



## Identification of *Armillaria* field isolates using isozymes and mycelial growth characteristics

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### Abstract

This research was conducted to develop procedures based on mycelial growth characteristics and patterns of esterase (EST) and polyphenol oxidase (PPO) production by diffuse mycelia for identification of *Armillaria* field isolates from *Quercus-Carya-Pinus* forests in the Ozark Mountains (central USA). The 285 isolates collected were first identified by standard diploid-haploid pairing tests as *A. gallica*, *A. mellea*, or *A. tabescens*. A strong PPO band was diagnostic for *A. gallica*. All *A. mellea* isolates tested and 91% of the *A. tabescens* isolates tested were distinguished based on production of EST bands in three standardized  $R_f$  ranges. A procedure based on mycelial growth and morphology on tannic acid medium (TA) at 24 °C and on malt extract medium (ME) at 33 °C correctly identified 98% of *A. gallica* isolates and all *A. mellea* and *A. tabescens* isolates. On TA, *A. gallica* grew slowest. On ME, *A. mellea* grew slowest: mycelial morphology differed among species; *A. gallica* typically stained the agar and produced an appressed/submerged growth pattern with concentric bands of decreasing hyphal density, *A. mellea* typically did not stain the agar and produced round mycelia with smooth margins and abundant aerial hyphae, *A. tabescens* typically stained the agar and grew appressed/submerged with very irregular margins and patchy hyphal density. These are the first published systems evaluating the potential for identifying *Armillaria* field isolates based on their mycelial growth characteristics and EST and PPO complements.

**Key words:** *Armillaria gallica*, *mellea*, *tabescens*, isozymes, mycelium

### Introduction

*Armillaria* (Fr. : Fr.) Staude is a globally distributed white-rot wood decay fungus genus (Agaricales, Tricholomataceae) comprising at least 40 species that vary in root pathogenicity contributory to stress-induced forest decline; approximately nine species occur in North America [1, 2]. Differentiation of *Armillaria* spp. in the field is often difficult due to similarities among their macroscopic features (mycelial fans, rhizomorphs, and basidiomata) [3]. *Armillaria* spp. differ considerably in ecology and geographic distribution, and the number of species encountered decreases as the search pattern becomes more focused [e.g., 4–8]. As the number of species to be distinguished is reduced, it may become easier to develop

identification systems, especially if the species to be distinguished are distantly related [9]. It is necessary to be able to distinguish the *Armillaria* spp. functioning in an ecosystem to reach an understanding of how the spatial distributions and ecological attributes of *Armillaria* individuals (genets, *sensu* Harper [10]) affect long-term forest community structure in response to natural as well as management-related perturbations [e.g., 8, 11, 12].

The usual means of identifying *Armillaria* field isolates to species are by pairing single-basidiospore isolates of known identity (tester isolates) with either field-collected single-basidiospore isolates (haploid-haploid matings [13]) or vegetative mycelium isolates (diploid-haploid pairings [14]). Unfortunately, both

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pairing techniques are laborious and time-consuming [15], occasionally producing ambiguous results necessitating repetition with additional testers. Also, field-collected basidiospores are not consistently available or inducible because *Armillaria* genets fruit infrequently and sporadically in nature and can not be dependably or rapidly fruited *in vitro* [16].

Isozyme analysis provides alternative procedures for characterizing fungal isolates [17]. Esterases (EST) and polyphenol oxidases (PPO) have been used to study *A. ostoyae* (Romagn.) Herink variability in British Columbia, Canada [18]. Total protein and EST patterns have been used to distinguish *A. ostoyae*, *A. gallica*, *A. calvescens* Bérubé & Dessureault, and *A. sinapina* Bérubé & Dessureault in Ontario, Canada [4]. Isozymes have been used to develop electrophoretic phenotypes for distinguishing certain *A. calvescens*, *A. gemina* Bérubé & Dessureault, and *A. ostoyae* genets in New Hampshire [19]. The likely representation of products of multiple genes in isozyme phenotypes may be an advantage over faster methods focusing specifically on rDNA analysis [e.g., 9, 20, 21].

The simplest type of technique for identifying field isolates to species might be based on the characteristics of laboratory-grown mycelia. Variables used to describe *Armillaria* cultures include characteristics of rhizomorph systems, mycelial growth rate and pattern, production and characteristics of aerial hyphae, and pigmentation of the agar substrate [22–27]. Cultural characteristics have been studied at different temperatures and pH levels [22, 25, 26, 28], as well as on various media [23, 25, 26, 29]. However, the reliability of these growth characters has not been evaluated, and geographically separated populations of *Armillaria* spp. differ in growth characteristics [e.g., 23, 25].

The purpose of this research was to develop additional methods for routine identification of *Armillaria* field isolates collected for studies of the spatial distributions and interactions of *Armillaria* genets influencing forest structure and decline in Ozark forests [8]. This is the first published description of systems developed and tested with large numbers of isolates for the reliable identification of *Armillaria* isolates based on their mycelial growth characteristics and their EST and PPO complements.

## Materials and methods

### *Fungal isolates*

EST and PPO isozyme profiles of 285 presumptively diploid [15] *Armillaria* field isolates (115 isolates representing 105 *A. gallica* genets, 136 isolates representing 121 *A. mellea* genets, and 34 isolates representing 24 *A. tabescens* genets), and mycelial growth characterization of a subset of these (51 isolates representing 44 *A. gallica* genets, 92 isolates representing 83 *A. mellea* genets, and 34 isolates representing 26 *A. tabescens* genets) were obtained. All isolates were obtained from rhizomorph, mycelial fan, or basidioma context tissue samples collected in upland *Quercus-Carya-Pinus* forests of Missouri's Ozark Mountains [8]. Isolates were identified to genet by somatic incompatibility tests [30], and genets were identified to species by standard diploid-haploid pairing tests [14, 31], using tester isolates provided by Dr. J.B. Anderson, Dept. of Botany, Erindale College, University of Toronto. All isolates were maintained under refrigeration on 2% (w/v) malt extract (ME) medium solidified with 2% (w/v) agar (2MEA). Field isolates representative of each species have been deposited with the Center for Forest Mycology Research, USDA Forest Products Laboratory, Madison, WI.

### *Isozyme studies*

Isolates were incubated for 11–16 days at 21 °C in the dark on 1.5% (w/v) ME solidified with 1.5% (w/v) agar (1.5 MEA). Approximately 2.0 cm<sup>3</sup> of agar culture from the growing margins of several colonies were macerated and transferred to 100 mL 1.5% (w/v) ME liquid medium containing 12.5 mM MES (2-[N-morpholino]ethanesulfonic acid) at pH 6.0. These cultures were incubated stationary at 21 °C for 29–33 days in the dark. Mycelia were collected under vacuum on Whatman No. 1 filter paper and crushed in liquid nitrogen. Before the sample thawed, 350–400 µL of 50 mM TRIS (pH 7.5) were added, and the sample was further ground for 2–3 min. The suspension was stored overnight at 4 °C and centrifuged at 13 200 × *g* for 10 min. The supernatant liquid was collected, and the protein concentration measured [32].

Samples were subjected to discontinuous polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions [33]. All gels were 8.5 cm wide, 5.5 cm tall, and either 1.0 or 1.5 mm thick. Gel thickness does not affect R<sub>f</sub> value; thicker gels were used

with larger samples (containing more protein, see below). Resolving gels for EST analyses contained 12% (w/v) polyacrylamide. Resolving gels for PPO analyses contained 7% or 12% (w/v) polyacrylamide. The buffer in the resolving gel was 0.375 M Tris, pH 8.8. All stacking gels contained 4% (w/v) polyacrylamide; the buffer in the stacking gel was 0.125 M Tris, pH 6.8. The electrode buffer was 0.025 M Tris, 0.2 M glycine, pH 8.3. Electrophoresis was carried out at 150 V until the bromophenol blue dye front reached the bottom of the gels (50–60 min).

Isoforms of EST were visualized with activity stain. Gels were briefly washed in distilled water several times and stained for EST activity following the colorimetric assay of Wendel and Weeden [34]. The staining solution was prepared by mixing substrate A (25 mg  $\alpha$ -naphthyl acetate) dissolved in 50 ml of 100 mM sodium phosphate buffer (pH 6.0) with substrate B (25 mg  $\beta$ -naphthyl acetate) dissolved in 1 ml of cold acetone and adding 50 mg fast blue RR salt. Gels were incubated in the staining solution for 10–15 min on a rotary shaker, thoroughly washed in distilled water and dried. Best results were obtained with 60  $\mu$ g and 40  $\mu$ g protein per lane in 1.5 mm and 1.0 mm thick gels, respectively; both gel thicknesses produced the same EST  $R_f$  values. The position of each band relative to the dye front as the dye front reached the bottom of the gel ( $R_f$ ) was measured before the gel was dried.

Although electrophoresis was always carried out until the bromophenol blue dye front reached the bottom of the gel, isoform mobility typically varies among gels to a greater extent than does the mobility of the bromophenol blue dye. Thus, for comparison of isoform mobility ( $R_f$  values) among gels, it is appropriate to include in each gel an internal standard (IS) "marker" isoform of the general class being studied (EST) which produces a band approximately in the center of the lane. By defining the average  $R_f$  value of the IS band as its standardized  $R_f$  ( $R_{fs}$ ) value, the  $R_{fs}$  value for any band can be calculated by linear interpolation from the raw  $R_f$  values of that band and the IS produced in the same gel. For this study, an EST isoform which produced a clear strong band with an average  $R_f$  value of approximately 0.55 was consistently produced by *A. gallica* isolate 3c6 (clone 2 [35]). The isoform producing this band was adopted as the IS, and this isolate was therefore included in each gel to provide the IS band. All  $R_f$  values reported have been standardized. A complete set of the raw  $R_f$  values and corresponding  $R_{fs}$  values associated with

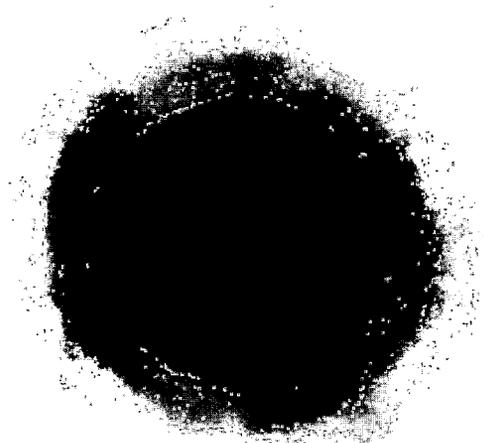


Figure 1. Representative *A. gallica* mycelium photographed with bright field and reflected illumination after 7 wk incubation in the dark on ME agar medium, demonstrating appressed/submerged growth and concentric bands of decreasing hyphal density. Inoculum plug was 3 mm diam.

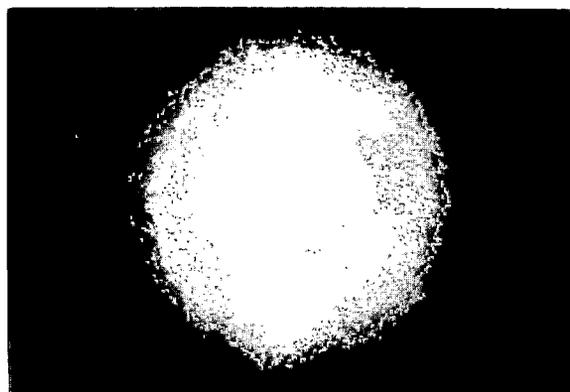


Figure 2. Representative *A. mellea* mycelium photographed with dark field and reflected illumination after 7 wk incubation in the dark on ME agar medium, demonstrating abundant aerial growth, round outline, and uniform hyphal density. Inoculum plug was 3 mm diam.

all isolates used in this study is available from the corresponding author.

Isoforms of PPO were visualized with a 10 mM DL-3,4-dihydroxyphenylalanine (DL-DOPA) solution (pH 7.0) [36]; PPO activity was assessed 15 min after staining. Clear binary PPO activity data were consistently produced with 20  $\mu$ g protein per lane. We defined strong PPO activity as the appearance of at least one broad (3–10 mm) orange band within 15 min of staining; these bands always occurred between  $R_{fs}$  0 and  $R_{fs}$  50.

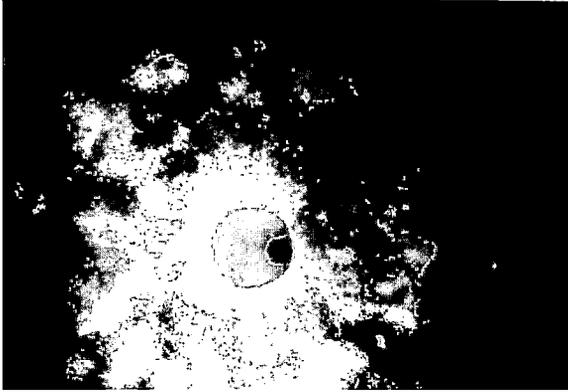


Figure 3. Representative *A. tabescens* mycelium photographed with dark field and reflected illumination after 7 wk incubation in the dark on ME agar medium, demonstrating patchy hyphal density and irregular margin. Inoculum plug was 3 mm diam.

Table 1. Numbers of *Armillaria* isolates producing selected isoform bands. Numbers of isolates tested are shown in parentheses for each species

Isoforms <sup>a</sup>	<i>A. gallica</i> (115)	<i>A. mellea</i> (136)	<i>A. tabescens</i> (34)
PPO	115	0	0
EST-g1	108	2	19
EST-m1	46	136	3
EST-t1	1	6	33
EST-t2	14	1	26

<sup>a</sup> PPO: appearance of at least one strong, broad band with standardized  $R_f < 0.50$  within 15 min of adding DOPA staining solution; selected EST bands: g1 = a 2–4 mm broad band within standardized  $R_f$  0.12–0.17; m1 = a strong, independent band within standardized  $R_f$  0.23–0.30; t1 = at least two bands within standardized  $R_f$  0.43–0.54; t2 = a single clear band within standardized  $R_f$  0.31–0.36.

### Mycelial growth studies

Isolates were initially incubated 11–16 days at 21 °C in the dark on 1.5 MEA. Agar plugs (3 mm dia) were then transferred from the growing margins of several mycelia to petri plates for 7 wk incubation, either on 1.5 MEA in the dark at 33 °C, or on tannic acid agar (TA) [29] in the dark at 24 °C. Colony diameter was then measured and the MEA mycelia were also evaluated for presence/absence of six morphological traits: (1) abundant aerial hyphae, (2) smooth, round mycelial margin, (3) staining of the agar medium, (4) zonate growth pattern, (5) patchy hyphal density, and (6) irregular mycelial outline (Figures 1–3).

## Results

### Isozyme studies

All isolates initially identified as *A. gallica* produced strong PPO activity (Table 1, Figure 4). *Armillaria tabescens* often produced faint PPO activity with 15 min staining. The 115 *A. gallica*, 136 *A. mellea*, and 34 *A. tabescens* isolates produced  $3.77 \pm 0.91$  (mean  $\pm$  standard deviation),  $3.95 \pm 1.22$ , and  $6.68 \pm 1.84$  EST bands, respectively. Several EST patterns were frequently observed (Table 1, Figure 4): for *A. gallica*, a 2–4 mm thick, smeared band at  $R_{fs}$  0.12–0.17, designated “g1”; for *A. mellea*, a strong independent band in the region of  $R_{fs}$  0.23–0.30, designated “m1”; for *A. tabescens*, at least two bands in the region  $R_{fs}$  0.43–0.54, together designated “t1”, and a single clear band in the region of  $R_{fs}$  0.31–0.36, designated “t2”. Nearly all *A. gallica* isolates produced a g1 band; all *A. mellea* isolates produced an m1 band; and all *A. tabescens* isolates produced t1 and/or t2 bands. The following key, based on PPO and EST patterns, correctly identified all *A. gallica* and *A. mellea* isolates, but misidentified 3 *A. tabescens* isolates (8.8%) as *A. mellea* because they produced m1 bands but lacked t1 and/or t2 bands.

A. Isolates with strong PPO activity	<i>A. gallica</i>
A'. Isolates without strong PPO activity	B
B. Isolates without an m1 EST band, with t1 and/or t2 bands	<i>A. tabescens</i>
B'. Isolates with an m1 EST band	C
C. Isolates producing either t1 or t2 bands, or neither	<i>A. mellea</i>
C'. Isolates producing both t1 and t2 bands	<i>A. tabescens</i>

In addition to the Ozark isolates, one *A. gallica* isolate from Michigan (Clone 2 [35]), two *A. gallica* isolates from Crittendon Co., Arkansas, three *A. tabescens* isolates from Columbia, Missouri, and four *A. tabescens* isolates from the Delta National Forest in Mississippi were also correctly identified using this system.

### Mycelial growth studies

Growth of *A. gallica* isolates on TA was slower than that of *A. mellea* or *A. tabescens* isolates (Figure 5). Mean diameter growth ranges were 0.0–16.3 mm (mean  $\pm$  sd,  $6.5 \pm 3.3$  mm) for 51 *A. gallica* isolates, 14.5–27.0 mm ( $20.2 \pm 2.8$  mm) for 92 *A. mellea* isolates, and 8.3–27.7 mm ( $17.8 \pm 3.8$  mm) for 34 *A. tabescens* isolates.

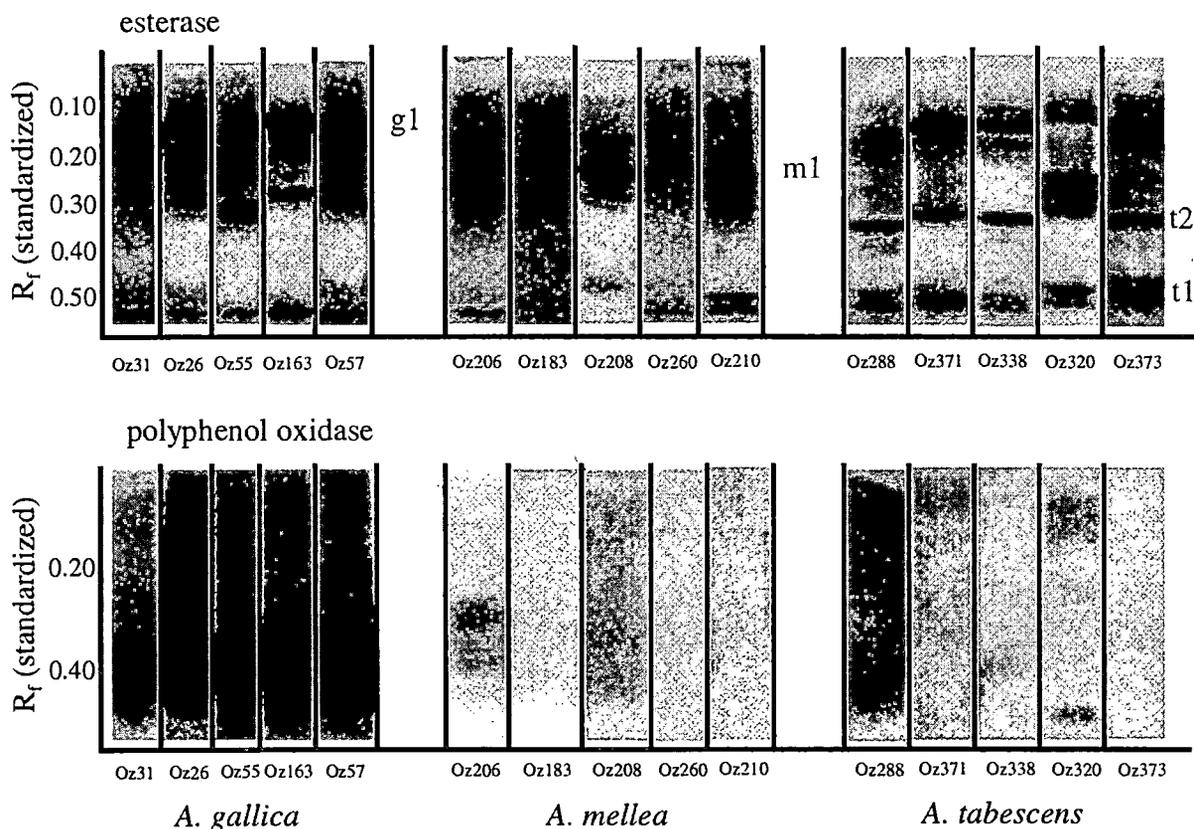


Figure 4. Representative EST and PPO patterns for *A. gallica*, *A. mellea*, and *A. tabescens*. EST band standardized  $R_f$  regions g1, m1, t1 and t2 are indicated. The cathode was at the top of each gel (above  $R_f$  0.0), and the anode was at the bottom of each gel (below  $R_f$  1.0).  $R_f$  values were standardized (see Methods) by linear interpolation relative to internal standard marker bands (EST defined  $R_f$  0.55; PPO defined  $R_f$  0.40) produced by *A. gallica* isolate 3c6, which was included in each gel. To produce this figure, selected lanes were digitized from gels using a flat bed scanner and Deskscan II software; Deskscan II files were read into WordPerfect 6.1 for configuration, where lanes were electronically stretched as necessary to accurately represent the standardized  $R_f$  values of the bands depicted.

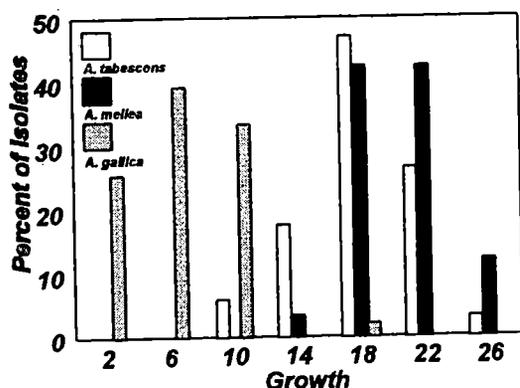


Figure 5. Distributions of diam growth (mm) after 7 wk incubation in the dark at 24 °C on tannic acid medium. *Armillaria gallica*, *A. mellea*, and *A. tabescens* were represented by 51, 92, and 33 field isolates, respectively. Growth of each isolate is the mean diameter extension from the inoculum plug for two perpendicular measurements of three mycelia in separate petri dishes.

Colony diameter ranges on 1.5 MEA at 33 °C were 9.7–30.9 mm ( $19.5 \pm 4.6$  mm) for *A. gallica*, 0.0–20.8 mm ( $7.9 \pm 4.6$  mm) for *A. mellea*, and 13.3–36 mm ( $25.9 \pm 6.0$  mm) for *A. tabescens*. Only 7.8% of the 51 *A. gallica* isolates, and none of the 34 *A. tabescens* isolates, grew less than 13.0 mm, compared with 87.0% of the 92 *A. mellea* isolates.

Mycelial morphology on 1.5 MEA at 33 °C differed among the species (Figures 1–3). Nearly all *A. gallica* (98.0%) and *A. tabescens* isolates (91.2%) produced appressed/submerged mycelia, whereas the mycelia of most *A. mellea* isolates (85.9%) produced abundant aerial hyphae. All *A. mellea* mycelia with little aerial development grew slowly. The mycelia of most *A. mellea* isolates (73.9%) were round with very smooth margins. The mycelia of nearly all *A. gallica* (96.1%) and most *A. tabescens* isolates (73.5%) stained 1.5 MEA brown; the few *A. mellea* isolates

(6.5%) producing mycelia which stained the agar grew slowly. Nearly all *A. gallica* isolates (94.1%) produced mycelia with concentric bands of decreasing hyphal density. No *A. mellea* or *A. tabescens* mycelia combined concentric banding with agar staining, whereas the mycelia of 90.2% of the tested *A. gallica* isolates did. Almost half of the *A. tabescens* isolates (44.1%) produced mycelia with randomly distributed, patchy hyphal aggregations, and most *A. tabescens* (76.5%) produced mycelia with very irregular margins. The following key correctly identified 176 of the 177 Ozark isolates considered. One *A. gallica* isolate was misidentified as *A. tabescens*; it grew 9.67 mm in the TA test and 13.8 mm in the MEA test, did not produce concentric bands of decreasing hyphal density in the MEA test, but did stain MEA.

A.	Diameter growth on TA < 8.0 mm; mycelia on 1.5 MEA at 33 °C at 7 wk not distinctly round in outline with smooth margin	<i>A. gallica</i>
A'	Diameter growth on TA ≥ 8.0 mm	B
B.	Mycelia on 1.5 MEA showing concentric banding of hyphal density and staining the agar	<i>A. gallica</i>
B'	Not as above	C
C.	Diameter growth on 1.5 MEA < 13 mm	<i>A. mellea</i>
C'	Diameter growth on 1.5 MEA ≥ 13 mm	D
D.	Diameter growth on 1.5 MEA ≤ 21 mm; mycelium margin not irregular, no patchy hyphal aggregations, and not staining the medium	<i>A. mellea</i>
D'	Not exactly as above, and mycelium on 1.5 MEA never with concentric banding of hyphal density	<i>A. tabescens</i>

In addition to the 177 Ozark isolates, one *A. gallica* isolate from Michigan (Clone 2 [35]), two *A. gallica* isolates from Crittendon Co., Arkansas, eight *A. gallica* isolates from eastern Tennessee [7], and four *A. tabescens* isolates from west-central Mississippi were tested. Of these 15 extra isolates, 14 were correctly identified. One Tennessee *A. gallica* isolate was misidentified as *A. mellea*; in the TA test it grew 11.8 mm, and in the MEA test it failed to produce concentric banding, did not stain the agar, and grew 17.2 mm. The tabulated growth measurements and characteristics of all isolates tested are available on request.

## Discussion

Because *Armillaria* mycelia grow slowly, we initially attempted to base our isozyme system on the EST and PPO contents of rhizomorphs grown on oranges autoclaved in water. Rhizomorphs growing submerged in water were generally white, lacking the characteristic melanization of the rhizomorph cortex that develops with oxidation. Rhizomorph initiation and growth on autoclaved oranges were often prolific and fast, but were also undependable, varying both among replicates of isolates and among isolates of each species. Also, the rhizomorph tissues produced from autoclaved oranges contained different EST complements than mycelia of the same isolate grown in ME. Rhizomorph production was much poorer on both MEA and TA than from autoclaved oranges. For these reasons, rhizomorph evaluations were discontinued.

Other studies have shown that isozyme analysis has potential for distinguishing *Armillaria* isolates to species [4, 30, 37–42] or even genet [19], and that *Armillaria* spp. vary in gross mycelial form *in vitro* [22–29, 37]. Nevertheless, this is the first study to present tested protocols based on isoform patterns and gross mycelial characteristics for the reliable identification to species of *Armillaria* field isolates from a geographic region. This endeavor was feasible because the study region represents a natural set of related environments (the upland oak-hickory-pine forests of Missouri's Ozark mountains) where only three *Armillaria* spp. appear to occur [8]. Further, these three *Armillaria* spp. are phylogenetically relatively distant [9], differing sufficiently in both *in vitro* EST and PPO complements and gross mycelial characteristics to permit development of rule sets for their dependable identification. Similar efforts including more species, more closely related species, or more of the global genetic diversity of the species considered could be more challenging [23, 25].

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tester isolates of North American *Armillaria* spp. The authors also gratefully acknowledge discussions with Mr. Mark T. Banik and Dr. Harold H. Burdsall, Jr., at the outset of the growth characteristics study.

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