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# Biomechanics of invasive growth by *Armillaria* rhizomorphs

Levi Yafetto [a,1], Diana J. Davis [a,b], Nicholas P. Money [a,*]

[a] Department of Botany, Miami University, Oxford, OH 45056, USA
[b] Department of Chemistry and Physical Science, College of Mount St. Joseph, Cincinnati, OH 45233, USA

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ABSTRACT

Rhizomorphs of wood-decay basidiomycetes are root-like structures produced by the coordinated growth of thousands of hyphae. Very little is known about their development nor the way that they penetrate soils and rotting wood. In this study, we applied techniques used in previous studies on hyphae to explore the mechanics of the invasive growth process in *Armillaria gallica*. Growth rate measurements were made in media with different gel strengths. The osmolyte composition of rhizomorph sap was determined spectroscopically and the forces exerted by growing tips were measured using a force transducer. Cultured rhizomorphs extended at much faster rates than unbundled hyphae (3.5 mm $d^{-1}$ versus 1.5 mm $d^{-1}$) and their growth accelerated in response to increased medium gel strength (to 7.4 mm $d^{-1}$). Measurements of rhizomorph osmolality provided a turgor pressure estimate of 760 kPa (7.5 atm.), and spectroscopic analysis showed that this pressure was generated by the accumulation of erythritol, mannitol, and KCl. Forces exerted by growing tips ranged from 1 to 6 mN, corresponding to pressures of 40–300 kPa (0.4–3.0 atm.). Pressures exerted by extending rhizomorphs are comparable to those produced by individual vegetative hyphae. This suggests that the mechanical behavior of hyphae is similar whether they grow as unbundled cells or aggregate to form macroscopic rhizomorphs.



## 1. Introduction

Rhizomorphs are among the most complex organs produced by fungi, consisting of a series of differentiated tissues each with distinctive hyphal orientation, size, and function. Extension growth of these invasive organs can occur through dry and nutrient-poor soils and rotting wood, and rhizomorphs can breach mechanical obstacles (Shaw and Kile, 1991; Fox, 2000). Rhizomorphs produced by pathogenic species allow fungi to travel between host plants. This process has contributed to the well-publicized spread of *Armillaria gallica* and *Armillaria ostoyae* over vast territories (Smith et al., 1992).

Active rhizomorphs are formed from many thousands of individual hyphae whose coordinated growth produces cylindrical structures with rounded tips (Fig. 1a). Anatomically, the rhizomorph is similar to a basidiome primordium (Hedger et al., 1993): the shaft of the organ is organized like a mushroom stipe, and the tip region resembles the outer surface of an immature cap (Fig. 1b). This structural affinity may reflect common developmental processes and shared evolutionary origin (Moore, 1998). Despite their ecological importance, and their role in plant disease, we know very little about the development of rhizomorphs and almost nothing about their mechanism of invasive growth. The ease of culturing *Armillaria* rhizomorphs lends them to the experimental analysis of many intriguing questions about the development of fungal multicellularity, hyphal communication, and tip growth. In this study, we have applied techniques perfected during earlier research on hyphal growth (Money, 2008) to understand the mechanics of invasive growth in *A. gallica* rhizomorphs.

## 2. Materials and methods

### 2.1. Organism and culture methods

*A. gallica* Marxm. & Romagn. strain DR-140 was obtained from Dr. Dana Richter (Michigan Technological University). The identity of the strain was confirmed by comparison of ITS sequences with GenBank data (accession no. FJ744699). The primer pair ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) was used for PCR and sequencing. This strain produced rhizomorphs on potato dextrose agar (PDA) and in potato dextrose broth (PDB). To aid in separation of rhizomorphs from PDA for spectroscopic analysis, cultures were grown on disks of sterilized cellophane (P25; Innovia Films, Tecumseh, KS) placed on the surface of the medium. After inoculation, cultures were stored in the dark to stimulate rhizomorph formation.

### 2.2. Scanning electron microscopy

Rhizomorphs were fixed in 2.5% (v/v) glutaraldehyde, 1% (v/v) formaldehyde in 0.05 M (v/w) sodium cacodylate buffer (pH 7.2)

* Corresponding author. Fax: +1 513 529 4243.
*E-mail address:* moneynp@muohio.edu (N.P. Money).
[1] Present address: Department of Organismic and Evolutionary Biology, Harvard University, Biological Laboratories, 16 Divinity Avenue, Cambridge, MA 02138, USA.

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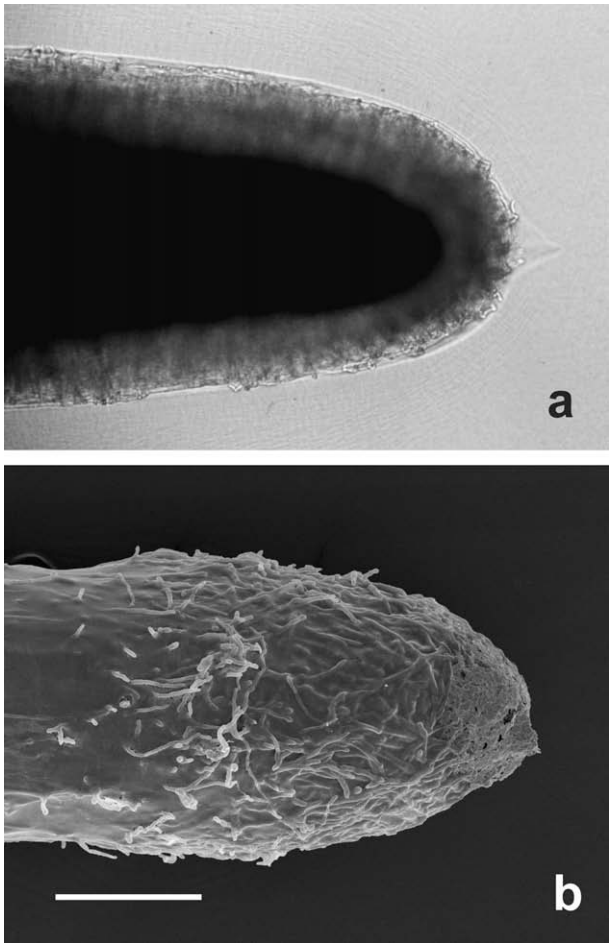


**Fig. 1.** Rhizomorph tips of *A. gallica*. (a) Light micrograph showing rhizomorph penetrating agar medium. Gas-filled central cavity appears as opaque core. Note medium compression around entire tip and fracture in advance of the extreme apex, which is analogous to the mechanism of crack propagation by burrowing invertebrates. (b) Scanning electron micrograph of rhizomorph tip showing peripheral hyphae embedded in mucilaginous extracellular matrix and prominent mucilage cap covering the extreme tip. Scale = 100 μm.

for 2 h at room temperature. Fixed samples were rinsed 3× with 0.05 M sodium cacodylate buffer (pH 7.2) at intervals of 10 min then dehydrated serially in ethanol. For freeze-fracture, samples were removed from 100% ethanol, plunged into liquid nitrogen, and pressed against a fracturing plate with a chilled razorblade. Fractured samples were then returned to 100% ethanol, then critical point dried, gold plated, and examined with a JOEL JSM-840A microscope.

### 2.3. Light microscopy and image analysis

Rhizomorph tips were viewed with a 10× objective lens on inverted microscopes (models IX70 and IX71; Olympus, Tokyo). CamWare software (version 2.10 and 2.21, PCO), was used to capture images of extending rhizomorphs at 1 min intervals from CCD video cameras (PixelFly and pco1600; PCO, Kelheim, Germany) and saved in 16-bit TIFF files. Files were then processed to adjust brightness and contrast, and saved in 8-bit TIFF format using ImageJ software (version 1.40g, NIH). Video clips were assembled using BTV Pro Carbon software (version 5.4.1, Ben Software, London). A customized Canny edge-detection analysis was performed using MATLAB (Version 7.6.0 R20008a; The MathWorks, Natick, MA). This highlighted details of rhizomorph anatomy, granules within the agar medium (that served as useful external positional markers), and compression of the agar.

### 2.4. Growth rate measurements

The rate of hyphal extension was determined from daily measurements of colony radius from six replicate cultures (made from two randomly-selected radii in each Petri dish). This simple method is just as effective at providing information on growth trends as more detailed measurements based upon the volume of individual hyphae (Davis et al., 2006). Rhizomorph extension was measured by marking the position of rhizomorph tips at daily intervals on the bottom of the Petri dishes ($n = 6$) with the aid of a dissecting microscope. The total length of rhizomorphs produced in single Petri dish cultures ($n = 4$) was determined from scanned images of cultures using Image-Pro Plus 4.5 software (Media Cybernetics, Bethesda, MD). The majority of the rhizomorphs formed within the agar, but a few were exposed on the surface of the medium.

### 2.5. Measurement of osmotic pressure and estimation of turgor

Osmometric measurements of rhizomorph osmolality were made according to the methods detailed in Money and Ravishankar (2005). Because each rhizomorph is composed of a series of concentric cylinders of differentiated hyphae, these data reflect the mean osmolality of the entire organ: some layers of hyphae may have a higher or lower osmolality than others. This is an inherent limitation of the method. Osmolalities were converted to osmotic pressures using the Van't Hoff equation and turgor pressure estimates were based upon average values for osmotic pressure and assumed maximum hydration. For this reason, turgor pressures of rhizomorphs growing on the surface of cultures probably varied in relation to dehydration caused by evaporation of water (see further discussion in Money and Ravishankar (2005)).

### 2.6. Quantitative spectroscopy

Samples of 5–10 individual rhizomorphs were harvested from cultures using sterile forceps and placed in pre-weighed microfuge tubes. After measuring sample wet weight, 150 μL of sterile DW was added to each tube and the rhizomorphs were crushed using a Teflon plunger to release sap from their constituent hyphae. Following disruption, the samples were centrifuged at 15,000*g* for 3 min to separate cell wall debris from the translucent supernatant of diluted sap. The dry weight of the pellets was determined by placing them on pre-weighed filter papers and drying in an oven at 80 °C for 24 h. Dry weight measurements were subtracted from the wet weights to provide estimates of the volume of fluid in the original samples. Supernatants from the microfuge tubes were then stored at −20 °C.

Sugars and sugar alcohols contributing to sap osmolality were identified and quantified using GC/MS. Samples were derivatized to produce alditol acetates of the sugar alcohols (Higgins et al., 1994). The dried samples were resuspended in 10 μL of chloroform and multiple injections of 1 μL, separated by blank runs of chloroform, were analyzed on a Varian CP-3800 GC/Saturn 2000 MS. Osmolytes were identified by comparison to GC/MS profiles of purified samples of alditol acetates, and mass spectra of alditol acetates obtained from the NIST/EPA/NIH Mass Spectral Library (NIST, Gaithersburg, MD). The concentrations of the major osmolytes were determined from standard curves produced by plotting the log of the concentration of standards versus the log of the ion intensity of a characteristic ion fragment for each osmolyte (103 for glycerol, 139 for mannitol, and 217 for erythritol). Separate sets of aqueous extracts from the rhizomorphs were further diluted to 10 mL and metals analysis was carried out using a Varian 800 series ICP-MS (Mulgrave, Victoria, Australia) controlled with Varian ICP-MS Expert software. Ion concentrations were determined from