Germination of suspended and settled conidia in aquatic fungi

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Kempt, E.D., A. Maamri & F. Bärlocher. Germination of suspended and settled conidia in aquatic fungi. – Sydowia 53(2): 200–210.

In 9 species of aquatic fungi, ≤ 4% of conidia germinated while suspended in distilled water. Supplementing the suspension with malt extract, or allowing the conidia to settle on membrane filters, resulted in ≥ 84% germination within 48 hours. Adding EDTA to suspended conidia released from pure cultures or collected from naturally formed stream foam increased germination rates to a lesser extent. When conidia trapped on membrane filters were supplied with malt extract, germination proceeded more quickly and more germ tubes per conidium were produced.

Keywords: germination, aquatic hyphomycetes, suspended and settled conidia, organic nutrients, EDTA.

Aquatic hyphomycetes dominate leaf decomposition in streams. Their mycelia can account for up to 17% of detrital leaf mass (Gessner, 1997) and their annual production per stream area is of the same order of magnitude as that of bacteria and macroinvertebrates (Suberkropp, 1997). Reproduction and dispersal within streams occurs primarily through conidia, which are formed at the tips of conidiophores projecting into the water. Mature conidia are carried away by the current, and are often tetraradiate, branched, or sigmoid (Bärlocher, 1992). These types of shapes, which have evolved in several phylogenetically distinct lineages, tend to increase the efficiency with which conidia are trapped on solid surfaces (Webster, 1959; Webster & Davey, 1984). While suspended in the water or trapped in foam, germination is rare (Nilsson, 1964); Webster & Descals (1981) state that spores may remain in foam for at least 1 month at 13 C without germinating. This dormancy is broken within 2–6 hours after conidia have become attached to a solid surface, and germination rates approach or exceed 90% within 24–48 hours (Read

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& al., 1992a-c; Au & al., 1996a-c). Attachment of a spore is facilitated by mucilage (primarily polysaccharides), secreted before or immediately following settlement. Physical contact causes a flattening of the cell wall; in algal carpospores this creates a calcium gradient and hyperpolarization of the basal spore membrane (Jones & Jones, 1986). This in turn leads to a reassortment of cell components, and the spore germinates.

Thus, initial conidial contact with a solid surface is known to stimulate both release of mucilage and germination of aquatic hyphomycete conidia. The nature of the actual trigger(s) is unknown; it may be a combination of chemical (e.g., higher nutrient concentration at interfaces) and physical (e.g., electrostatic charges) factors (Read & al., 1992a; Jones & O'Shea, 1994; Au & al., 1996a-c). The objectives of the current study were to determine whether conidium/substrate contact is in fact an essential prerequisite, or whether germination can be induced in suspended, unattached conidia. One potentially relevant factor may be availability of organic nutrients, which favors vegetative growth over reproduction (Sridhar & Bärlocher, 1997). We therefore compared germination of suspended conidia with or without nutrient amendments. Another prerequisite for spore germination may be changes induced in the cell wall or membrane by a thigmotropic response (Jones & Jones, 1986). Such changes likely involve some rearrangement or selective removal of wall constituents. We therefore examined the effects of EDTA on conidial germination. EDTA is known to capture bivalent cations (such as Ca^{2+}) and leaches proteins (Ziola & al., 2000) and polymeric substances similar to mucilage (Battin & al., 2001) from microbial cell walls and membranes.

Materials and methods

The following species, all isolated from single conidia and provided by Dr. L. Marvanová (Czech Culture Collection, Brno) were used: *Anguillospora longissima* (Sacc. & Syd.) Ingold (CCM F-00680), *Clavariopsis aquatica* De Wild. (CCM F-10491), *Cylindrocarpon destructans* (Zins.) Scholten (CCM F-50), *Fusarium aquaeductuum* Lagh. (CCM F-13997) *Heliscus lugdunensis* Sacc. & Thérry (CCM F-109), *Tetracladium furcatum* Descals (CCM F-11883), *T. marchalianum* De Wild. (CCM F-312), *T. maxilliforme* (Rostrop) Ingold (CCM F-14286), and *T. setigerum* (Grove) Ingold (CCM F-20987). Several species belonging to *Cylindrocarpon* and *Fusarium* are often found on leaves decaying in streams, but are not generally considered to be typical aquatic hyphomycetes (Bärlocher, 1992). Cultures were maintained on 1% malt extract agar (MEA, 1.5% agar) at 18 C.

To induce sporulation, 3–4 agar plugs (5 × 5 mm) were cut from the growing edge of a colony and transferred to 500 ml Erlenmeyer flasks

with 250 ml sterile, distilled water. The flasks were aerated for 2–3 days at 18 C, and sub-samples examined for suspended conidia. When sufficient numbers were present (≥ 50/ml), the agar plugs were removed and conidial suspensions were used for experiments. In a control experiment, aeration of spores suspended in distilled water was continued. In a parallel experiment, conidial suspensions were first provided with sterile malt extract (SIGMA M-0383; final concentration: 1% ME), or, in some cases, with EDTA (SIGMA E-1644, final concentration: 0.1 or 0.01%). After 72 hours, the suspensions were filtered through 1 μm nucleopore filters (polycarbonate) and stained with aniline blue in lactophenol. Under the microscope, proportions of germinated spores were determined, counting at least 100 conidia per filter. Generally, proportions were determined on 3 individual filters, and averages calculated. Arcsin transformed data for each species were analyzed with one-way ANOVA, followed by Tukey's multiple comparison test (SYSTAT, Version 5.3.1 for Macintosh).

In a second series of experiments, conidia were filtered through 1 μm membrane filters (nitrocellulose). The filters were then placed on top of Whatman filter paper (rinsed in sterile distilled water for 48 hours) soaked in distilled water or malt extract broth with final concentrations of 0.25, 0.5 and 1%. After 0, 2, 4, 8, 24 and 48 h of incubation at 18 C, the membrane filters with the conidia were fixed and stained (aniline blue in lactophenol). Proportions of germinated spores and number of germ tubes per germinated conidium were determined for the various treatments. Since germination and number of germ tubes vs. time appeared to follow a rectangular hyperbolic function (identical to equilibrium binding or enzyme kinetics), we estimated maxima (B_{max}) and the time at which 50% of these maxima was reached (K_d). The effect of nutrient concentrations on these values was analysed by ANOVA (GraphPad Prism, Macintosh version 3.0; Ratkowsky, 1983; Motulsky, 1999; Motulsky & Ransnas, 1987).

To determine the effects of gravity on germination parameters, conidia collected on membrane filters and placed on filter paper pads were incubated in the normal, horizontal position, as well as in a vertical and upside-down position. The proportions of germinated spores and number of germ tubes were determined, and again analyzed with ANOVA.

On 9 August, 11 September, and 15 November, 2000, foam was collected from Allen Creek (Sackville, N.B., Canada; for site description, see Bärlocher, 1987). Subsamples of 1–2 ml were diluted with 250 ml of sterile, distilled water, with or without EDTA (final concentration: 0, 0.01 or 0.1%). The suspensions were aerated for 72 h at 18 C, and filtered through nuclepore filters. Trapped conidia were stained with aniline blue in lactophenol, and germination rates were determined and analyzed by ANOVA.

Results

We confirmed that conidia suspended in distilled water had very low germination rates: percentages among the 9 species varied from 0 to 4% (Tab. 1). By contrast, when conidia were allowed to settle, germination percentages increased to ≥ 84%.

Tab. 1. – Effect of Malt Extract (ME) and EDTA supplements on germination of suspended and settled conidia. – N = 3, ± SD. Arcsin transformed values for each species were evaluated separately with One-Way ANOVA, followed by Tukey's multiple comparison test. Values followed by different letters are significantly different at 1% level.

	Suspended			Settled	
	None	1% ME	0.01% EDTA	None	1% ME
A. longissima	1 ± 1^{a}	99.5 ± 0.1^{d}	16 ± 4^{b}	89 ± 6^{c}	99.4 ± 0.3^{d}
C. aquatica	4 ± 2^{a}	98.6 ± 0.1^{c}	25 ± 9^{b}	99 ± 1^{c}	99.3 ± 0.1^{c}
C. destructans	0^{a}	99.1 ± 0.2^{c}	20 ± 9^{b}	91 ± 4^{c}	99.5 ± 0.1^{c}
F. aquaeductuum	0^{a}	99.6 ± 0.2^{d}	23 ± 4^{b}	84 ± 4^{c}	99.4 ± 0.2^{d}
H. lugdunensis	1 ± 1^{a}	99.7 ± 0.1^{c}	33 ± 12^{b}	91 ± 6^{c}	99.6 ± 0.2^{c}
T. furcatum	2 ± 2^{a}	99.6 ± 0.1^{d}	14 ± 4^{b}	89 ± 6^{c}	99.2 ± 0.1^{d}
T. marchalianum	1 ± 1^{a}	99.2 ± 0.1^{c}	22 ± 8^{b}	98 ± 1^{c}	99.7 ± 0.2^{c}
T. maxilliforme	4 ± 2^{a}	99.6 ± 0.2^{c}	27 ± 10^{b}	97 ± 2^{c}	99.3 ± 0.3^{c}
T. setigerum	3 ± 1^{a}	99.6 ± 0.2^{c}	31 ± 5^{b}	95 ± 3^{c}	99.4 ± 0.3^{c}

Addition of malt extract increased germination rates in both settled and suspended conidia; however, at a p-value of 1%, the differences were only significant with suspended conidia. The addition of EDTA also had a significant, though less pronounced, effect. This was confirmed with conidia trapped in foam samples collected on 2 of 3 occasions (Tab. 2). The dominant species in these mixtures were *Alatospora acuminata* Ingold, *Articulospora tetracladia* Ingold, *Clavariopsis aquatica*, and *Tetrachaetum elegans* Ingold.

Tab. 2. – Effect of EDTA supplement on germination of suspended conidia, collected from foam in Allen Creek. – N = 3, ± SD. Arcsin transformed values were evaluated with One-Way ANOVA, followed by Tukey's multiple comparison test. Values followed by different letters are significantly different at 1% level.

Date	EDTA (%)	Germination (%)
15 July	0	7 ± 3^{a}
	0.01	59 ± 6^{b}
	0.1	63 ± 14^{b}
15 August	0	8 ± 4^{a}
	0.01	11 ± 5^{a}
	0.1	9 ± 3^{a}
19 November	0	4 ± 6^{a}
	0.01	12 ± 6^{a}
	0.1	16 ± 5^{b}

Although adding ME to settled conidia did not significantly influence the final germination percentages (Tab. 1), it clearly affected the speed of germination. Figs. 1 and 2 illustrate the time course of germination and the number of germ tubes for three species: *A. longissima* (aquatic hyphomycete with sigmoid conidia), *T. marchalianum* (aquatic hyphomycete with tetraradiate conidia) and *C. destructans* (not considered a true aquatic hyphomycete). Estimated B_{max} and K_d values for 8 species are summarized in Tab. 3 (insufficient conidia were produced by *T. furcatum*). In *C. aquatica*, *C. destructans* (Fig. 1) and *H. lugdunensis*, initial germination rates (within first 8 hours) were low, suggesting a logistic germination pattern.

Tab. 3. – Estimated parameters of germination curves (interpreted as rectangular hyperbola), as influenced by Malt Extract (ME) supplements. – First line: K_d (hours) for germination percentages; second line: B_{max} for germ tube numbers; third line: K_d (hours) for germ tube numbers. Data were analyzed by One-Way ANOVA, followed by Tukey's multiple comparison test. Values followed by different letters are significantly different at 1% level. Estimated maximum germination percentages (not shown) did not differ significantly at p = 0.01.

		ME in %				
		0	0.25	0.5	1	p
A. longissima	K_d	38^a	4.8^b	3.6^b	3.5^b	0.0002
	B_{max}	2.1^a	3.8^b	2.6^a	3.5^a	0.03
	K_d	8.7	8.6	2.6	3.5	0.36
C. aquatica	K_d	43^a	0.8^b	0.3^b	1.0^b	0.02
	B_{max}	2.2^a	3.5^{ab}	4.0^{ab}	3.8^{ab}	0.04
	K_d	1.6	1.1	0.9	1.8	0.34
C. destructans	K_d	75^a	4^b	4^b	6^b	0.0001
	B_{max}	1.8^a	2.0^a	2.9^{ab}	3.7^b	<0.0001
	K_d	1.4	0.7	2.4	1.1	0.29
F. aquaeductuum	K_d	33^a	1.4^b	0.4^b	0.4^b	<0.0001
	B_{max}	2.1^a	2.0^a	2.7^{ab}	3.3^b	0.005
	K_d	2.9	0.9	2.9	2.4	0.33
H. lugdunensis	K_d	83^a	3.6^b	4.0^b	6.3^b	<0.0001
	B_{max}	2.2	2.1	2.7	3.3	0.19
	K_d	4.3	1.0	2.8	2.3	0.23
T. furcatum	K_d	40^a	5^b	4^b	4^b	0.0001
	B_{max}	2.7^a	4.3^{ab}	4.0^{ab}	5.6^b	0.002
	K_d	2.0	6.0	3.5	2.7	0.17
T. marchalianum	K_d	52^a	4^b	3^b	2^b	0.005
	B_{max}	1.8^a	4.2^b	4.6^b	3.8^b	<0.0001
	K_d	3.5	1.5	1.3	2.4	0.35
T. setigerum	K_d	61^a	4^b	6^b	2^b	0.008
	B_{max}	2.3^a	4.9^b	5.3^b	4.4^{ab}	0.007
	K_d	3	8	5	4	0.19

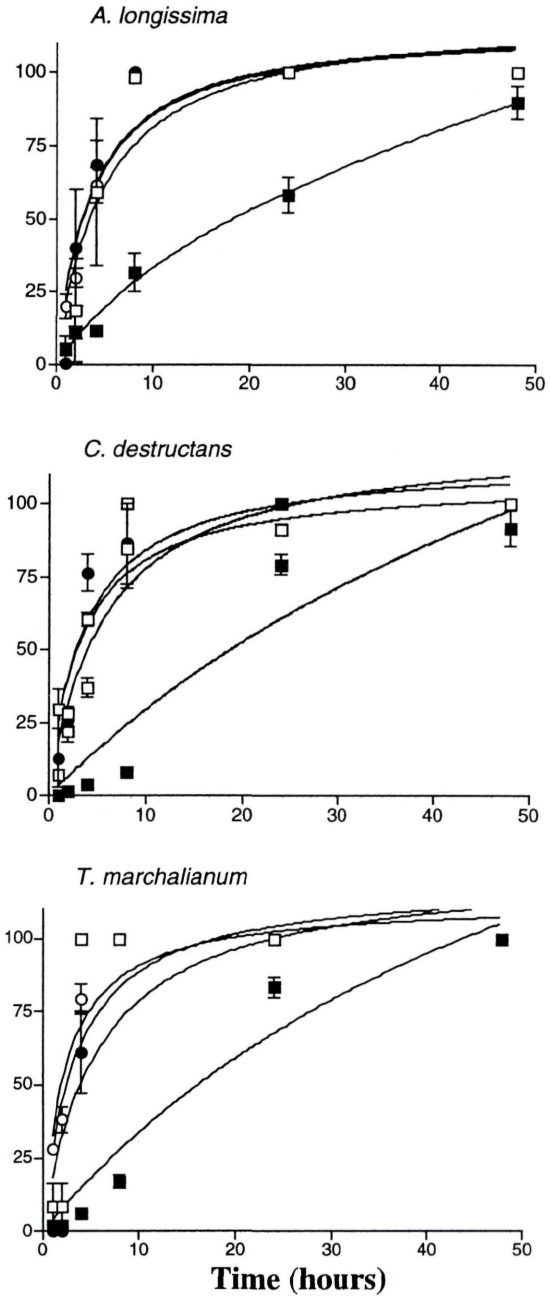

Fig. 1. – Germination percentages of conidia settled on membrane filters and supplemented with Malt Extract (ME). – N = 3, ± SD. Curves show best fit of rectangular hyperbola.
■ 0% ME; □ 0.25% ME; ● 0.5% ME; ○ 1% ME.

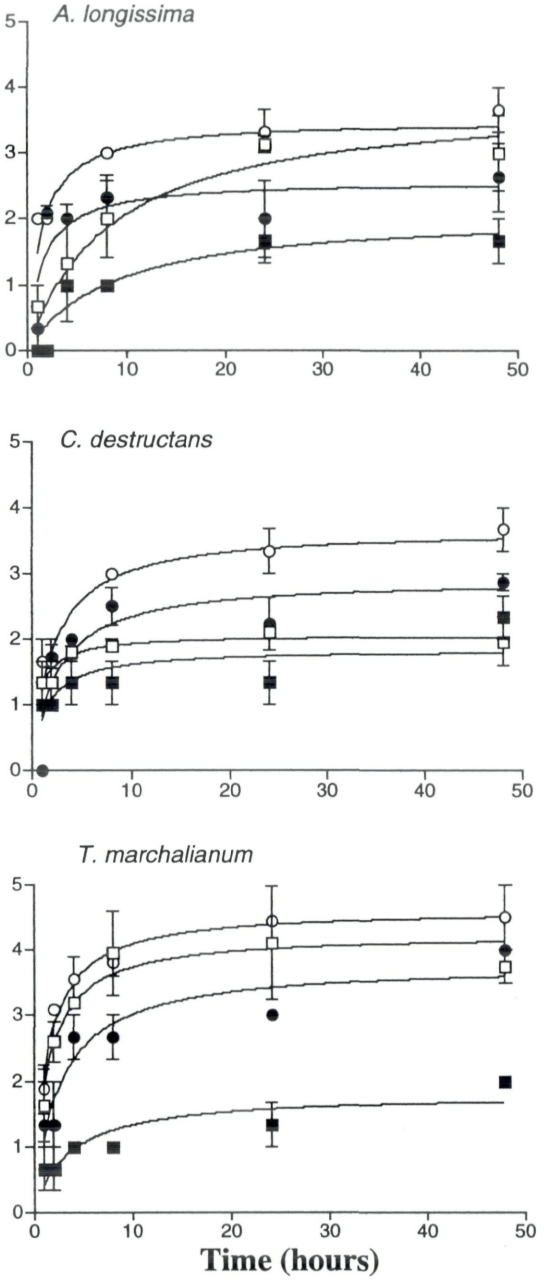

Fig. 2. – Number of germ tubes of conidia settled on membrane filters and supplemented with Malt Extract (ME). – N = 3, ± SD. Curves show best fit of rectangular hyperbola.
■ 0% ME; □ 0.25% ME; ● 0.5% ME; ○ 1% ME.

Orientation of conidia settled on membrane filters (without added nutrients or EDTA; horizontal, or after 90 or 180° rotation) had no significant effect on germination properties (data not shown; see Kempt, 2000).

Discussion

Many morphological and physiological traits are believed to have contributed to the success of aquatic hyphomycetes in the stream environment. These include the ability to grow and reproduce at temperatures down to 0 C, effective mechanisms of dispersal and colonization of new substrates, and the production of a wide range of enzymes active against plant polysaccharides (Ingold, 1966; Chamier, 1985; Bärlocher, 1992; Dix & Webster, 1995). It has long been recognized that the conidial shape facilitates early attachment to potential substrates after liberation of the spore (Webster, 1959; Webster & Davey, 1975). Secure attachment is further ensured by mucilage already present on the suspended spore or secreted upon settling on a substrate, and by appressorium formation (Read & al., 1991, 1992a–c; Au & al., 1996a-c). Once attached, conidia of aquatic hyphomycetes germinate rapidly (within a few hours) and at high rates (close to 100%; Read & al., 1992a). The processes (mucilage secretion) and structures (mucilage, appressoria) involved in attachment and germination have been well characterized by light and electron microscopy (Read & al., 1992a–c; Au & al., 1996a–c).

Our study confirmed that germination rates of newly released conidia of aquatic fungi are high (upon settlement) and exceed those of typical terrestrial fungi (Kendrick, 1992; Read & al., 1992a; Dix & Webster, 1995). However, without added nutrients, several species took 24–48 hours to reach final germination rates rather than 12 hours as reported by Read & al. (1992a). A distinct initial lag period was observed in *C. aquatica*, *C. destructans*, *T. marchalianum* and *H. lugdunensis*. A similar delay in *Anguillospora crassa*, *Tumularia aquatica* and *H. lugdunensis* was attributed to the presence of glycogen reserves in the conidia of these fungi (Read & al., 1992a). Glycogen may allow the conidia to remain viable for longer periods of time; on the other hand, its mobilization, and therefore germination, is relatively slow. We have no data on internal reserves of the species we studied; however, one (*H. lugdunensis*) was identical to the ones studied by Read & al. (1992a), a second its close relative (*C. destructans*, Rossman & al., 1999), and the third has a conspicuous central bulge (*C. aquatica*). Furthermore, the lag period disappeared when simple, external nutrients were supplied (Fig. 1, Tab. 3). Read & al. (1992a) suggest that the reliance on internal nutrients is more common in species colonizing wood (nutrient poor, long-lasting substrates) rather than leaves (richer, ephemeral substrates).

Germination of suspended conidia in distilled water was low, but contrary to a widely held belief, settlement and attachment are not essential prerequisites. We identified two factors that can induce germination in suspended spores: availability of external nutrients and EDTA. It is possible that the same mechanism triggered germination in both cases, i.e., some rearrangement of cell wall or membrane structures. EDTA selectively removes certain compounds such as ions, protein, or polysaccharides (Ziola & al., 2000; Battin & al., 2001), while nutrient uptake in fungi generally involves a combination of facilitated diffusion and active transport, both of which may change membrane properties (Kendrick, 1992). More specific responses, however, may play a role as well: in pathogenic fungi, spore germination depends on the quality as well as on the quantity of externally supplied soluble compounds, some of which are inhibitory, while others are stimulatory (Inyang & al., 1999).

The settlement of spores has been reported to flatten the cell wall (Read & al., 1992a), which, in algae, may create gradients within the membrane and trigger germination (Jones & Jones, 1986). If this mechanism plays a role in aquatic hyphomycetes, it is not influenced by gravity (which may control the degree of flattening) in settled spores.

In contrast to soils, persistent and essentially static litter layers are rare in running waters, and the capture of new resources by fungi probably occurs primarily through settlement, attachment and germination of conidia dispersed by water currents (Bärlocher, 1992). Regardless of which environmental cues trigger these processes, natural selection is likely to have ensured their correlation with the presence of suitable substrates. To begin with, settlement and attachment are selective (Bärlocher & al., 1977), and generally less pronounced on artificial substrates (Read & al., 1992a, b). Rapid colonization of newly submerged leaves seems to be vital for the build-up of biomass and spore production (Sridhar & Bärlocher, 2000); the more easily accessible nutrients decline within a few days to weeks. The first phase of decomposition generally involves some leaching of soluble substances (phenolics, sugars, amino acids); depending on pretreatment of the leaves (e.g., drying or freezing), this process may last from hours to days (Taylor & Bärlocher, 1996). Increased concentrations of simple nutrients may therefore indicate the presence of a freshly immersed leaf, when rapid germination, superficial growth and invasion (facilitated by increased number of germ tubes) seem a highly appropriate response. Leaching is delayed in conifer needles or leaves with thick, waxy cuticles (Bärlocher, 1982; Canhoto & Graça, 1999); fungal growth on such leaves seems to occur primarily within the mesophyll. To reach it, conidia germinate with a few 'scouting' hyphae that enter the leaf's interior through

stomata (Canhoto & Graça, 1999). Thus, the rate and pattern of germination seem to be strongly influenced by local conditions, and the availability of simple nutrients may be crucial.

Acknowledgments

Financial support from the Natural Science and Engineering Research Council of Canada to FB, and from Societas Internationalis Limnologiae to A. M., is gratefully acknowledged.

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(Manuscript accepted 20th May 2001)