
MORPHOLOGICAL CHARACTERISTICS OF INCOMPATIBILITY REACTIONS AND EVIDENCE FOR NUCLEAR MIGRATION IN ARMILLARIA MELLEA

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ABSTRACT

A new technique was developed to distinguish the four incompatibility interactions in Armillaria mellea sensu stricto. The technique revolves confronting two single-basidiospore isolates on oak wood extract agar and then transplanting a strip from the colonies including the interaction zone onto squeezed orange juice agar. A compatible (A*B+) interaction results in a change of the colony morphology along the entire agar strip. Morphological characteristics of compatible interactions are highly variable. They may be expressed as appressed or crustose secondary mycelium. In a common B (A*B=) interaction, such a change occurs only in the interaction zone. The ends of the agar strip remain white and fluffy. Both the common A (A=B*) and common AB (A*B=) interactions remain fluffy and unchanged. The genotype can be deduced after identifying the A+B+ and A+B- interactions. In addition, pairing mycelium produced by the confrontations with known monosporous tester isolates showed that nuclear migration occurs in A+B+ and A+B- interactions but does not occur in A*B= interactions.

Key Words: Armillaria, incompatibility genotype, nuclear migration

Armillaria mellea (Vahl: Fr.) Kummer sensu stricto is an important pathogen of the roots of many woody perennial plants (Wargo and Shaw, 1985; Rishbeth, 1985; Farr et al., 1989). Although A. mellea has been considered to have a wide host range and worldwide distribution, this plant pathogen apparently is confined to hard-wood hosts in Japan, North America, and Europe. Armillaria root disease in parts of Asia, Africa, Australia, and on conifers in North America and Europe is caused by several other species of Armillaria (Kile and Watling, 1988; Mohammed et al., 1989; Mwangi et al., 1989). In North America, seven species of Armillaria have been delimited recently (Bérubé and Des- sureault, 1989), including a nonannulate species. A. tabescens (Scop.: Fr.) Dennis. No species designations have been made for two North American biological species. NABS IX and NABS X.

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Biological species classification (Anderson, 1986) is based on interspecific incompatibility mating reactions, but serological and molecular techniques have also been employed for the identification of Armillaria species (Fox and Hahne, 1989; Smith and Anderson, 1989; Burdsall et al., 1990). Identification based on basidiome morphology is difficult because of the variability of morphological characteristics among the biological species (Watling et al., 1991). However, by combining the characteristics of macromorphology and micromorphology (e.g., lack of clamp connection at the base of basidia), cultures, and host associations, A. mellea can be consistently and accurately identified. Specimens and isolates used in this study were so identified.

Hyphal confrontation between two haploid monosporous isolates is used to identify biological species and to distinguish incompatibility genotypes. This technique is usually performed by placing two inocula 0 to 2 mm apart on malt extract agar (MEA) (Korhonen, 1978; Ullrich and Anderson, 1978; Anderson and Ullrich, 1979). Two isolates are considered compatible if hyphal
confrontations result in the development of a crustose or more appressed colony but are incompatible if the colony remains white and fluffy (Hintikka, 1973; Ullrich and Anderson, 1978; Guillaumin et al., 1991). However, this technique makes results of hyphal confrontations difficult to interpret. Fortunately, several species of *Armillaria* form clamp connections in interaction zones of compatible confrontations. Observing the absence or presence of clamp connections in confrontations among such species makes species determination simpler and more reliable (Larsen et al., 1991). However, the absence of clamp connections in *A. mellea* makes this technique inapplicable.

Incompatibility in *A. mellea* is governed by two independent multiallelic loci, arbitrarily named A and B (Ullrich and Anderson, 1978). Thus, each basidiome produces basidiospores of four different incompatibility genotypes. Confronting single-spore isolates from these basidia in all possible combinations results in $A = B\neq$, $A \neq B\neq$, $A \neq B\neq$, and $A = B\neq$ interactions. The $A = B\neq$ interaction has been reported to form a crustose interaction zone occasionally (Korhonen, 1978). Guillaumin et al. (1991) proposed such an interaction as $A \neq B\neq$. In other basidiomycetes, the $A \neq B\neq$ interaction is characterized by a condition in which nuclear migration is blocked or occurs only a limited distance from the junction (Swiezynski and Day, 1960; Raper, 1966). Such evidence has not been confirmed for species of *Armillaria*. In addition, consistent characteristics of the $A \neq B\neq$ interaction have not been reported.

In this paper we describe a technique to identify all the incompatibility genotypes in *A. mellea*. The morphological variability of compatible interactions and evidence of nuclear migration in the $A = B\neq$ interaction are also discussed. A preliminary report has been published (Darmo and Burdsall, 1990).

**MATERIALS AND METHODS**

**Media preparation.** - Two different media were used in this experiment, oak wood extract agar (OWE) and squeezed orange juice agar (SOJ). To prepare OWE, boil 50 g of oak wood sawdust in 1 L of distilled water for 60 min; strain the extract through two layers of cheesecloth, autoclave separately 200 ml of oak wood extract and 20 g agar in 1 L of distilled water. Mix oak wood extract and agar together after cooling (40-45 C). Pour this mixture into 60-mm-diam plastic Petri plates to form a 5-mm-thick agar substrate. To prepare SOJ, squeeze juice of fresh oranges with a household electric juicer strain the extract through two layers of cheesecloth; autoclave separately 200 ml of squeezed orange juice and 20 g agar in 1 L of distilled water. Mix squeezed orange juice and agar together after cooling (40-45 C). Pour this mixture into 60-mm-diam plastic Petri plates to form a 5-mm-thick agar substrate. Neither of these media will solidify if the constituents are mixed before autoclaving. The use of canned frozen orange juice and other commercial orange juice products to prepare SOJ resulted in slow colony growth.

**Fungal isolates and single-spore isolation.** - Cultures were isolated from eight collections of *A. mellea* in Wisconsin; two collections were from Madison (Dane County), five from Natural Bridge State Park (Sauk County), and one from the Aldo Leopold Reserve (Sauk County). The minimum distance between these three collection sites is 35 km. All isolates were derived from basidiomata collected from the base of dead or dying red oak trees, *Quercus rubra* L. All isolates and basidiomata are on deposit at the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wisconsin. To obtain single-basidiospore isolates, an aqueous suspension was prepared from spore prints deposited in Petri plates. Suspensions were spread on MEA (15 g of malt extract, 20 g of Difco Bacto agar, and 1 L of distilled water) supplemented with 100 mg/L of streptomycin. After incubation for 3 da at 22-24 C with variable light, individual germinated basidiospores were removed manually with the aid of a dissecting microscope at 65x and transferred onto fresh MEA. The resulting cultures were stored at 4 C on MEA tubes. Compatibility among the isolates from all three collecting areas was demonstrated using the technique described in this work.

**Monospore isolate confrontations.** - All monosporous isolates were used 2 to 3 wk after isolation. Pairs of monosporous isolates were placed 10 mm apart on OWE in 60-mm-diam Petri plates and incubated in polyethylene bags at 22-24 C with variable light. After 10 to 14 da, a strip about 2 by 35 mm was cut across the two colonies perpendicular to the interaction zone. This strip, composed of one colony at either end and the interaction zone, was transferred onto SOJ in 60-mm-diam Petri plates and incubated in a polyethylene bag at 22-24 C for an additional 10 to 14 da at variable light (FIG. 1). Twelve to 20 monosporous isolates from each of the eight basidiomes were paired in all possible combinations.

To investigate nuclear migration, inocula about 2 by 2 mm were removed from different sites along the hyphal periphery of colonies growing from subcultured agar strips. These inocula were confronted with appropriate tester isolates of known genotype from the same basidiome, as indicated for previous confrontations.

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RESULTS AND DISCUSSION

OWE and SOJ media. – Armillaria spp. usually form rhizomorphs in agar media. The growth rate of rhizomorphs is not uniform, and they often grow faster than the surface mycelial colony. This type of in vitro growth hinders the accurate interpretation of monosporous hyphal confrontations. Such interference is reduced by placing inocula close to each other; however, this technique often prevents distinguishing between $A^+B^-$ and $A^+B^+$ interactions or between $A^\rightarrow B^-$ and $A=B^+$ interactions. In addition, the use of media that inhibits rhizomorph development allows contact to occur only between mycelia at the peripheries of the confronted colonies.

In more than 1000 culture plates of A. mellea observed, no single-spore isolate produced rhi-
though there was some variability in two of these morphologies, they were always easily distinguished from each other (Figs. 3, 4). Two of them (Figs. 3, 4) occurred in ratios expected in crosses of a species with a bifactorial mating system (Fig. 5), and the other (Fig. 2a) was present in approximately twice the expected frequency. This led to the question of whether the morphology could be used to identify mating types of the interacting single-spore isolates in such genetic confrontations.

Evidence for nuclear migration. — The B incompatibility locus in basidiomycetes has historically been designated as the locus that controls nuclear migrations (Raper. 1966). The following experiments allowed us to determine which phenotype was associated with inhibited nuclear migrations. To determine the extent of nuclear migration in A=B+, A*B=, and A*B≠ interactions, inocula taken from sites a, b, and c (Fig. 6) of the subculture colonies on SOJ were confronted with isolates of each genotype. Representative results using genotypes A,B, and A,B, are illustrated (Fig. 6). The isolates were confronted using the same OWE-SOJ agar method previously described and replicated five times for each of the eight isolates. Nuclear migration in an A=B= interaction cannot be observed with this technique, because both sides of the subcultured colonies have the same genotype. In A≠B= interactions, confronting inocula taken from site a (Fig. 6) with A1B1 and A2B2 inocula resulted in the formation of compatible and fluffy colonies, respectively. Confronting inocula taken from site c with A1B1 and A2B2 inocula resulted in the formation of fluffy and A+B= type colonies, respectively, thus indicating that the A2B2 nucleus does not migrate to site c. Similarly, A1B2 does not migrate to site a. Nuclear migration is confined at the interaction zone. Inocula taken from site b formed fluffy and A+B= type colonies with mycelia of genotypes A1B1 and A2B2, respectively. In this interaction zone, A,B, is more prevalent than A,B, but from our experience, the prevalence of one nucleus over another in this zone seems to be a random phenomenon.

Nuclear migration in A=B≠ is extensive and bidirectional. Nuclei A,B, and A,B, are present at sites a, b, and c (Fig. 6): therefore, inocula taken from sites a, b, and c are compatible with mycelia of genotypes A,B, and A,B, (data not shown). Inocula from sites a, b, and c formed...
fluffy colonies with \( A_B \) mycelium, typical of either \( A=B \) or \( A=B \) interactions.

Inocula taken from sites a, b, and c of the resulting compatible interaction (Fig. 6) were compatible with mycelia of genotypes \( A_B \) and \( A_B \). Compatible-like interactions were also found when these inocula were confronted with monosporous isolates having genotypes \( A_B \) or \( A_B \) (data not shown). This type of reaction has been reported previously (Ullrich and Anderson. 1978). However, this evidence does not unequivocally indicate that extensive nuclear recombi-
nation takes place in those colonies. Such reactions may be due to an assayed A=B interaction that is indistinguishable from an A=B interaction or that the A=B nuclear condition dominates the phenotype reaction.

All incompatibility genotypes of monosporous isolates of *A. mellea* maintained a fluffy morphology when confronted with vegetative isolates of eight unrelated North American species of *Armillaria* (Fig. 7), indicating that nuclear migration does not occur in interspecies interactions. Therefore, further testing of the OWE-SOJ technique might demonstrate its utility for identifying *Armillaria* species.

**A=B** and **A=B** interactions. — **A=B** interactions resulted in the formation of fluffy colonies along the entire agar strip on SOJ, like that found in self confrontations, A=B (Fig. 2). Thus, A=B = (A,B, × A,B, or A,B, × A,B,) interactions cannot be differentiated morphologically from A=B = (A,B, × A,B,). A,B, × A,B, × A,B, × A,B, or A,B, × A,B,) interactions.

**A=B** interactions. — In **A=B** = (A,B, × A,B, or A,B, × A,B,) interactions, the confronted colonies remained fluffy at the ends of the agar strip but were appressed or crustose and constricted at the interaction zone (Fig. 2). The constriction at the interaction zone was highly consistent (Fig. 3) in *A. mellea* and proved useful for identifying the genotype of confronted monosporous isolates from a single basidiome. It was also useful for identifying compatible interactions having atypical morphology. The constricted interaction zone was often produced rhizomorphs. The rhizomorphs were several centimeters long and became crustose when they reached the agar surface. Subculturing to SOJ from the interaction zone resulted in a stable appressed or crustose colony that was morphologically indistinguishable from the compatible interaction. Such stability may not occur in nature.

**A=B** interactions. — It has been reported that **A=B** interactions result in the formation of crustose colonies (Fig. 2), indistinguishable from a colony originated from stipe or cap tissue (Hinrikka, 1973; Ullrich and Anderson, 1978). How-
**Interaction type** | **Mycelium source** | **Tester isolate**
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A₂B₂ x A₁B₂ (Common B)

| a | Compatible | Fluffy |
| b | Fluffy | Common B |
| c | Fluffy | Common B |

A₁B₁ x A₂B₂ (Common A)

| a | Fluffy | Compatible |
| b | Fluffy | Compatible |
| c | Fluffy | Compatible |

A₁B₁ x A₂B₂ (Compatible)

| a | Compatible | Compatible |
| b | Compatible | Compatible |
| c | Compatible | Compatible |

Fig. 6. Representation and results of confrontation of mycelia from colonies of common B (A₁B₂=), common A (A₂B₁=), and compatible A₂B₂ interactions with two monosporous tester isolates to verify nuclear migration in *Armillaria mellea*.

However, in this study the morphological characteristics of compatible interactions (A₂B₂ x A₁B₁, or A₂B₁ x A₁B₂) were highly variable (Fig. 4). A crustose colony was not formed in every compatible interaction. The resulting colonies usually become appressed, although some colonies remained fluffy but possessed other morphological changes such as short aerial mycelium, hyphal discoloration, or rhizomorph proliferation.

**Differentiation of basidiospore genotypes.** - The ability to recognize A₁B₂= and A₂B₁= interactions (Figs. 3, 4) allowed the determination of the other genotypes from a single basidiome of *A. mellea*, even though the genotypes were morphologically identical (Fig. 5). Monosporous self confrontations consistently resulted in the formation of fluffy colonies. Confrontation of monosporous isolates 1 and 3 (Fig. 5) resulted in the formation of an appressed colony along the agar strip. If isolate 1 is arbitrarily assigned genotype A₁B₂, then isolate 3 must be A₂B₁, because confrontation of these two isolates resulted in the formation of an A₁B₂= interaction. Confrontation of isolate 3 (A₂B₁) and isolate 2 resulted in the formation of an interaction; thus, isolate 2 must be A₁B₂. Confrontation of isolate 1 (A₁B₁) and isolate 2 (A₂B₁) resulted in
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LITERATURE CITED


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