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Age-Dependent Differential Responses of *Saprolegnia* Hyphal Tips to a Helical Growth-Inducing Factor in the Agar Substitute, Gellan

SUSAN G. W. KAMINSKYJ[1] AND I. BRENT HEATH

Department of Biology, York University, 4700 Keele Street, North York, Ontario, M3J 1P3 Canada

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KAMINSKYJ, S. G. W., AND HEATH, I. B. 1992. Age-dependent differential responses of *Saprolegnia* hyphal tips to a helical growth-inducing factor in the agar substitute, gellan. *Experimental Mycology* 16, 230–239. *Saprolegnia ferax* produces more-or-less straight, subapically branched, hyphae when growing in liquid or agar-solidified media, with abundant aerial mycelium on the latter. In contrast, the same medium solidified with gellan gum induced helical growth with reduced branching and almost no aerial mycelium. Helical growth induction was gellan concentration-dependent, peaking at 0.4–0.6% (w/v), when about 60% of tips were helical. Gellan-induced helices showed concentration-dependent inhibition by agarose and polyethylene glycol. Colonies on gellan–agarose, where helices were inhibited, reverted to having aerial mycelium, whereas those on gellan–polyethylene glycol did not. Branches on helical hyphae were initially linear, but converted to helical growth after about 2 h of extension. This transition was often marked by a branch, thus branch and helix competency appeared to be related. Germinating cysts took twice as long as hyphal inocula before producing helical hyphae, reinforcing the suggestion that helix competence was age-related. *Achlya*, but not *Phytophthora*, also showed gellan-induced helical growth and aerial mycelium suppression. These results showed (a) that morphogenic regulators of hyphal growth responded to gelling agents, probably high-molecular-weight polysaccharides, (b) that all growing hyphal tips were not equivalent, and (c) that hyphal tips underwent age-related changes in their response to the environment. The gellan-related differences in aerial mycelium mimic hydrophobin-based mycelium behavior and may thus indicate environmental regulation of hydrophobin production.

INDEX DESCRIPTORS: oömycetes; *Saprolegnia;* gellan; helical growth; hyphal morphology; morphogens; hydrophobins.

Gellan gum ("Gel-Gro") is an optically superior substitute for agar and is considered to be metabolically inert (Kang *et al.*, 1982). Thus, it may be superior for diverse cytological studies of fungi, as recently suggested by Aist *et al.* (1991). During evaluation of this product, we discovered that it induced morphological changes in two species of hyphal organisms. This report presents a documentation and partial characterization of this effect, which has revealed unexpected differences in the behavior of presumably comparable growing hyphal tips.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Cultures and Media

Saprolegnia ferax (Gruith.) Thuret, ATCC No. 36051, *Achlya ambisexualis* Raper, ATCC No. 11400, and *Phytophthora infestans* (Mont.) de Bary were routinely cultured on an organic medium designated OM (Heath and Greenwood, 1968).

All media were prepared in liquid OM and are described only by the concentration (% w/v) of their gelling agents. Tested compounds included agar (Difco Bacto, Detroit, MI), gellan gum (Gel-Gro, lots 26398.01 and 30530.01: ICN, Cleveland, OH), agarose (BRL, Gaithersburg, MD),

0147-5975/92 $5.00

and polyethylene glycol 8000 (PEG: Sigma, St. Louis, MO).

Some hyphal inocula were placed on dialysis tubing strips (Fisher Spectrapor, pore size 12–14 kDa) (Heath, 1987), overlying gellan. Of these, some plates were covered with a volume of sterile distilled water or liquid OM similar to that of the gellan.

Zoospores were induced using the dilute salts technique of Holloway and Heath (1974), stored at 4°C overnight, and harvested by centrifugation (5 min at ~500*g*) before use.

Thin slabs of 0.6% gellan were made by pipetting molten medium between sterile 25 × 60-mm coverslips, with No. 1 coverslips as spacers. The upper coverslip was removed after the gel had set.

Observation Systems

Hyphal morphology was examined in sectors cut from colony margins. Images were recorded with 35-mm film and videotape and measured from tracings off the video monitor, where 1 mm was equivalent to 5.18 μm.

Hyphal Characteristics

The length along the helix coil per turn relative to the linear extension was defined as the helix index, $HI = (T^2 + \pi W^2)^{1/2}/T$, where T and W are helix turn length and width, respectively. Straight hyphae have a *HI* of 1. The degree of branching of hyphae was defined as the branching index, *BI* = number of branches per 5180 μm (1000 mm on the video monitor). Unbranched hyphae have a BI of 0.

Statistical Analyses

Statistical analyses were performed according to the procedures of Zar (1984) aided by the statistical software package Statview SE + Graphics 1.02 (Abacus Concepts, Inc., Berkeley, CA). Results were plotted with Cricket Graph 1.3.1 (Cricket Software, Malvern, PA). Results were pooled when they were not significantly different ($P > 0.05$) by factorial ANOVA. Hyphal characteristics are presented as mean ± SD; branching indices as mean ± SE. Values are followed by the results of specified statistical analyses.

RESULTS

Helical Growth

Saprolegnia hyphae were straight or undulating on liquid or agar-solidified OM (Fig. 1a). In contrast, OM solidified with gellan induced regular, three-dimensional, helices (Figs. 1b and 2). Helical growth was seen in about 60% of the tips at the colony margin in optimum levels of gellan (Fig. 3) and was induced by two different batches of gellan.

Helical growth was most abundant at between 0.4 and 0.6% gellan and decreased on either side of this peak (Fig. 3). The phenomenon was gellan-dependent as opposed to gel strength-dependent, since 0.25, 0.5, and 0.75% agarose, which subjectively covered the same range of hardnesses as the gellan gels (assessed when mounting colony sectors, about 1 × 2 cm in size), did not induce helical growth. When present, helical growth was an ongoing process because hyphae could grow helically for at least 20 turns with approximately constant pitch. However, for quantitative analysis, hyphae were considered helical if they had at least two turns with similar width and length. On different gellan concentrations, helices varied in both width and distance along the axis of the helix to complete one helical turn (turn length). These parameters were correlated with each other ($r = 0.62$), and each was linearly negatively correlated with gellan concentration (width, $r = 0.74$; turn length, $r = 0.70$) (Fig. 4). The slopes of the regression lines differed from zero (each, $P = 0.0001$, ANOVA), but not from each other ($P > 0.50$, *t* test).

Colony growth rates were measured as described in Kaminskyj *et al.* (1992) and are

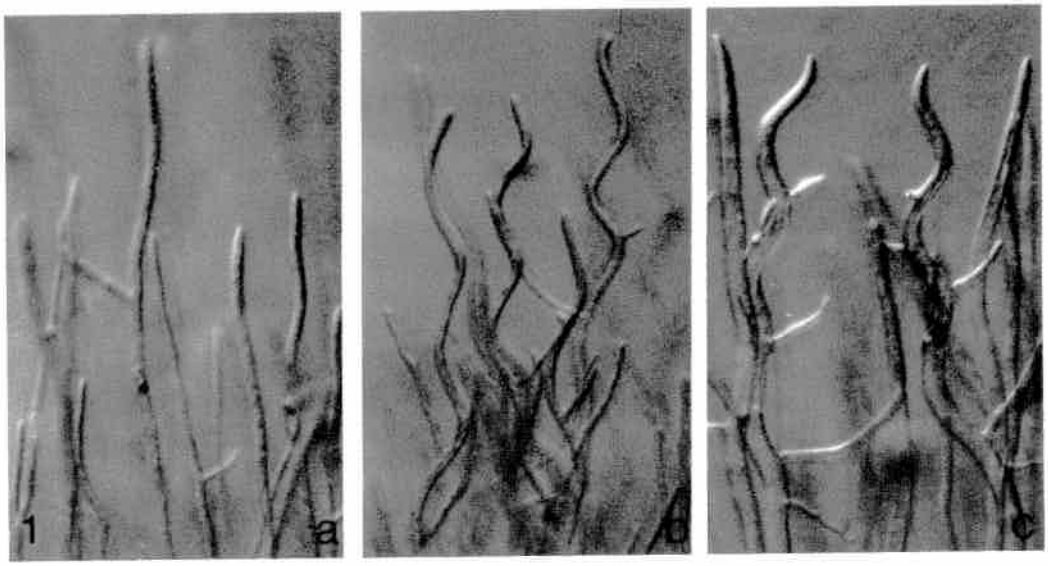

FIG. 1. Bright-field images of hyphal morphology on OM solidified with agar or gellan. (a) *Saprolegnia* on 1.5% (w/v) agar after 16 h. (b) *Saprolegnia* on 0.4% (w/v) gellan after 16 h. (c) *Achlya* on 0.7% (w/v) gellan, after 8 days. ×100.

given in Fig. 3b. Colony growth rate and occurrence of helical hyphae were linearly negatively correlated on gellan media (r = 0.97). This does not imply that helical hyphae were growing more slowly than linear ones, since helical growth is not equivalent to linear extension. At ≥0.4 gellan, the pooled *HI* was 1.7 ± 0.3 (n = 206); for each gellan concentration it was 1.7–1.8. Thus on average, helical hyphae were growing 1.7-fold further than colony extension rates indicated. Assuming that the proportion of helical tips at a colony margin (from Fig. 3a) reflected the time that a typical hypha was growing helically rather than linearly, colony growth rates can be corrected by multiplying them by the average *HI* value for that gellan concentration. Raw and corrected growth rates are shown in Fig. 3b; the slope of the regression through the latter did not differ from zero (P = 0.3543, ANOVA), thus showing that helical tip growth rates are similar to normal rates.

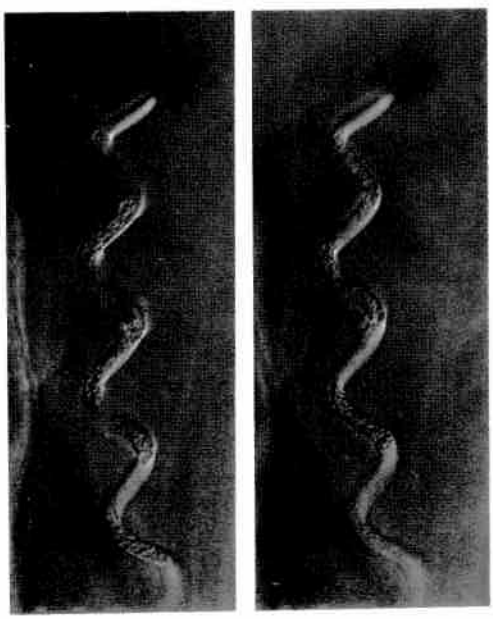

FIG. 2. Differential interference-contrast images of two focal levels of a helical *Saprolegnia* hypha on 0.6% (w/v) gellan. ×200.

Helical hyphae occasionally reverted to linear growth (Fig. 5), more commonly seen after mounting living hyphae (11% reversion, n = 164) than after mounting fixed hyphae (4% reversion, n = 335) (P = 0.0039, contingency test); few reversions were seen in colonies examined undisturbed in the petri dish.

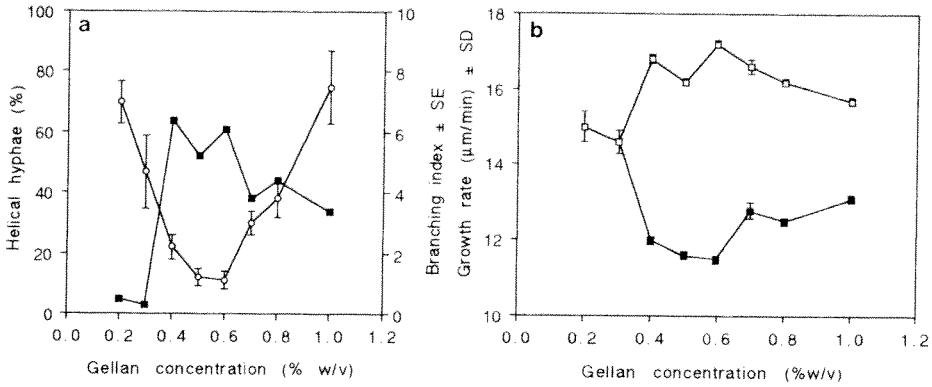

FIG. 3. Effect of gellan concentration on (a) occurrence of helical growth (solid squares) and branching index (open circles) of *Saprolegnia*. (b) Colony radial growth rate (solid squares) and growth rate corrected for helix index (open squares).

On agar-solidified OM, *Saprolegnia* produced abundant aerial mycelium, beginning near the inoculum about 24 h after inoculation (Fig. 6a). In contrast, on 0.4–0.6% gellan, aerial growth was substantially reduced so that colonies of a similar age appeared uniformly flat (Fig. 6b). On petri plates with alternating strips of agar- and gellan-solidified OM, colonies had bands of aerial mycelium restricted to the OM-solidified strips (Fig. 6c).

Concentrations of 0.6–1.0%, but not 0.4%, gellan induced helical growth and partly suppressed aerial mycelium production in *Achlya* after 8 days (Fig. 1c), but none of these concentrations induced any helical hyphae in the more distantly related *Phytophthora* even after 29 days. All subsequent data refer to *Saprolegnia*.

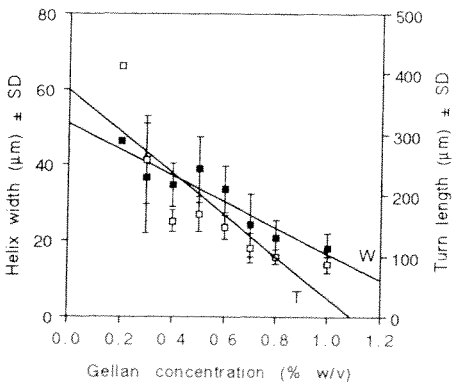

FIG. 4. Effect of gellan concentration on helix width (solid squares) and turn length (open squares) of *Saprolegnia*. Error bars for 0.2% gellan are covered by the symbols. Correlation coefficients for the regression lines are $r = 0.74$ for width (W) and $r = 0.70$ for turn length (T).

Factors Which Inhibit Helical Growth

Agarose was an inhibitor of gellan-induced helical growth. Its addition to helix-inducing 0.4% gellan reduced the occurrence of helical hyphae in a near-linear, dose-dependent manner ($r = 0.99$), with 1% agarose giving complete inhibition; colonies were normal with respect to growth rate and hyphal morphology. Helices on gellan–agarose media had agarose-dependent reductions in width (regression slope significantly different from zero: $P = 0.0001$, ANOVA) but turn length was unaffected (regression slope not different from zero: $P = 0.7459$, ANOVA). The helix inhibition of 1.0% agarose in 0.4% gellan was not overcome by increasing the concentration of gellan to as high as 0.7%. Colonies on 0.4% gellan with 1% agarose had aerial mycelium, but those on 0.4% gellan with ≤0.5% agarose had none.

Colonies growing on 0.4% gellan containing 10.0–20.0% PEG had normal hyphal

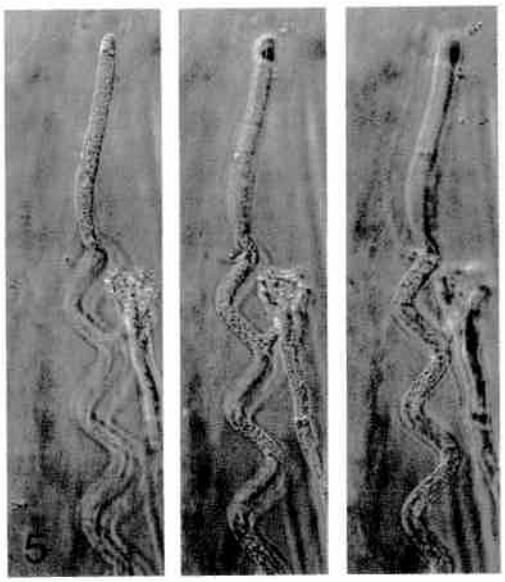

FIG. 5. Differential interference-contrast images of three focal levels of a *Saprolegnia* hypha, mounted while living, which reverted from helical to linear growth. ×200.

morphology but lacked helical hyphae, and yet these media were not subjectively stiffer than 0.4% gellan alone. Helical growth was not affected by 0.5–2.0% PEG. None of these colonies had aerial mycelium.

At least for gellan vs liquid or agarose media, the inducing and/or inhibiting factor(s) did not diffuse rapidly because hyphae growing in 0.4% gellan did not continue helical growth in a trough of liquid OM which had been in contact with the gellan for ~2 h, and they resumed helical growth upon reentering the gellan. Hyphae growing on plates with alternating strips of 0.4–0.6% gellan and 1.5% agar were characteristic of the gelling agent (Fig. 7a) when these media had been in contact for ~16 h, and hyphae changed form when they grew from one medium to the other (Figs. 7b and 7c). This change became less distinct after the media had been in contact for 24 h. Consistent with the implication that the inducing and/or inhibiting factor(s) might therefore be slowly diffusing between gellan and agar, OM–agar inocula did not produce aerial hyphae when placed on 0.6% gellan (Fig. 6b) as they would have done on agar (cf. Fig. 6a).

Hyphae growing on either surface of the gel were not helical. We did not include surface-growing hyphae in any of our analyses in order to avoid this problem. Also, colonies on 0.4% gellan which grew together did not have helical hyphae at their interface.

Hyphae growing on top of dialysis tubing on 0.4–1.0% gellan were not helical after 16 h (there were helical hyphae in the underlying medium), even when covered with distilled water or liquid OM to counteract surface inhibition of helical growth. In the latter case, volumes of gellan and liquid were similar so that a diffusible inducer should not have been diluted below the optimal concentration with ≥0.8% gellan.

Branching

Even with optimal media, only ~60% of tips were helical because branches of helical hyphae were initially linear (Fig. 8). Branches growing on thin slabs of 0.6% gellan were linear for 1610 ± 130 μm (n = 27), about 2 h of growth at rates given in Kaminskyj *et al.* (1992), before growing as helices. While linear, these branches were at the same focal level as helical hyphae, so a surface effect would not explain this helix inhibition.

Hyphae in 0.4–0.6% gellan often (64%, n = 44) branched at the point where they first began to grow helically (Fig. 8), although the branch would have been produced after helical growth had begun. Hyphae in 0.4–0.6% gellan which branched without also being helical were uncommon (12%, n = 346). The fact that branches first grew linearly suggested that there was a developmental component to the helical growth phenomenon so, to examine this further, we examined the growth form of cysts germinating on 0.4% gellan.

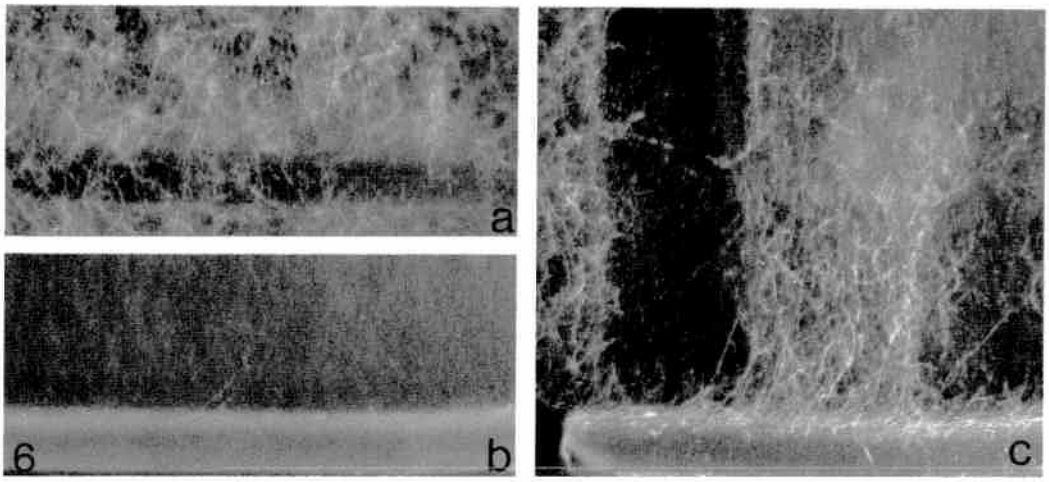

FIG. 6. Colony growth form after >24 h on (a) 1.5% agar, showing abundant aerial mycelium, (b) 0.6% gellan, without aerial mycelum even on the agar inoculum strip, (c) colony growth form on strips of agar-solidified and 0.4% gellan-solidified medium showing abundant aerial mycelium on agar medium only. ×6.

Cysts were inoculated in a streak on a 0.4% gellan plate, and the development of helical growth was compared to that of colonies from hyphal inocula (Fig. 9). Cyst-derived colonies took much longer to exhibit helical growth than hypha-derived ones; both were similar by 13.5 h. Cysts were also inoculated by swirling into 35°C 0.4% gellan (0.05- to 0.25-ml cyst suspension in 50 ml gellan). Germination and growth were similar to streak-inoculated cultures, but there was no helical growth even at 25 h. Cyst germlings branched by 8.5 h whether inoculated as a streak or a dispersion.

At peak levels for inducing helical growth, gellan also seemed to inhibit branching (Fig. 3). Hyphae on 0.4–0.6% gellan had significantly lower *BI* (1.5 ± 0.2, $n = 203$) than those on all other gellan concentrations (*BI* 5.3 ± 0.4, $n = 237$) ($P = 0.0001$, ANOVA). As shown in Table 1, the pooled *BI* for colonies growing on gellan was significantly lower than that for those on agarose or gellan–agarose media. Hyphae growing on 0.4–0.6% gellan, agarose, or gellan–agarose media had different characteristic branching patterns, both for tip-to-first branch distance and for distance between branches (Table 1).

DISCUSSION

Some component of gellan gels induced a consistent, concentration-dependent, change in hyphal morphology. This component could be either a physical or a chemical property of the gel. The former seems unlikely since the matrices of gellan and agarose gels are similar (Kang *et al.*, 1982), and pure agarose and some PEG-containing gellans of similar firmness to inducing gellans were not helix-inductive. Thus, we favor the chemical hypothesis. Since hyphal growth was distinctly different on (1) gellan and liquid media at 2 h and (2) juxtaposed gellan and agar media at 16 h, but less so after 24 h, and since hyphae were linear on top of a dialysis membrane with a pore size that would allow passage of a hexose 65-mer, the inducer must be large (thus diffusing slowly) or relatively immobile within the gellan. We suggest that the helix inducer is chemical, with a polysaccharide the most likely type of molecule.

Fungal hyphae apparently respond with

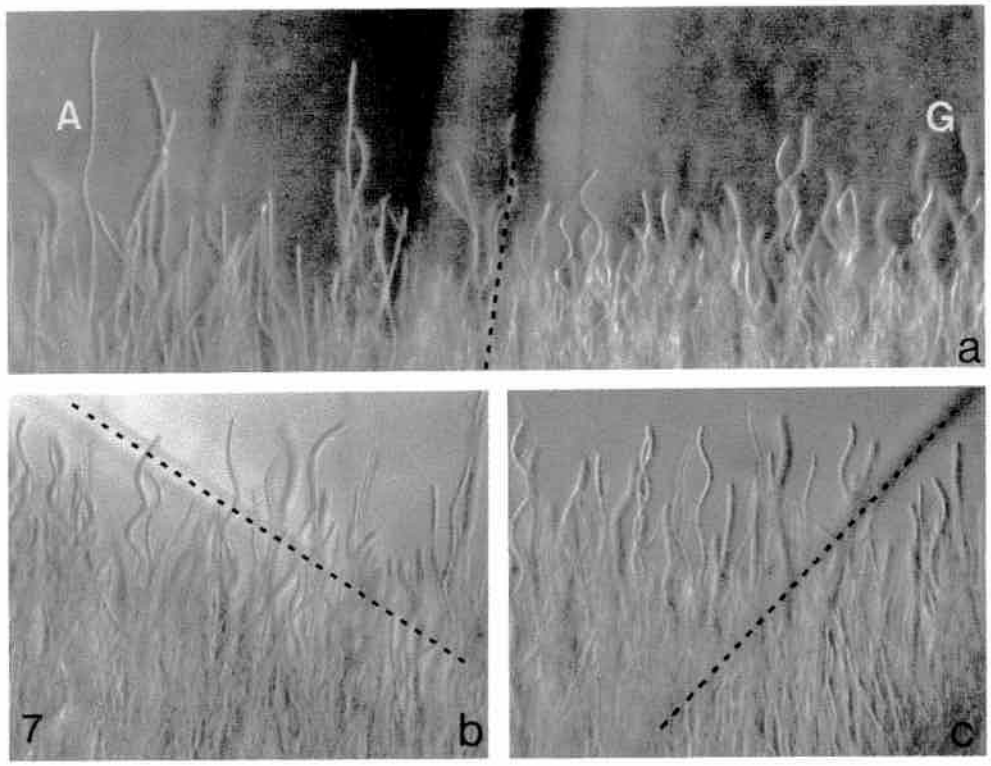

FIG. 7. Bright-field images of growth form transition between 0.4% gellan and 1.5% agar media. The dashed line indicates the interface between the media. (a) Hyphae growing on agar-solidified (A) and 0.4% gellan-solidified (G) medium. (b) Hyphae growing from gellan to agar. (c) Hyphae growing from agar to gellan. ×100.

altered morphology to contact with cell wall components in numerous situations, e.g., mating responses, best characterized in yeasts (Cross *et al.*, 1988; Madden *et al.*, 1992). Helical growth may be a similar type of response, triggered by a polysaccharide(s) in gellan. If so, inhibition of gellan-induced helices by other carbohydrates, agarose and PEG, could be due to masking. The observation that gellan caused correlated changes in both helix width and turn length, whereas agarose affected helix width but not turn length, suggests that these characteristics can be uncoupled and may indicate two inducers.

The helix inducer(s) may be comparable to oligosaccharins (Albersheim and Darvill, 1985; Ryan and Farmer, 1991). However, it differs in that oligosaccharins are typically small (≤hexose 20-mers), mobile, effective at very low concentration (10^{-10} *M*), and not readily repressed by other components of the medium.

Gellan-induced helical growth in *Saprolegnia* is intriguingly similar in helical hyphal morphology, reduced hyphal branching, and suppression of aerial mycelium to that of the *thn* mutant of *Schizophyllum commune* (Schwalb and Miles, 1967). The *thn* mutation prevents expression of hydrophobins, small hydrophobic proteins essential for production of aerial mycelium and fruiting bodies in *Schizophyllum* (Wessels *et al.*, 1991a,b). Hydrophobic proteins and peptides required for normal development and aerial mycelium are described also for *Aspergillus* (Stringer *et al.*, 1991) and *Streptomyces* (Willey *et al.*, 1991), respec-

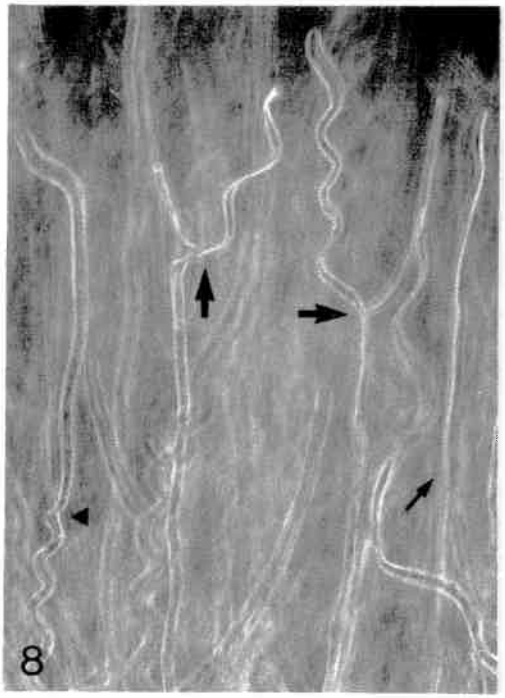

FIG. 8. Phase-contrast image of a *Saprolegnia* colony on 0.4% gellan, mounted while living. Branching at the transition from linear to helical growth (large arrows). Reversion of a helical hypha to linear growth (arrowhead). Branch which has not yet become helix competent (small arrow). ×100.

tively. Thus, hydrophobic molecules appear to be a necessary feature of aerial structures in widely separated taxa. The gellan/agarose-related change in abundance of aerial mycelium in *Saprolegnia* suggests

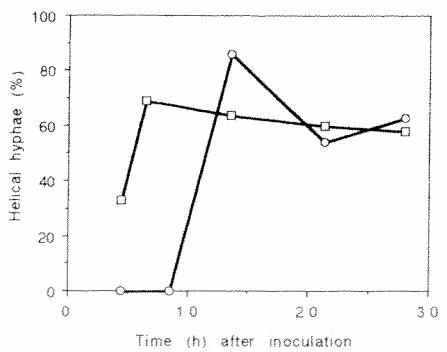

FIG. 9. Development of helical growth in *Saprolegnia* colonies produced from streak-inoculated cysts (circles) and hyphal inocula (squares).

that hydrophobin production may be another response to environmental polysaccharides, these features possibly having a common regulatory pathway.

Gellan-induced morphology of *Saprolegnia* differs from that induced by the *thn* mutation of *Schizophyllum* because the latter is a heritable, apparently irreversible, trait (Wessels *et al.*, 1991b) rather than being substrate-induced. In addition, since helix induction and aerial mycelium inhibition in *Saprolegnia* were correlated on gellan–agarose media, but not on gellan–PEG media, hydrophobins alone cannot explain our results. The gellan-induced lack of aerial mycelium in *Saprolegnia* differs from the heritable, peptide-related, features of *Streptomyces* because the colonies of the latter with blocked aerial hypha formation could be rescued by juxtaposition with normal colonies (Willey *et al.*, 1991).

Saprolegnia colonies also show the spiral colony growth form and the negative autotropic response described by Trinci *et al.* (1979). We have not investigated these phenomena with respect to the effect of gellan, but spiral colonies were seen on gellan- and agar-solidified media. Unlike the photoresponse of *Phycomyces* sporangiophores (Shropshire, 1963), the gellan response was seen in vegetative hyphae, was not photosensitive, and was seen in tip-growing cells.

The gellan effect is also reminiscent of griseofulvin-induced curling (Aytoun, 1956; Bent and Moore, 1966; Napier *et al.*, 1956). However, after griseofulvin treatment (1) curling was seen with many taxa, but was specifically not found with oömycetes, (2) the abnormal growth was irregular along hyphae and was not similar between hyphae, (3) curling was seen in germlings, and (4) hyphae often had irregular swellings, as well as curling. So, any resemblance between these growth forms seems spurious.

Since branching of *Saprolegnia* on gellan often occurred where helical growth began, tip morphogenesis and branch initiation may be interrelated phenomena. This was

TABLE 1
Effect of Gelling Agent on Branching of *Saprolegnia* Hyphae

Gelling agent	Gellan[a] (*n*)	Agarose[b] (*n*)	Agarose + gellan[c] (*n*)
Branching index[d] mean ± SE	3.5 ± 0.3[e,f] (440)	7.3 ± 0.4[g] (216)	6.7 ± 0.4[g] (220)
Distance to first branch (μm) mean ± SE	530 ± 30[f] (86)	370 ± 10[g] (174)	320 ± 10[h] (108)
Distance between branches (μm) mean ± SE	480 ± 80[f] (15)	180 ± 10[g] (115)	190 ± 10[g] (54)

[a] Pooled results for all gellans (branching index) or for 0.4–0.6% gellan (distances). Branching indices for 0.4–0.6% gellans and for other gellan concentrations are in text.
[b] Pooled results for 0.25, 0.5, 0.75, and 1.0% agarose, 1.5% agar.
[c] Pooled results for 1.0% agarose plus 0.4–0.6% gellan.
[d] Defined in text.
[e] Values in any row followed by different letters (*f–h*) are significantly different at $P < 0.05$, ANOVA.

not unexpected, but its demonstration in a simple system provides a new avenue for investigation of the relationship. Branching of *Saprolegnia* hyphae was inversely correlated with helical growth on gellan media (Fig. 3); it was also affected by the gelling agent, regardless of whether growth was helical (Table 1). On gellan, germlings from streak-inoculated cysts branched long before they were helical, and germlings from dispersed cysts did not form helices, although they branched at a similar time to streak-inoculated ones. Therefore, branching and helical growth are not correlated under all conditions. This shows that branching in *Saprolegnia* is a complex interaction of responses to hyphal volume (Trinci, 1973), developmental stage, and external factors; it may be analogous to branch regulation in *Fusarium* (Wiebe *et al.*, 1992).

Irrespective of the nature of the inducer, the present results show the unexpected fact that all morphologically similar tips of hyphae at a colony margin are not equal and that germ tube tips are not equal to hyphal tips in their response to morphogenic stimuli. This age-related change in responsiveness by morphologically similar hyphal tips is a significant novel finding.

ACKNOWLEDGMENTS

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