

## Interfertility among isolates of *Armillaria tabescens* in North America

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Darmono, T.W., H.H. Burdsall, Jr. & T.J. Volk (1992). Interfertility among isolates of *Armillaria tabescens* in North America. – *Sydowia* 42(2): 105–116.

Haploid–haploid hypha confrontations and the di–mon reaction were performed to investigate the interfertility of North American isolates of *Armillaria tabescens*. Isolates derived from collections from northeastern Ohio, Maryland, South Carolina, Georgia, southern Illinois, Florida, and Louisiana were all interfertile as indicated by the formation of clamp connections and the compatible type of colony morphology, subsequent basidioma production in haploid–haploid confrontations, and morphological changes in the mat character when using the di–mon reaction. The evidence indicates that *A. tabescens* in the eastern United States is a single biological species and supports a previous conclusion that *A. tabescens* is bifactorially heterothallic.

Keywords: *Armillaria*, interfertility, basidioma, North American species, hap–hap, di–mon.

*Armillaria tabescens* (Scop.: Fr.) Dennis, Orton & Hora is virulent on many hardwood and coniferous tree species, particularly in southern Europe (Rishbeth, 1985) and in southeastern North America (Farr & al., 1989). It is distinguished from other species of *Armillaria* by the absence of an annulus. However, variation in morphology led numerous North American mycologists to believe that more than one exannulate *Armillaria* species occurs in eastern North America (O.K. Miller, W.J. Sundberg, J.W. Kimbrough, personal communication, Sept. 1985). Such is also the case in Europe where both *A. ectypa* (Fr.) Lamoure and *A. tabescens* are formally recognized (Watling & al., 1991). The purpose of this research is to establish whether isolates collected from widely dispersed localities east of the Mississippi River belong to a single biological species by using haploid–haploid hypha confrontations (hap–hap) and vegetative–haploid hypha confrontations (di–mon). Because only isolates from secondary mycelium (from basidioma, rhizomorphs or mycelium fans) are available in many cases, we are also interested in comparing the di–mon with hap–hap confrontations to determine the reliability of the di–mon reaction in delimiting this species of *Armillaria*.

Secondary mycelium is defined as mycelium resulting from plasogamy in the primary (haploid) mycelium (Hawksworth & al., 1983). In the di-mon reaction, a secondary mycelium isolate is confronted with a monosporous haploid isolate. The use of the di-mon confrontations was attempted by others, but they reported that interactions are difficult to interpret (Guillaumin & al., 1989; Mohammed & al., 1989). Interfertility studies in *Armillaria* spp. are limited by the inability of most species and isolates to fruit readily *in vitro*. However, many isolates of *A. tabescens* fruit readily *in vitro*, so that fresh monosporous isolates are often available.

### Materials and methods

Isolates of *A. tabescens* from northeastern Ohio, Maryland, South Carolina, Georgia, southern Illinois, Florida, and Louisiana (Tab. 1) were used in this study. All isolates were maintained on malt extract agar (MEA) slants at 4 C and were transferred onto fresh medium yearly. To obtain monosporous cultures, isolates collected from Louisiana, Florida, and southern Illinois were induced to produce basidiomata *in vitro* by culturing on chopped orange medium (Guillaumin & al., 1989). *In vitro* basidiomata of MB II isolate were obtained by crossing two haploid collections from Louisiana, MB 2018-1 and MB 2112-2. Monosporous isolates of TJV-9 and TJV-2 were obtained from basidiomata collected from nature. The method for collecting single spore isolates was described by Darmono & Burdsall (1992).

Mating types of monosporous isolates from each basidioma were determined by confronting hyphae of 12 to 20 monosporous isolates on oak wood extract-squeezed orange juice agar (OWE-SOJ) as described by Darmono & Burdsall (1992). Factors A and B were arbitrarily assigned. Whenever possible, monosporous isolates representing different mating types were chosen from each basidioma and were crossed among each other and with isolates from different geographical regions. Clamp connections were observed by removing 10 agar plugs, ca. 2 by 2 mm, from different sites in each of the 3-week-old colonies resulting from hap-hap confrontations, placing the inocula on fresh SOJ, incubating the cultures in a polyethylene bag at 22 C to 24 C under variable light for 3 days, removing agar plugs from the cultures, and observing with a compound microscope. Colonies resulting from hap-hap confrontations were further incubated for 5 weeks on SOJ to promote the formation of basidiomata.

Four monosporous isolates of TJV-9 and of TA 1-12 and two monosporous isolates of MB II used in the hap-hap confrontations were used as haploid testers in the di-mon reaction. These haploid isolates were confronted with secondary mycelium isolates of differ-

ent geographical origins. The di-mon reaction was performed on OWE-SOJ as indicated for hap-hap confrontations. Morphological changes of the haploid testers as a result of hypha confrontation with a secondary mycelium isolate were recorded.

Tab. 1.— Collection data for isolates of *Armillaria tabescens* used in this study.

Isolate	Substrate	State
TJV-2	<i>Acer saccharum</i>	Ohio
TJV-3	<i>Fagus grandifolia</i>	Ohio
TJV-5	<i>Fraxinus</i> sp.	Ohio
TJV-6	<i>Fagus grandifolia</i>	Ohio
TJV-7	Unknown	Ohio
TJV-8	<i>Acer saccharum</i>	Ohio
TJV-9	<i>Acer saccharum</i>	Ohio
HHB 162	<i>Pinus</i> sp.	Maryland
SC 87-3	<i>Prunus persica</i>	South Carolina
SC 87-S	<i>Prunus persica</i>	South Carolina
SC 87-6	<i>Prunus persica</i>	South Carolina
SC 87-7	<i>Prunus persica</i>	South Carolina
GA 90-54	<i>Prunus persica</i>	Georgia
92485-1	Dead stump	Illinois
92485-2	Undetermined shrubs	Illinois
TA 1-12	<i>Quercus nigra</i>	Florida
MB 2018-1	Unknown	Louisiana
MB 2112-2	<i>Ulmus rubra</i>	Louisiana

## Results

### Compatible characters in hap-hap confrontations

Appressed and slow hypha growth and clamp connection formation consistently occurred in the compatible ( $A \neq B \neq$ ) interactions. The culture mats of the  $A \neq B \neq$  interactions occasionally became crustose and dark brown on SOJ. In some  $A \neq B \neq$  interactions, tufts of mycelium, 0.5 to 2.0 mm in diameter and 1 to 2 mm high, developed (Fig. 1).

Basidiomata were formed in the  $A \neq B \neq$  interactions on SOJ (Fig. 1), although not every  $A \neq B \neq$  interaction produced basidiomata. Culturing mycelium from the  $A \neq B \neq$  interactions that were capable of producing basidiomata on SOJ, onto chopped orange medium in flasks under blue light, resulted in the formation of normal-size basidiomata. Basidiomata were formed in hap-hap confrontations between monosporous isolates obtained from the field (TJV-9), as well as in hap-hap confrontations between monosporous isolates of the *in vitro* basidioma of TA 1-12. No haploid cultures formed basidiomata *in vitro*.

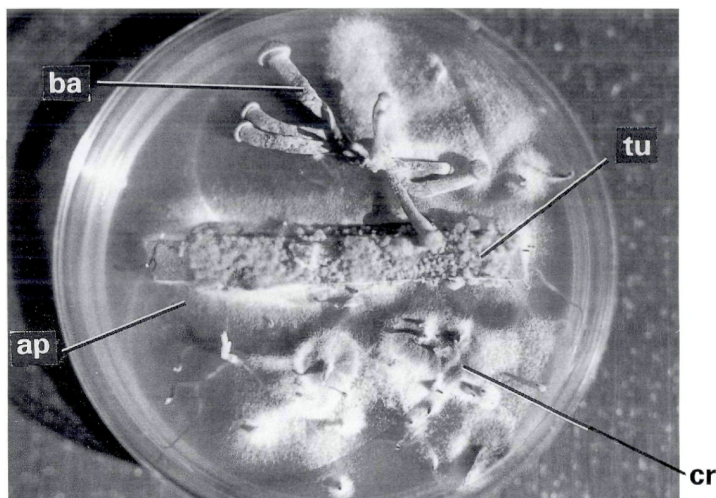


Fig. 1.— Result of crossing two monosporous isolates of *Armillaria tabescens* in a compatible interaction that is characterized by the appearances of appressed growth (ap), crustose (cr), tufts (tu) of mycelium, and basidiomata (ba).

In typical hap–hap confrontations, incompatible ( $A=B=$ ) interactions resulted in the formation of fluffy colonies. Common A ( $A=B\neq$ ) and B ( $A\neq B=$ ) type of interactions were morphologically indistinguishable from the incompatible interactions. Neither clamp connections nor basidiomata were formed in these interactions.

Monosporous isolates from the basidioma TJV–2 collected from nature produced atypical colonies. They were fluffy, but became appressed after transferring them onto a fresh medium. Intra-basidioma hypha confrontations among these monosporous isolates produced uninterpretable results (Tab. 2). Thus, the genotypes of these isolates could not be determined.

Two phenotypes of monosporous isolates, appressed and fluffy, were recovered from a basidioma of MB II produced *in vitro*. The fluffy phenotype was composed of two compatible mating genotypes. The appressed monosporous isolates possessed clamp connections indicating they were derived from binucleate spores.

#### Monosporous hypha confrontations

In monosporous hypha confrontations between isolates of different geographical origins, all isolates tested were interfertile; i.e.,



Tab. 2.— Results of haploid-haploid hypha confrontations among monosporous isolates of *Armillaria tabescens* from different geographical origins based on clamp connection formation.\*

Isolate	TJV-9				TJV-2				MB II		TA 1-12				92485-1	
	1	2	3	4	1	2	3	4	1	2	1	2	3	4	1	2
TJV-9																
1	-															
2	-	-														
3	-	+	-													
4	+	-	-	-												
TJV-2																
1	+	+	+	+	?											
2	+	+	+	+	?	?										
3	+	+	+	+	?	?	?									
4	+	+	+	+	?	?	?	?								
MB II																
1	+	+	+	+	+	+	+	+	-							
2	+	+	+	+	+	+	+	+	+	-						
TA 1-12																
1	+	+	+	+	+	+	+	+	+	+	-					
2	+	+	+	+	+	+	+	+	+	+	-	-				
3	+	+	+	+	+	+	+	+	+	+	-	+	-			
4	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	
92485-1																
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

\*Presence of clamp connections was consistently associated with a change in culture mat morphology; -, incompatible; +, compatible; ?, uninterpretable.

Tab. 3.— Results of the Buller reaction in *Armillaria tabescens* between secondary mycelium isolates from northeastern Ohio and 10 haploid testers.

Secondary mycelium isolates	Haploid testers*									
	TJV-9				MB II		TA 1-12			
	1	2	3	4	1	2	1	2	3	4
TJV-2	-	-	+	+	-	+	-	-	+	+
TJV-3	-	-	+	+	-	-	+	-	-	+
TJV-5	+	+	+	+	-	-	+	-	+	+
TJV-6	-	-	+	+	-	-	-	-	-	-
TJV-7	-	-	+	+	-	+	-	-	+	+
TJV-8	-	-	+	+	-	-	-	+	-	-
TJV-9	-	-	+	+	-	-	-	-	-	+

\*-, incompatible; +, compatible.

all possible combinations of hypha confrontations resulted in the formation of the  $A \neq B \neq$  type of colony morphology that produced clamp connections (Tab. 2). This included the confrontations with monosporous isolates of TJV-2. Repeating the confrontations with an additional 25 monosporous isolates from TJV-2 against monosporous haploid testers of TJV-9, MB II, and TA 1-12 gave identical results. Many colonies derived from the  $A \neq B \neq$  interactions between progeny of 92485-1 and MB II produced basidiomata *in vitro* when they were grown on chopped orange medium. The control haploid colonies did not form basidiomata.

### Di-mon reaction

In the di-mon reaction, compatible interactions resulted in the mat of the formerly fluffy confronted haploid culture becoming appressed (Fig. 2), sometimes followed by the formation of crustose surface mycelia. Such morphological changes occurred far beyond the interaction zone or hypha periphery of the secondary mycelium isolates. The haploid colonies in incompatible interactions, including self confrontations, remained fluffy even after prolonged incubation (Fig. 2). In several cases, the haploid portion of the culture mat in the compatible interactions remained fluffy with a smooth surface for several days, then became wooly with a rough surface (Fig. 3). Secondary mycelium isolates from different geographical origins were not compatible with mating genotypes of any haploid testers (Tab. 3 and 4). However, all of them, including secondary mycelium isolates from northeastern Ohio, were compatible with monosporous isolates 3 and 4 of TJV-9 (Tab. 3). As expected, the secondary mycelium isolate of TJV-9 was compatible with only two of the mating genotypes from TJV-9 basidioma. However, the secondary mycelium isolate of TJV-9 was not compatible with monosporous isolates of MB II and was compatible with only one out of four mating genotypes or haploid isolates of TA 1-12 (Tab. 3). Secondary mycelium isolates of TJV-5 and HHB 162 were compatible with all four genotypes of TJV-9. An irregular pattern of interactions in the di-mon reaction was found when haploid testers of MB II and TA 1-12 were used.

### Discussion

In determining mating genotypes of individual monosporous isolates in *A. mellea* (Vahl.: Fr.) Kummer, the  $A \neq B =$  type of interactions can be distinguished readily from the  $A = B \neq$  and  $A = B =$  types of interactions (Darmono & Burdsall, 1992). However, in *A. tabescens*,  $A \neq B =$  interactions were indistinguishable from  $A = B \neq$  and  $A = B =$  reactions. In the absence of a distinguishable  $A \neq B =$  type of

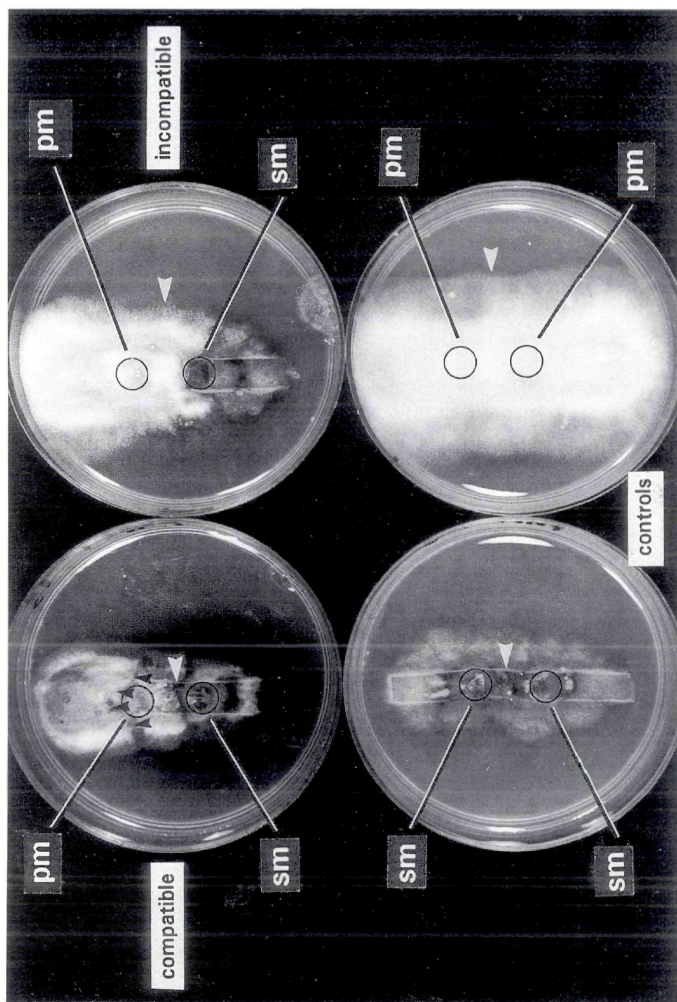


Fig. 2.— Crossing between a secondary mycelium (sm) and a primary mycelium (pm) in *Armillaria tabescens*. Compatible interaction is characterized by the appearance of appressed hypha growth occurring from the interaction zone (white arrow) toward the end of the primary mycelium (black arrows). The primary mycelium remains fluffy in the incompatible interaction.

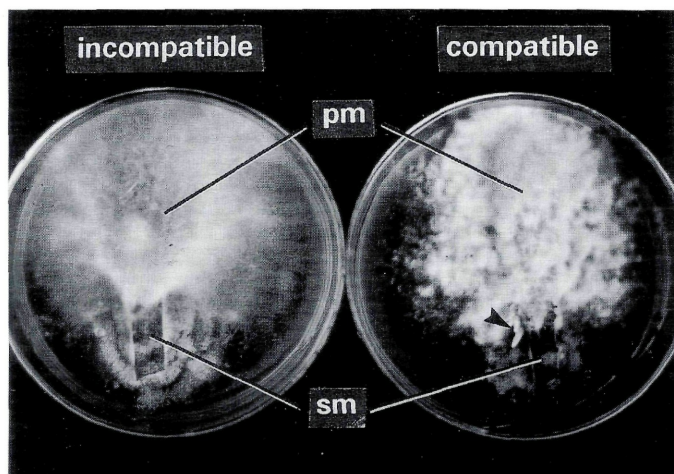


Fig. 3.— Woolly appearance of the formerly fluffy colony in the compatible interaction between a primary mycelium (pm) and a secondary mycelium (sm) in *Armillaria tabescens*.

interaction, the presence of clamp connections and subsequent basidioma production in *A. tabescens* on SOJ proved to be useful for determining the  $A \neq B \neq$  type of interaction. We always found an association between the formation of clamp connections and the development of appressed mycelium in the  $A \neq B \neq$  interaction in *A. tabescens*, as reported by Anderson (1982).

A crustose colony surface or pseudosclerotial plate (Lopes-Real, 1975) was formed on SOJ (Fig. 1) in some colonies of the  $A \neq B \neq$  interactions. This is the first report that such a structure is formed by *A. tabescens* on agar medium. Previous reports using other media indicated that *A. tabescens* does not form a crustose colony surface (Anderson, 1982; Intini & Gabucci, 1987).

Another characteristic of the  $A \neq B \neq$  interaction is the formation of tufts of mycelium in some interactions. This characteristic is usually observed along the OWE agar strip on SOJ (Fig. 1). The tufts also are formed on SOJ in cultures of the secondary mycelium isolates, but are absent in culture mats of the  $A = B \neq$ ,  $A \neq B =$ , and  $A = B =$ .

Basidiomata are formed only in the  $A \neq B \neq$  interactions, although not every  $A \neq B \neq$  interaction produces basidiomata. Our



Tab. 4.— Results of the Buller reaction in between *Armillaria tabescens* secondary mycelium isolates from Maryland, southern Illinois, South Carolina, and Georgia and six haploid testers.

Secondary mycelium isolates	Haploid testers*					
	TJV-9				MB II	
	1	2	3	4	1	2
HHB 162	+	+	+	+	-	+
92485-1	-	-	+	+	-	+
92485-2	-	-	+	+	-	+
SC 87-3	-	-	+	+	-	+
SC 87-5	-	-	+	+	-	-
SC 87-6	-	-	+	+	-	+
SC 87-7	-	-	+	+	-	-
GA 90-54	-	-	+	+	-	-

\* -, incompatible; +, compatible.

data support a previous conclusion (Anderson, 1982) that *A. tabescens* possesses bifactorial heterothallism. Although TJV-2 does not display the typical tetrapolar system, not enough evidence is present to demonstrate that homothallism exists in *A. tabescens*, because no monosporous cultures ever produced basidiomata *in vitro*.

Two different types of monosporous isolates, fluffy and appressed, were produced by a basidioma of MB II produced *in vitro*. From the population or the fluffy monosporous isolates, two mating genotypes were recovered. The appressed type of monosporous isolates were compatible with both mating genotypes of fluffy monosporous isolates, indicating that in addition to haploid spores, dikaryotic spores may be produced *in vitro*. However, cytological observation needs to be done to confirm this evidence. Dikaryotic spores were observed in *A. mellea* using DAPI staining and fluorescent microscopy (Darmono & Burdsall, unpublished data).

In determining interfertility in *A. tabescens*, hap-hap confrontations were more reliable than was the di-mon reaction. This supports the conclusion of Guillaumin & al. (1989). In our study, all possible combinations of hap-hap confrontations between isolates from different geographical origins resulted invariably in the formation of the  $A \neq B \neq$  type of colony morphology and clamp connections, indicating that they are fully compatible. Compatibility among these isolates was further confirmed in this study by the ability of some crosses to produce normal basidiomata *in vitro*.

Based on the hap-hap confrontations, TJV-9 and TJV-2, both from the same small park in northeastern Ohio, are fully interfertile (Tab. 2). This suggests that they belong to different clones. However,

the same conclusion cannot be drawn from the results of the di-mon reaction (Tab. 3). Thus, the di-mon reaction is of questionable value for clonal determination in *A. tabescens*.

Secondary mycelium isolate TJV-9 is compatible with only two mating genotypes of monosporous isolates obtained from its own basidioma (Tab. 3). However, it is not known if such a pattern also occurs in other isolates of *A. tabescens*. In *A. mellea*, secondary mycelium isolates are usually compatible with all mating genotypes of monosporous isolates obtained from the same basidioma (Ullrich & Anderson, 1978; Darmono & Burdsall, 1992).

An irregular pattern of interactions was found when haploid testers of MB II and TA 1-12 were used in the di-mon reaction (Tab. 3 and 4). Monosporous isolates of MB II and TA 1-12 were obtained from basidiomata produced *in vitro*; whereas, monosporous isolates of TJV-9, which showed a regular pattern of interactions, were obtained from a basidioma collected from nature. This suggests the possibility that the use of wild-type isolates as haploid testers may be preferable to the use of haploid testers produced *in vitro* and that several monosporous isolates must be used to confront any secondary mycelium isolates.

The secondary mycelium isolate of TJV-9 appears to be incompatible with MB II and is compatible with only one out of four mating genotypes of haploid isolates of TA 1-12 (Tab. 3). However, based on the hap-hap confrontations, they are fully interfertile with each other (Tab. 2). Thus, despite the fact that the secondary mycelium isolate of TJV-9 caused morphological changes with only one of four mating genotypes of haploid isolates of TA 1-12, it must be considered compatible with TA 1-12. Thus, secondary mycelium isolates of 92485-1, 92485-2, SC 87-3, SC 87-5, SC 87-6, SC 87-7, and GA 90-54 are also compatible with TJV-9. However, di-mon crosses can experience much interference. Thus, haploid testers used in the di-mon reaction must represent several mating genotypes. With the di-mon reaction, one positive reading indicates compatibility, or conspecificity. Even then negative readings must be considered as inconclusive.

We conclude that all North American isolates of *A. tabescens* used in this study are interfertile. These include isolates collected from northeastern Ohio, Maryland, South Carolina, Georgia, southern Illinois, Florida, and Louisiana. Only one isolate was collected from pine; others were from hardwoods (Tab. 1). Guillaumin & al. (1989) found a single biological species in Europe, despite the fact that *A. ectypa* is recognized (Watling & al., 1991). Preliminary data using hap-hap confrontations between North American and Italian isolates suggest that North American isolates and European isolates are interfertile (Darmono & Burdsall, unpublished data), although a

previous report using the di-mon reaction (Guillaumin & al., 1989) indicates that they are intersterile.

### Acknowledgments

We thank Drs. W.J. Sundberg, Southern Illinois University, M. Blackwell, Louisiana State University, J. W. Kimbrough, University of Florida, and P. L. Pusey, USDA-ARS Byron, Georgia, for providing some of the cultures used in this study.

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(Manuscript accepted 5th June 1992)

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Autor(en)/Author(s): Darmono T. W., Burdsall Jr. Harold. H., Volk T. J.

Artikel/Article: [Interfertility among isolates of \*Armillaria tabescens\* in North America. 105-116](#)