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# Influence of conidial traits and leaf structure on attachment success of aquatic hyphomycetes on leaf litter

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**Abstract:** Attachment of conidia on leaves is a critical first step in the life cycle of aquatic hyphomycetes in streams. In a first series of microcosm experiments, attachment success of three common aquatic hyphomycete species differing in conidial shape (compact, filiform and tetra- or polyradiate) was determined on two leaf species, black alder and downy oak. Fungal species identity and leaf surface structure significantly affected conidial attachment after 24 h. The lower sides of oak leaves with extensive tufts trapped 2.4–8.8 more conidia than the upper sides of oak leaves and both sides of alder leaves. In a second experiment with seven fungal species, attachment success of two species with tetra- or polyradiate conidia was much greater than that of two other tetra- or polyradiate and three compact conidia, which all had similar attachment success. The species with the largest spores was also the most successful, but this pattern was not consistent across the size range of tested conidia. These results highlight the importance of leaf surface structure, possibly conidial shape and size and additional properties of aquatic hyphomycete conidia in determining attachment success on leaves and they point to the potential role of these factors in structuring fungal communities on decomposing leaves in streams.

**Key words:** aquatic hyphomycetes, attachment, conidial shape, decomposition, leaf litter, stream

## INTRODUCTION

Aquatic hyphomycetes are important drivers of leaf litter decomposition in streams, a fundamental ecosystem process in forested catchments (Bärlocher and Kendrick 1974, Suberkropp and Klug 1976, Gessner and Chauvet 1994, Hieber and Gessner 2002, Pascoal and Cássio 2004). Colonization of leaves and other substrates by aquatic hyphomycetes may occur through three pathways: by direct contact as a result of hyphal outgrowth from a colonized leaf touching another leaf or piece of wood; at a distance by either detached hyphal fragments (Park 1974, Knudsen and Stack 1991) or asexual spores called conidia (Read et al 1992) that land on a leaf surface. The last mechanism appears to be the predominant one to colonize distant leaf patches (Sridhar and Bärlocher 1997).

Once settled on a substrate, conidia of aquatic hyphomycetes germinate within hours, or less (Read et al 1992; Au et al 1996), by developing one or more germ tubes that generally form appressoria on the substratum (Read et al 1992). These structures ensure a solid adherence to the colonized surface (Webster and Davey 1984) and subsequently permit the fungus to penetrate the plant tissue (Hatzipapas et al 2002). At the beginning of colonization, hyphal extension in leaf tissue can be rapid, with growth rates measured in leaf litter of up to  $0.72 \text{ d}^{-1}$  (Suberkropp and Weyers 1996, Gessner and Chauvet 1997, Pascoal and Cássio 2004). In contrast to most other fungi, aquatic hyphomycetes typically start to produce and release conidia soon after initial colonization of a new substrate, and the high sporulation activity of up to 7000 conidia produced per mg leaf dry mass per day (Bärlocher 1982, Suberkropp 1991, Gessner and Chauvet 1994, Gulis and Suberkropp 2003, Pascoal et al 2005) results in conidial concentrations in headwater streams that can reach several thousands per liter during autumn leaf fall (Webster and Descals 1981, Bärlocher 1992). Conidia of aquatic hyphomycetes released into flowing water may be carried from a few hundred meters to a few kilometers (Thomas et al 1990) and can maintain their ability to germinate for several days (Iqbal and Webster 1973, Sridhar and Bärlocher 1994). However, unlike conidia of many other fungi, those of aquatic hyphomycetes are not designed as resting stages, and their delicate structure suggests that prolonged maintenance of viability is limited (Sridhar and Bärlocher 1994). Furthermore

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there is evidence that early establishment on a freshly fallen leaf confers early colonizers a competitive advantage over fungi arriving at a later stage (Bärlocher and Schweizer 1983, Suberkropp and Chauvet 1995). Thus efficient attachment to a suitable fresh substratum is a most critical point in the life cycle of these fungi.

In contrast to most terrestrial fungi, aquatic hyphomycetes show a variety of characteristic conidial shapes, which range from compact to filiform to branched. Tetraradiate shape as the dominant branched type and filiform shape are the most common, with experimental evidence suggesting that more complex shapes result from adaptation to turbulent aquatic environments (Webster 1959, Webster and Davey 1984, Webster 1987). Accordingly conidia with tetraradiate shape are trapped on surfaces most efficiently, making simultaneous contact with the tips of three of their branches. Filiform conidia are often sigmoid (i.e. twisted in three dimensions), which ensures two contact points, whereas compact forms only have one and consequently should be least efficient at attaching to surfaces, especially in turbulent conditions. Differences in the surface roughness of leaves, both among species and between the upper and lower leaf side, might further influence attachment success. This idea led Bärlocher (1992) to suggest that some observed difference in community structure of aquatic hyphomycetes on different leaf species in the same stream (Thomas et al 1992, Gulis 2001) might be caused partly by varying success among species during the initial conidial settlement and germination phases.

The aim of this study was to determine the variability in attachment success on natural leaf surfaces among the three most common types of conidia (branched/tetraradiate, filiform/sigmoid and compact) belonging to widespread aquatic hyphomycete species. To this end we first compared attachment success on two types of leaves of three aquatic hyphomycete species, each exhibiting one of the three basic conidial shapes. We used black alder (*Alnus glutinosa* [L.] Gaertn.) and downy oak (*Quercus humilis* Miller) to test whether leaf surface structure influences conidial attachment by comparing attachment success on the upper (adaxial) and lower (abaxial) sides of leaves. Leaf blades of both species have smooth upper and rougher lower sides, the latter resulting from protruding veins and more or less numerous and extensive tufts. Given the greater importance of tufts on oak leaves, we expected roughness to be greatest on the lower side of oak leaves and lowest on upper leaf surfaces. Thus we hypothesized that (i) differences in surface structure between leaf sides influence conidial attach-

ment; (ii) simple compact conidia are less influenced by surface structures than filiform unbranched and tetraradiate conidia, which are more likely to get trapped by tufts; and (iii) filiform and tetraradiate conidia attach more efficiently than conidia with simple compact shape, even on smooth surfaces. In a second experiment we tested with a greater number of fungal species whether tetraradiate conidia are more efficient at attaching to leaves than compact conidia.

#### MATERIALS AND METHODS

*Production of conidia.*—All fungal strains were obtained from single conidia isolated from river foam and grown on 2% malt agar (Merck 1.11929). *Tetrachaetum elegans* Ingold (*Te*, CERR28-1457), *Flagellospora curvula* Ingold (*Fc*, CERR81-150) and *Heliscus lugdunensis* Saccardo & Thérrey (*Hl*, CERR28-1453) were chosen for the first experiment as representatives of tetraradiate, filiform and compact conidial shapes, respectively (FIG. 1). Five additional species were used in the second experiment. These were *Alatospora acuminata* Ingold (*Aa*, CERR28-1464), *Articulospora tetradia* (*At*, CERR28-1458) and *Tetracladium marchalianum* de Wild. (*Tm*, CERR28-1454) with tetraradiate conidia, and *Goniopila monticola* (Dyko) Marvanová & Descals (*Gm*, CERR28-1455) and *Tumularia aquatica* (Ingold) Descals & Marvanová (*Ta*, CERR28-1461) with compact conidia (FIG. 1).

Sporulation of aquatic hyphomycetes was induced by soaking strips of 7–10 d old colonies on 2% malt agar in microcosms designed for studying fungal leaf decomposition in streams (see Suberkropp 1991 for details). Forty mL of nutrient solution containing per liter 100 mg  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ , 10 mg  $\text{MgSO}_4 \cdot 2 \text{H}_2\text{O}$ , 0.5 g 3-morpholinopropane-sulfonic acid (MOPS), 100 mg  $\text{KNO}_3$  and 5.5 mg  $\text{K}_2\text{HPO}_4$ , with pH adjusted to 7.0 were added to each microcosm (Dang et al 2005). Microcosms and nutrient solutions of cultures were autoclaved before inoculation. Air flow through microcosms was adjusted to  $80 \text{ mL min}^{-1}$  during experiments and temperature was maintained at 15 °C. Conidia produced after 24–48 h were used in experiments.

*Experimental design.*—Leaf disks (1 cm diam) were cut from freshly fallen alder and oak leaves with a cork borer, autoclaved, air dried and stored. Although autoclaving and drying of leaves may affect surface structure of leaves, we assumed such changes would be small and that any effects on attachment would vary little across fungal species. Six disks of each leaf species were introduced into each of three replicate microcosms (Suberkropp 1991) and incubated with aeration for 24 h before adding a conidial suspension of a single species. In a first series of experiments about 10 000 conidia for *F. curvula* and *H. lugdunensis* or 2000 for the much larger *T. elegans* were used. Number of conidia in the inoculum was verified by staining and counting the conidia of three additional inocula on membrane filters (Gessner et al 2003). The same protocol

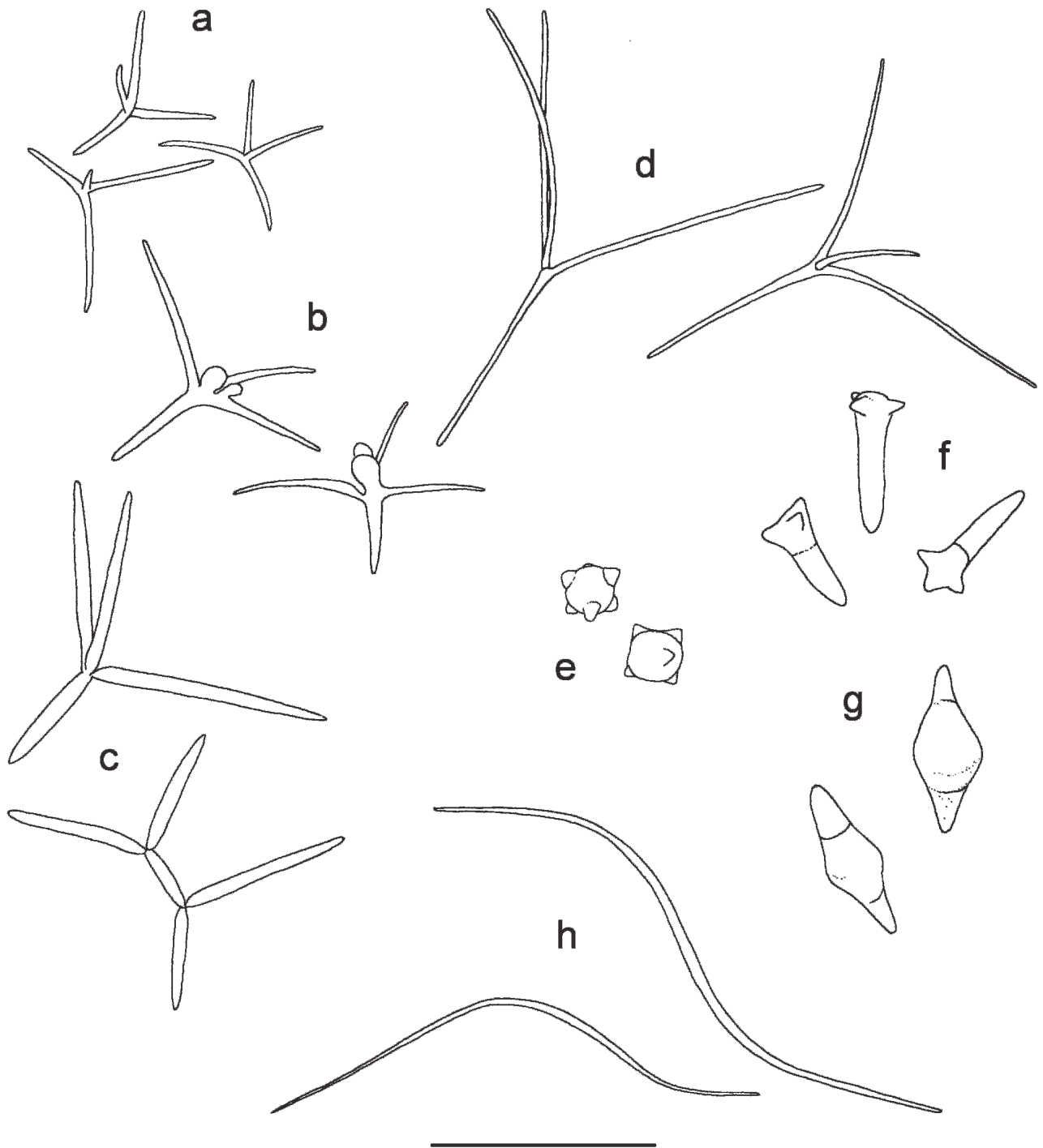


FIG. 1. Conidia of *Alatospora acuminata*. a. *Tetrachidium marchalianum*. b. *Articulospora tetracladia*. c. *Tetrachaetum elegans*. d. *Goniopila monticola*. e. *Heliscus lugdunensis*. f. *Tumularia aquatica*. g. *Flagellospora curvula*. h. Bar = 50  $\mu$ m (a-c, e-h) or 100  $\mu$ m (d). After Chauvet (1990).

was followed in the second experiment, except that microcosms were inoculated simultaneously with all seven species and that each of three replicate microcosms received 12 alder leaf disks and ca. 17 000 conidia of each fungal species.

*Experimental procedures.*—After 24 h incubation the microcosms were drained and the solution filtered through membrane filters (5.0 mm pore size, nitrocellulose, Whatman International Ltd., Maidstone, UK). Conidia on filters were stained with trypan blue (0.1%) in lactic acid (60%)

and counted (see below). Leaf disks were removed carefully from microcosms with tweezers, stained with trypan blue and placed between a slide and cover slip. In the first series of experiments conidial attachment was checked by scanning the entire surface area of both sides of all leaf disks under a Zeiss Axioplan microscope at 160 $\times$  to obtain an exhaustive account of all settled conidia. Conidia first were counted on the leaf side facing the cover slip, then the slide was turned upside down and conidia counted on the side facing the slide. In the second experiment to assess success of the two extreme conidial shapes, a total of three lower and three upper sides of the 12 leaf disks per microcosm were scanned.

**Data analyses.**—Attachment success was assessed as the percentage of conidia attached to each side of all leaf disks from a given microcosm. Because a conidium can settle only on one side of a given disk in a microcosm, conidial attachment events are not statistically independent and analyses with parametric statistical tests are inappropriate (Sridhar et al 2001). However conidial attachment events are independent among replicate microcosms, which were treated as blocks in analyses. The influence of leaf surface structure (i.e. leaf species and side) and fungal species on conidial attachment success was analysed by two-way randomized ANOVA (cf. Bärlocher 2005) after arcsine-square root transformation of data; the routine was written in Matlab 6.5 for PC. To maintain independence among blocks, permutations were restricted to within blocks (i.e. microcosms) by shuffling all data (Sridhar et al 2001). After each shuffling (permutation), F-statistics were calculated as in a normal two-way ANOVA. Based on 10 000 permutations significance was inferred from the number of F-values as extreme as, or more extreme than, the initial F-value (i.e.  $F_0$  obtained from the empirically observed data). When the test was significant, differences between pairs were tested by Tukey's HSD test with the same permutation method. In the second experiment, the influence of species on attachment success was analyzed by one-way randomized ANOVA on arcsine-square root transformed data followed by Tukey's HSD test, using similar procedures as in Experiment 1. Finally Spearman's correlation was calculated between effective volume of conidia and attachment success to assess whether conidial size could have influenced attachment success in the second experiment. Effective volume of conidia was approximated by a sphere in the case of tetra- radiate conidia and *G. monticola*, a cone for *H. lugdunensis* and a double cone for *T. aquaticus* (FIG. 1).

## RESULTS

Microscopic observations revealed that all conidia settled on the leaves had germinated. Leaf side had a strong effect on conidial attachment ( $F = 68.5$ ,  $P < 0.0001$ ), with the lower side of oak trapping significantly more conidia of *T. elegans* and *F. curvula* than both the upper side of oak and either side of alder leaves (FIG. 2, TABLE I). Microscopic observations showed that when a branch of a tetra- radiate conidium

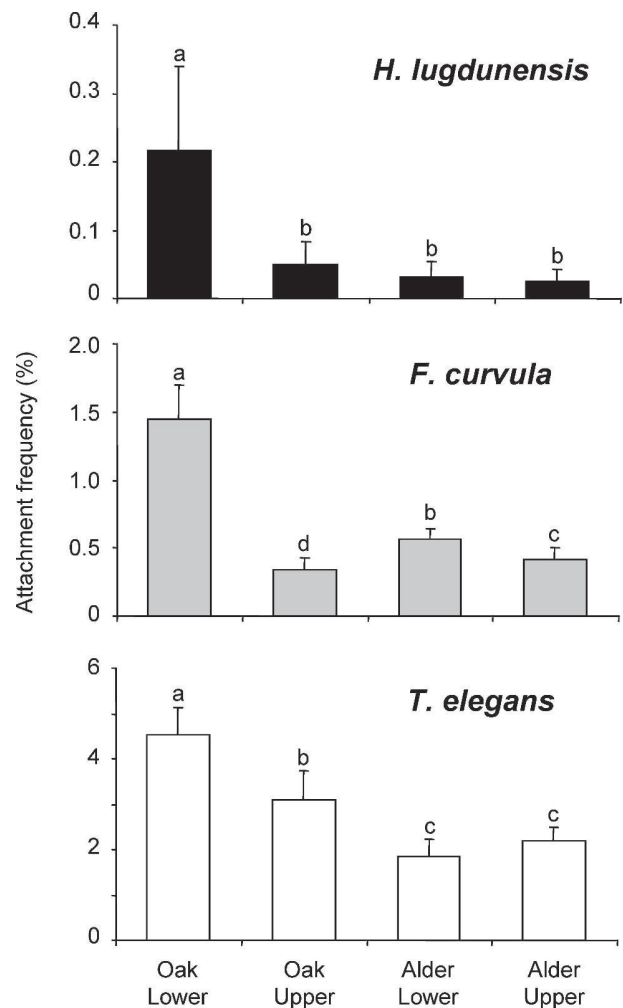


FIG. 2. Attachment success of three aquatic hyphomycete species on leaves expressed as frequency of inoculated conidia settled on one side of one leaf disk in microcosms (mean  $\pm$  1 SE,  $n = 18$ ). Alder and oak leaves as well as upper and lower sides of leaves were considered separately. Black, gray and white bars indicate species with compact, filiform and tetra- radiate conidia respectively. Leaf species/ side combinations that were significantly different according to randomized Tukey's test are indicated by lowercase letters.

or a filiform conidium encountered a hair on the lower side of an oak leaf (FIG. 3) the conidium got entangled and remained trapped and developed one or more germ tubes that often reached the leaf surface. Even the compact conidia of *H. lugdunensis* were significantly more abundant on the lower side of oak leaves (FIG. 2).

Conidial attachment varied greatly among the three fungal species with a total attachment frequency (all conidia attached to the two leaf species combined) of  $70 \pm 12\%$  (mean  $\pm$  1 SD) for the large

TABLE I. Results of two-way randomized ANOVA testing for effects of fungal species and leaf side on attachment success by aquatic hyphomycete conidia

Factor	SS	df	MS	F	P
Fungal species	0.77	2	0.39	882.5	<0.0001
Leaf side	0.090	3	0.030	68.5	<0.0001
Fungal species × leaf side	0.026	6	0.0043	9.8	<0.0001
Error	0.089	204	0.00044		

tetraspore conidia of *T. elegans*,  $16.6 \pm 5.4\%$  for the filiform conidia of *F. curvula* and  $1.9 \pm 2.4\%$  for the compact conidia of *H. lugdunensis*. In contrast to the other species *H. lugdunensis* never developed appressoria on leaf surfaces. Few conidia of *T. elegans* remained suspended after 24 h ( $2.7 \pm 1.9\%$ ), whereas about half ( $53 \pm 19\%$ ) of the *H. lugdunensis* conidia remained in suspension. Suspended conidia of *F. curvula* were not counted because they were not clearly distinguishable from hyphal fragments once they had germinated. Indeed after 24 h conidial suspensions of all species contained a high number of hyphal fragments, although inocula were almost free of hyphae. Inspection of the glass walls of microcosms under a dissecting microscope revealed a substantial number of attached conidia; however, because of the shape of microcosm, it was impossible to obtain reliable counts of these conidia.

Attachment success of the seven fungal species used in Experiment 2 is shown (FIG. 4). Species identity

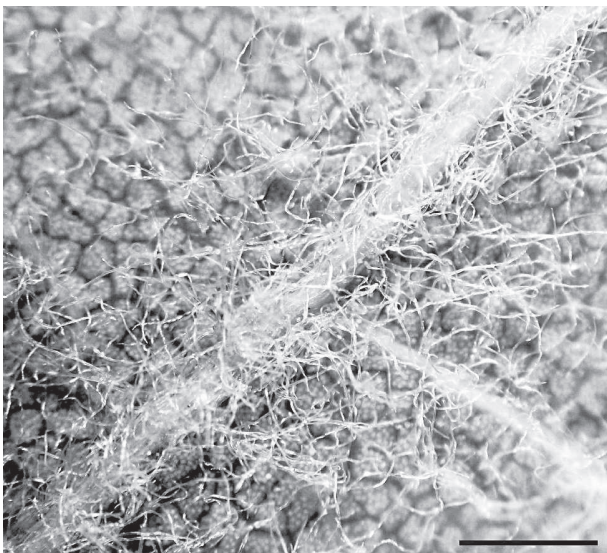


FIG. 3. View of the lower side of a downy oak leaf showing tuft that might be instrumental in increasing attachment success of aquatic hyphomycete conidia on leaf surfaces. Bar = 1 mm.

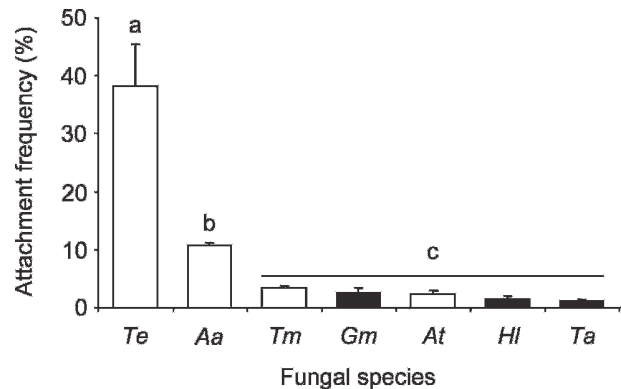


FIG. 4. Attachment success of seven aquatic hyphomycete species on leaves expressed as the frequency of inoculated conidia settled on leaf disks in microcosms (mean  $\pm$  1 SE,  $n = 3$ ). *Tetrachaetum elegans* (Te), *Alatospora acuminata* (Aa), *Tetracladium marchalianum* (Tm), *Goniopila monticola* (Gm), *Articulospora tetradia* (At), *Heliscus lugdunensis* (Hl) and *Tumulularia aquatica* (Ta). White bars indicate species with tetraspore conidia, gray bars species with compact conidia. Species significantly different according to a randomized Tukey's test are indicated by lowercase letters.

had a highly significant effect ( $F = 68.3$ ,  $P < 0.0001$ ), confirming results from Experiment 1 that large differences in attachment success exist among individual fungal species. There were also significant differences between fungal shapes, with two tetraspore species exhibiting the highest attachment frequencies (FIG. 4). In accordance with results of Experiment 1 *T. elegans* conidia were much more successful in attaching to leaves than all other species ( $38 \pm 13\%$ ) (FIG. 4). The second tetraspore species (*A. acuminata*) had significantly lower attachment frequencies of  $10.8 \pm 0.8\%$ , and attachment success of the five other species (tetraspore *T. marchalianum*  $3.4 \pm 0.7\%$  and *A. tetradia*  $2.6 \pm 0.4\%$ , and compact *G. monticola*  $2.7 \pm 1.1\%$ , *H. lugdunensis*  $1.3 \pm 1.1\%$  and *T. aquatica*  $1.1 \pm 0.6\%$ ) were similar. As a result multiple comparisons by Tukey's test following randomized ANOVA (TABLE II) differentiated three groups (FIG. 4).

An apparent relationship between size (i.e. effective volume) of conidia and attachment success (FIGS. 1 and 4) was driven strongly by a single species with large conidia and high attachment success (*T. elegans*) at one extreme and a cluster of smaller species at the other end. As a result effective volume and attachment success were not significantly correlated when assessed as Spearman's rank correlation ( $r_s = 0.54$ ,  $P = 0.22$ ). Further, when this large species was removed from the analysis, the previously apparent relationship disappeared altogether ( $r_s =$

TABLE II. Summary of pairwise comparisons (P values) made by randomized Tukey's test of aquatic hyphomycete attachment success on leaves. *Alatospora acuminata* (Aa), *Articulospora tetracladia* (At), *Goniopila monticola* (Gm), *Tetracladium marchalianum* (Tm), *Heliscus lugdunensis* (Hl), *Tumulularia aquatica* (Ta) and *Tetrachaetum elegans* (Te)

	Aa	At	Gm	Tm	Hl	Ta
At	0.005					
Gm	0.004	0.97				
Tm	0.016	0.37	0.36			
Hl	<0.001	0.35	0.38	0.21		
Ta	<0.001	0.29	0.32	0.17	0.90	
Te	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

0.26,  $P = 0.62$ ), although effective volumes of the remaining species still differed by a factor of 17.

#### DISCUSSION

To our knowledge this study is the first to estimate the attachment success of aquatic hyphomycete conidia on leaf litter. Webster (1959) found that conidial shape significantly influences attachment on collodion-coated objects submerged in flowing water. The present results lend partial support to Webster's (1959) conclusion that tetracladia conidia confer a competitive advantage because conidia of two tetracladia species attached to leaf surface more efficiently than compact forms. However species with tetracladia conidia exhibited a large range of attachment frequencies, with no statistically detectable difference between the two least efficient tetracladia and three compact conidia. This suggests that factors other than shape are also important in determining attachment success of aquatic hyphomycetes on leaves.

Higher attachment rates of tetracladia conidia have been attributed to the greater number of contact points that settling conidia make with the substratum. The rationale is that the tetracladia shape ensures simultaneous contact at three points and thus results in better adherence on surfaces than the single contact point of compact conidia and two made by sigmoid conidia (Webster and Davey 1984, Read et al 1991, Bärlocher 1992). Why then did conidial attachment success vary markedly among species with conidia of similar shape? Cox (1983) suggested that effective size is important because size is proportional to the probability that a spore hits a target object. This hypothesis is consistent with the high attachment success of *T. elegans*, the species in our experiments with by far the largest conidia. However *A. acuminata*, the second most successful species, has conidia of

intermediate size only (FIG. 1). Thus, although it cannot be ruled out that conidial size plays a role in determining attachment success, additional factors appear to be influential as well. One additional factor, unrelated to conidial shape or size, might be length of the germ tube that is formed before an appressorium is developed because quick development of appressoria at the end of germ tubes ensures firm attachment of newly settled conidia colonizing leaf surfaces (Webster and Davey 1984, Read et al 1991; Au et al 1996). In contrast to *G. monticola* and the tetracladia species, which produced short germ tubes before forming appressoria, conidia of *T. aquatica* usually developed long tubes before an appressorium was formed. This delay in appressorium formation might explain the particularly low attachment frequency of *T. aquatica* compared to all other species tested.

*H. lugdunensis* never developed appressoria on leaves in the present study, nor have these structures been observed on either glass surfaces or membrane filters in a previous investigation (Read et al 1991). Lack of an effective holdfast before hyphae penetrate the plant tissue may result in high vulnerability of settled conidia to turbulence, which could wash away conidia that are only loosely attached, consistent with our observation that more than half of the *H. lugdunensis* conidia added to microcosms remained in suspension after 24 h. The notable absence of appressorium formation in *H. lugdunensis* might be related to the regular occurrence of this hyphomycete in terrestrial habitats (Sridhar and Bärlocher 1993), from where it first was described (Saccardo 1880) and where firm attachment is less critical than in flowing water. Furthermore *H. lugdunensis*, like *T. aquatica*, has been reported from submerged wood (Shearer 1992), a much less ephemeral resource than leaves, suggesting that attachment success might play a less critical role in the life cycle of these species compared to other aquatic hyphomycetes. Finally the weaker ability of both *H. lugdunensis* and *T. aquatica* to attach to leaf surfaces might be related to the presence of glycogen as dominant carbon storage product (Ingold 1975), which has been suggested to reduce germination potential (Read et al 1992).

In addition to conidial shape and other traits of aquatic hyphomycetes, attachment success of conidia was affected significantly by leaf surface structure. Both filiform and tetracladia conidia were trapped most efficiently by tufts on the lower side of oak leaves (FIG. 3), which functioned like a comb and thus increased settlement rates. We had expected that compact conidia would be less responsive to differences in leaf surface structure. However compact conidia, such as those of *H. lugdunensis*, also were

trapped preferentially by tufts on the lower side of oak leaves (FIG. 3), an observation also made with conidia of *G. monticola* (data not shown). Microscopic observation suggested that this could be due to the fact that conidia are no longer compact once they have germinated and therefore might get entrapped with their extended germ tubes in a similar way as filiform conidia. Because attachment success was evaluated only 24 h after inoculation of microcosms it is not clear from the present results whether conidia germinated before or after entrapment. However, if they germinated in suspension, it would support Cox's (1983) suggestion that alteration of conidial shape by germination might render attachment to leaf surfaces more efficient.

Another critical factor to consider in assessing attachment success of aquatic hyphomycete conidia is thickness of the boundary layer on leaf surfaces. Given the small size even of large conidia (FIG. 1) conidia simply might get trapped in this boundary layer where flow is greatly reduced (Statzner et al 1988). Leaf surface structures such as tufts are likely to increase thickness of the boundary layer and thus increase chances of conidia of all sizes and shapes to be trapped, irrespective of a comb effect of tufts.

The weak attachment of conidia in the present experiments is likely to be related to the high turbulence prevailing in stream microcosms, which are aerated from underneath (Suberkropp 1991). However such turbulent conditions typically are encountered in running water, even though specific hydraulic characteristics may differ between microcosms and the field. Attachment of aquatic hyphomycetes is a critical stage in the colonization of fresh substrata in flowing water. Indeed aquatic hyphomycetes in streams are likely to experience large losses of conidia that never germinate on a suitable substratum. This is because conidia are constantly transported downstream with the unidirectional flow of water and have a tendency to attach to any solid substratum they encounter, such as a leaf or piece of wood, but also to mineral surfaces (Read et al 1992). Additional losses during transport occur through consumption by filter-feeding invertebrates (Bärlocher and Brendelberger 2004). In view of the rapid colonization of freshly fallen leaves in streams by aquatic hyphomycetes, it appears that the enormous number of conidia released by these fungi (Bärlocher 1992) is sufficient to compensate for these losses and inefficiencies in attaching to suitable substrates.

In conclusion the results of this study show that species identity clearly affects attachment success of aquatic hyphomycetes on leaf surfaces. Shape of conidia may partially determine success, whereas the importance of size is not clear, suggesting that other

characteristics of conidia are also influential. In addition to conidial traits leaf surface structure plays an important role in determining attachment success, particularly the presence of tufts in which conidia can get entrapped and which are likely to increase thickness of the boundary layer at the leaf-water interface. Given that settling of conidia on, and attachment to, leaf surfaces is a critical step in the life cycle of aquatic hyphomycetes, marked differences in attachment success might have implications for structuring aquatic hyphomycete communities on decomposing leaves in streams. For example *T. elegans* was highly efficient in the present study at attaching to alder leaves, consistent with the frequent record of this fungus as a dominant species during early stages of leaf colonization in natural streams (e.g. Chamier and Dixon 1982, Bärlocher 1991a, Gessner et al 1993). However traits of aquatic hyphomycetes, such as efficiency of resource capture, growth rate, competitive ability (Bärlocher 1991b, Yuen et al 1999, Treton et al 2004), and resistance to predation (Arsuffi and Suberkropp 1989), are likely to be additional determinants that shape aquatic hyphomycete community structure. Considerably more information clearly is needed to elucidate the factors governing establishment of aquatic hyphomycete communities on leaf litter in streams.

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