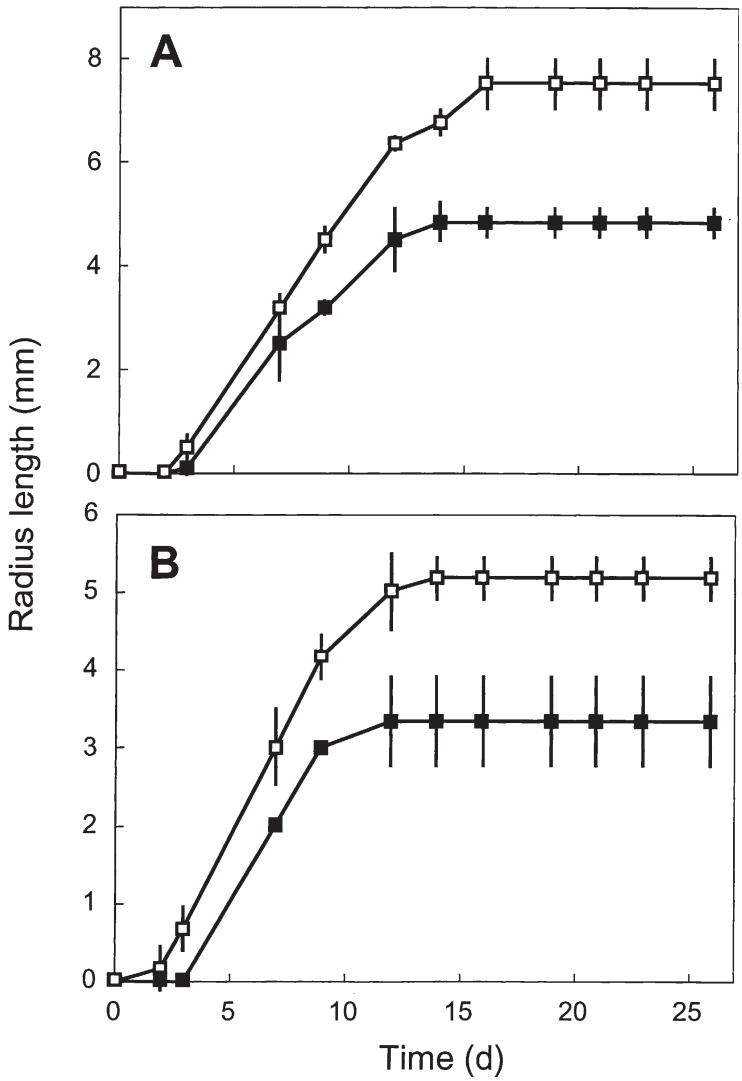

Figure 4. *Flagellospora curvula* and *Tetrachaetum elegans.* Growth kinetics from plain plugs placed 10 mm apart in a Petri dish containing MEA (A) and Agar (B) media. ■: *F. curvula* and □: *T. elegans.* Vertical bars indicate ± 1 SD (n = 3).

that observed for *F. curvula* (0.66 compared to 0.42 mm day^{-1}; ANCOVA, $P < 0.00001$).

When a plug of *Flagellospora curvula* was placed at a distance of ~1 cm from a plug of *Tetrachaetum elegans* on MEA medium, the growth of the dense mycelium of each species stopped (Fig. 4) just before the hyphae became intermingled (distance between the mycelia < 0.5 mm). On Agar medium, the distance between the dense mycelia was greater (~2 mm) while a few hyphae of each species were closer. In both cases, growth of *F. curvula* stopped before that of *T. elegans*.

Using two half-plugs of the same species placed side by side in the center of the Petri dish the mycelia grew 180° round each half plug: the mycelium growing from one half-plug did not invade the area on which the mycelium from the other half-plug was growing (data not shown). When half-plugs of *Flagellospora curvula* and *Tetrachaetum elegans* were placed side by side, the growth measured by the radial length of the mycelium growing from the half-plugs resulted in similar kinetics to those shown in Fig. 3 (data not shown). However, as shown in Fig. 5, *T. elegans* progressively reduced the area on which *F. curvula* could grow in the two media; this effect was greater on Agar (Fig. 5B) than on MEA (Fig. 5A) media.

Discussion

In the present study, the time lag before conidium production on alder leaf discs was 10–12 days for the two species studied, *Flagellospora curvula* and *Tetrachaetum elegans*, when microcosms were inoculated with 3000 or 10,000 conidia (Fig. 1). Under identical conditions, the same period without conidium production was observed on oak (*Quercus pubescens* Wild.; data not shown). These results can be compared to those of Chauvet and Suberkropp [9], who found a lag period of 7–9 days with eight species of aquatic hyphomycetes inoculated on yellow poplar (*Liriodendron tulipifera* L.) under similar

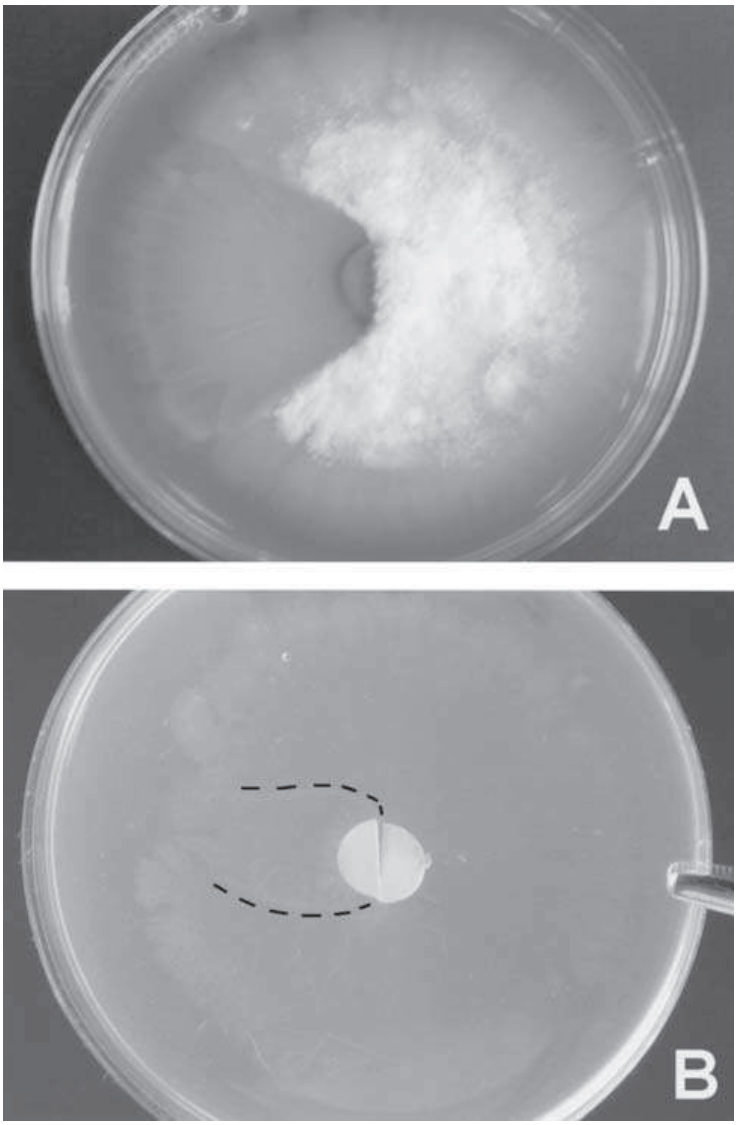

Figure 5. *Flagellospora curvula* and *Tetrachaetum elegans.* Mycelial growth on MEA (A) and Agar (B) media from two half-plugs placed side by side in the center of a Petri dish. The half-plug of *F. curvula* is on the left and *T. elegans* inoculum on the right. In B, the faint white limit between the two mycelia has been delimited with a dashed line. Photographs were taken after 50 days (A) and 64 days (B).

conditions. In our study it appeared that changing the inoculum size resulted in a change in the length of this lag time. Namely, the period before conidial production was inversely related to the inoculum size from 100 to 3000 conidia for both species (Fig. 1). This result suggests that conidium production on alder is triggered by the interaction between the colonies: a longer time is required to produce larger colonies before they can interact when conidia are inoculated at low surface densities. This seems to be related to the quality of the alder leaf since using oak leaf disks we found that the lags before conidial production of *T. elegans* were very similar (10–12 days) whatever the inoculum size, whereas the reproductive biomass was lower on oak than on alder (data not shown). As alder leaf litter decomposes seven-fold faster than oak [11], we may hypothesize that interactions between colonies mainly triggers conidial production on easily degradable litter, whereas the difficulty of decomposing the substrate may be the primary factor that induces aquatic hyphomycetes to produce conidia on refractory litter. Other experiments with other leaf litter and aquatic hyphomycete species will allow precise determination of the importance of litter quality in species interactions.

After the inoculum sizes that correspond to maximal conidial production had been determined, we studied the combination of the two species. On the one hand, the increase in the percentage of detrital leaf mass loss observed when both *Flagellospora curvula* and *Tetrachaetum elegans* were growing on the same leaf disks (Table 1) suggests that the two species worked in synergy to degrade the substrate. Assessment of the synergy requires approaches such as the construction of isobolograms (graphs of equally effective inoculum pairs, i.e., isoboles, for a single effect level) based on the equation of Loewe additivity [15]. The greater performance of the combination of the two species as compared to single species to degrade alder leaves may be due to differences in exoenzyme activities produced by *F. curvula* and *T. elegans*. For example, Suberkropp et al. [25] reported that *F. curvula* exhibits ~8-fold more pectin lyase activity than *T. elegans* and a protease activity that is not present in the latter. In contrast α-D-galactosidase and carboxymethylcellulose activities of *T. elegans* are 11- and 7-fold higher than those produced by *F. curvula*. The levels of exoenzyme activities produced in the litter may be quite different than the potential activities determined *in vitro* on pure substrate because of differential inhibition by the constitutive compounds of the leaf. Hence, the substrate quality may modulate the interactions between species. On the other hand, the reproductive output of both species was depressed in the combination, *F. curvula* being more affected than *T. elegans* (Table 1). *F. curvula* produced only 3-fold more conidia than *T. elegans* as compared to the 6- to 7-fold when each species was grown separately on alder leaf disks after inoculation of 3000 or 10,000 conidia per microcosm. Hence the dominance of *F. curvula* conidia in the river may be partly controlled by *T. elegans* when high numbers of conidia of the latter are present in the water flow.

Understanding the interaction observed between *Flagellospora curvula* and *Tetrachaetum elegans* on alder leaf disks was the next objective of this study; it was achieved by measuring their germination potential and mycelial growth. In mixed cultures, germ tube production by *F. curvula* conidia was strongly delayed and inhibited by *T. elegans* whereas only slight effects of *F. curvula* on *T. elegans* were observed. Our data strongly suggest that *T. elegans* produced a compound secreted in the medium, but we cannot rule out that germ tube

production by *F. curvula* on the leaf disk was inhibited by previous contact in the medium with *T. elegans* conidia. In the river, inhibition by a secreted compound is unlikely because of the continuous leaching and dilution, while contacts at random between conidia of both species or between *F. curvula* conidia and mycelium fragments of *T. elegans* are probably rare events. But on the leaf, when conidia of *F. curvula* settled on an area colonized by *T. elegans*, we may expect some inhibition of germ tube production by *F. curvula* conidia. *T. elegans* mycelium grew faster than *F. curvula* on poor but not on rich medium (Fig. 3). This demonstrates the advantage of using two media of very different richness. It is surprising that these species can grow on purified agar as it has been shown that biotin, at least, is required at a rather elevated concentration for the growth of two *Anguillospora spp.* [17]. Given the chemical quality and the heterogeneity of leaf litter it is likely that mycelial growth rates of both species in nature will be within the range reported here on artificial media. The results of the experiment in which facing plugs were used indicate that no obvious remote inhibition of mycelial growth occurred between the two species (Fig. 4). By contrast, *T. elegans* reduced the area in which *F. curvula* could grow on the two media when half-plugs of each species were placed side by side (Fig. 5). Although such a situation cannot occur on the leaf, these results suggest that *T. elegans* may limit the growth of *F. curvula* in particular cases. This in turn would result in decreased conidium production, as a close relationship between growth and sporulation has been shown in aquatic hyphomycetes [22].

It thus appears that whereas *Flagellospora curvula* potentially produces much more conidia than *Tetrachaetum elegans*, the latter seems to have developed strategies to counterbalance this handicap for a group of fungi depending on multiple substrate units that can be highly dispersed. In the streams from which the strains of the two species were isolated, conidia of *F. curvula* are present in substantial numbers in all seasons, whereas *T. elegans* is clearly a dominant species in autumn but almost disappears in winter [8]. This observation can be explained by a better adaptation of *F. curvula* to the range of temperatures recorded in these streams (3.5–18°C; [8]): The conidium production of *F. curvula* decreases whereas that of *T. elegans* increases when the temperature shifts from 15 to 25°C [9]. It is worth noting that the temperature of 16°C used in our study is more favorable to *F. curvula*. But it is possible that the temperature optima of both species studied here change in mixed cultures as described for two other aquatic hyphomycete species, *Tricladium chaetocladium* Ingold and *Lunulospora curvula* Ingold [28], although the experimental conditions were different between the two studies. Moreover, the interactions with several other species present in natural assemblages, in addition to *F. curvula* and *T. elegans*, will most probably modulate the interaction between the two species studied. Overall, one can expect that many subtle interactions which take account of all the parameters, including water nutrients [24], will influence the community structure.

In conclusion, we report that a decreased reproductive output of two aquatic hyphomycete species can result in an increase in the process in which they are involved. This suggests that competition between several species, while regulating their relative dominance, can have a positive effect on the ecosystem functioning. In the ecological group of fungi studied here, other experiments will have to be carried out with other species and substrates to provide a better understanding of the community dynamics and structure.

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