Mycologia, 84(2), 1992, pp. 214–218.

CLAMP CONNECTIONS IN NORTH AMERICAN *ARMILLARIA* SPECIES: OCCURRENCE AND POTENTIAL APPLICATION FOR DELIMITING SPECIES

MICHAEL J. LARSEN, MARK T. BANIK, AND H. H. BURDSALL, JR.

U.S. Department of Agriculture, Forest Service, Forest Products Laboratory,[1]
One Gifford Pinchot Drive, Madison, Wisconsin 53705-2398

ABSTRACT

Clamp connections were observed in basidiospore dilutions from each of four North American biological species of *Armillaria* (*A. gallica, A. sinapina, A. ostoyae,* and *A. calvescens*) following spore germination and hyphal anastomosis. The occurrence of clamp connections in intrabasidioma pairings of single-germinated basidiospores and of excised margin-hyphae of *Armillaria ostoyae* and *A. gallica* was that expected of a bifactorial incompatibility system. All intraspecific pairings between basidiospores or excised margin-hyphae from actively growing haploid cultures from two different basidiomata of *A. ostoyae* and of *A. gallica* produced clamp connections. The development of the crustose colony morphology was almost fully coincident with the formation of clamp connections in compatible matings with either *A. ostoyae* or *A. gallica.* Results indicate that clamp connections occur predictably and provide an additional precise and useful criterion for recognizing biological species of *Armillaria.* For unexplained reasons, haploid isolates paired more than 3 mm apart failed to produce clamp connections.

Key Words: *Armillaria,* basidiospores, biological species, clamp connections

The formation of clamp connections by compatible haploid isolates, indicative of dikaryon formation, is a useful *in vitro* tool for basidiomycete systematics. However, for many basidiomycetes, including *Armillaria* species, clamp connections reportedly do not occur commonly *in vitro* (Ullrich and Anderson, **1988).** The lack of clamp Connections in *Armillaria* has been attributed to the prevailing diploid nature of its vegetative mycelia (Korhonen and Hintikka, **1974).** However, clamp connections have been reported in culture for several species of *Armillaria.* Korhonen and Hintikka **(1974)** observed clamp connections in young cultures obtained from immature gills of *A. mellea sensu lata* Hyphae of older cultures did not possess clamp connections. They also reported the brief production of clamp connections after fusion of compatible haploid isolates indicating a transient dikaryophase. Anderson et al. **(1980)** reported the presence of clamp connections and degraded septa in a number of pairings between European and North American single-basidiospore isolates of *Armillaria* species. However, clamp connections were not formed in all pairings that were considered compatible. Clamp connections are known to occur in confrontations between haploid isolates of the exannulate *A. tabescens* Scop. & Bres. (Anderson, **1982)** and are correlated with a reduction in the amount of aerial mycelium produced. In a preliminary report, Banik et al. **(1990)** noted that clamp connections were formed in Petri plates containing basidiospore dilutions and between compatible pairings of germinated basidiospores of North American biological species (NABS) I, III, V, and VII.

The reported inconsistent occurrence of clamp connections has led to their being ignored as a criterion for delimiting biological species of *Armillaria.* Rather, the development of a "crustose" colony-morphology in compatible pairings, indicative of nuclear migration and fusion, has served as the standard for biological species identification (Hintikka, **1973;** Anderson and Ullrich, **1979).** This technique has led to the recognition of at least nine NABS (Ullrich and Anderson, **1988).** In spite of the utility of the technique, its full effectiveness has been doubted because Anderson **(1986)** redesignated NABS IV and **VIII** to V and VI, respectively. Partial incompatibility between NABS V, X, and XI and European *A. cepistipes* Velen. (Ullrich and Anderson, **1988)** has also made the distinctions

[1] The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article was written and prepared by U.S. Government employees on official time, and it is therefore in the public domain and not subject to copyright.

TABLE I

FIELD DATA FOR *ARMILLARIA* COLLECTIONS FROM WHICH BASIDIOSPORES WERE OBTAINED

Isolate number	Date collected	Location	Host	Species	North American biological species group
BP-4	9-16-89	Baraga Co., Mich.	*Picea glauca* (Moench) A. Voss	*A. ostoyae* (Romagn.) Herink	I
EL-4	9-19-89	Iron Co., Mich.	*Acer rubrum* L.		
ME-1	9-02-89	Menominee Co., Mich.	*Thuja occidentalis* L.		
PR-1b	9-18-89	Alger Co., Mich.	*Abies balsamea* (L.) Mill.		
PR-8	9-18-89	Alger Co., Mich.	*Abies balsamea* (L.) Mill.		
PR-10	9-18-89	Alger Co., Mich.	*Betula alleghaniensis* Britt.		
FFC-6	9-16-89	Baraga Co., Mich.	*Acer saccharum* Marsh.	*A. calvescens* Berube & Des.	III
FFC-7	9-17-89	Baraga Co., Mich.	Hardwood		
FFC-8	9-16-89	Baraga Co., Mich.	*Populus* sp.		
PR-1a	9-18-89	Alger Co., Mich.	*Ulmus* sp.		
PR-3	9-18-89	Alger Co., Mich.	*Acer saccharum* Marsh.		
PR-4	9-18-89	Alger Co.. Mich.	*Acer saccharum* Marsh.		
PR-5	9-18-89	Alger Co., Mich.	*Abies balsumea* (L.) Mill.	*A. sinapina* Berube & Des.	
PR-7	9-18-89	Alger Co., Mich.	*Thuja occidentalis* L.		
EL-1	9-19-89	Dickinson Co., Mich.	*Betula papyrifera* Marsh.	*A. gallica* Marx. & Romagn.	VI
EL-2	9-19-89	Dickinson Co., Mich.	*Quercus rubra* L.		
EL-3	9-19-89	Dickinson Co., Mich.	*Populus* sp.		
EL-4	9-19-89	Dickinson Co., Mich.	*Betula papyrifera* Marsh.		
EL-5	9-19-89	Dickinson Co., Mich.	*Populus* sp.		
EL-6	9-19-89	Dickinson Co., Mich.	*Quercus rubra* L.		
EL-8	9-19-89	Dickinson Co., Mich.	*Quercus rubra* L.		
EL-9	9-19-89	Dickinson Co., Mich.	*Populus* sp.		
EL-10	9-19-89	Dickinson Co., Mich.	*Betula papyrifera* Marsh.		
EL-11	9-19-89	Dickinson Co., Mich.	Unknown		
MA-1	9-20-89	Marathon Co., Wis.	*Acer saccharum* Marsh.		
ME-2	9-20-89	Menominee Co., Mich.	*Abies balsamea* (L.) Mill.		
ME-4	9-20-89	Menominee Co., Mich.	*Abies balsamea* (L.) Mill.		
ME-5	9-20-89	Menominee Co., Mich.	*Abies balsamea* (L.) Mill.		
ME-6	9-20-89	Menominee Co., Mich.	*Quercus rubra* L.		
OC-1	9-20-89	Oconto Co., Wis.	*Quercus rubra* L.		

among these species less definite. However, existing evidence suggests that these three NABS are incompatible populations. The delimitation of NABS has been confused further by the recent report that NABS II, III, and IX are partially compatible with the European *A. borealis* Marx. & Korh. (Rayner and Boddy, **1988).** However, this is in error; *A. borealis* is incompatible with all NABS of *Armillaria* (J. Anderson, University of Toronto, pers. comm.).

We propose that the presence or absence of clamp connections, a morphological response of mycelia involved in haploid pairings, provides a more precise means for identifying *Armillaria* NABS. Our purpose here is two-fold: 1) to report the consistency of clamp connection occurrence associated with randomly assorted or paired germinated basidiospores and paired margin-hyphae in some NABS of *Armillaria*. and 2) to compare incompatibility results based on the occurrence of clamp connections with that of existing culture morphology criteria.

Our studies show that clamp connections occur predictably in *Armillaria ostoyae* (Romagn.) Herink and *A. gallica* Marxm. & Romagn. under conventional *in vitro* conditions. Less exhaustive studies indicate that *A. calvescens* Berube & Des. and *A. sinapina* Berube & Des. react similarly. In addition, compatibility was noticeably coincident between haploid isolates as expressed by presence of clamp connections and development of a crustose colony morphology.

MATERIALS AND METHODS

Experiment 1: Identification of Armillaria *NABS and occurrence of clamp connections in* mass *basidiospore dilutions.* —To determine the NABS identity of the 30 *Armillaria* collections used in this study (TABLE I), basidiospores of each collection, obtained from small squares of hymenial tissue suspended over 2% water agar (WA) containing 0.01% streptomycin (ST) were transferred lo sterile distilled water and frozen for later experimentation. After thawing, 0.02 ml of suspension, containing ca 500 basidiospores, was diluted with 0.5 ml sterile water, spread with a sterile glass rod on the surface of WA + ST in Petri plates, and was incubated 24 to 48 h at 24 C. Single-germinated basidiospores were observed with the aid of a dissecting microscope at 60x and aseptically transferred onto plates of 2% agar, 1.5% malt extract, 0.01% ST, and incubated at 24 C. Two of the 10 haploid cultures thus derived from each collection were used for biological species identification with the aid of known tester isolates (Anderson, **1986)** using the protocol of Anderson and Ullrich **(1979)** and Anderson **(1986).** Isolates were paired on enriched medium (EM) (2% agar, 4% glucose, **3%** malt extract, 0.5% peptone) and incubated at 24 C for 3 wk. After incubation, the confrontations were scored as positive (crustose, compatible) or negative (fluffy, incompatible).

To determine the presence or absence of clamp connections in mass basiodiospore dilutions from the 30 *Armillaria* collections, basidiospores remaining in dilution plates were examined microscopically at 2 or 8 wk for the presence or absence of clamp connections.

Experiment 2: Clamp connection formation resulting from pairings of germinated basidiospores. —To determine the degree of incompatibility among intrabasidioma basidiospore populations. numerous single basidiospores from the same basidiome were paired from two collections each of *A. ostoyae* (PR-8, PR-10) and *A. gallica* (MA-I, ME-2). Intra-NABS incompatibility was determined by pairing numerous single basidiospores of PR-8 with PR-10 (A. ostoyae) and MA-1 and ME-2 (*A. gallica*). Inter-NABS incompatibility was assessed by pairing basidiospores of *A. ostoyae* (PR-10) with those of *A. gallica* (MA-1).

Single-germinated basidiospores were transferred from spore dilution plates onto WA + ST and paired 1 to 3 mm apart. A sterile cover slip was placed over each pair and examined microscopically. The basidiospore pairs were incubated at 24 C and monitored for growth; only those pairings where both germinated basidiospores continued to grow were examined further. Basidiospore pairings were observed weekly until clamps were observed.

Experiment 3: Clamp connection formation restilting from pairings of haploid margin-hyphae. —To determine if clamp connections form in compatible pairings of actively growing mycelia, margin-hyphae from three haploid isolates each of MA-1 and ME-2 (*A. gallica*) and three each of PR-8 and PR-10 (*A. ostoyae*) were paired intraspecifically. These isolates, previously stored for 4 months at 6 C in screw cap test tubes containing 1.5% malt extract and 2% agar, were transferred onto WA in Petri plates and incubated at 24 C for 11 days. Margin-hyphae approximately 1 mm long were then excised and paired on WA plates, using the protocol for basidiospore pairings described in Experiment 2. Each pairing was replicated five times, incubated at 24 C for 7 days, and observed microscopically for the presence or absence of clamp connections.

Experiment 4: Clamp connection formation, associated colony morphology, and determination of incompatibility system from intrabasidioma haploid margin-hyphae pairings. —To assess clamp connection formation, margin-hyphae from 15 haploid isolates of PR-10 (*A. ostoyae*) and 16 haploid isolates of ME-2 (*A. gallica*) were paired in all combinations following the protocol in Experiment 3. Pairings were incubated at 24 C and observed for clamp connections as previously described and the incompatibility system determined.

To study the colony morphology associated with haploid isolate interaction, inocula plugs were excised from these same isolates, grown on 1.5% malt/1.5% agar (w/v) and paired on EM. Two test inocula, one from each isolate, were paired near the center of the plate. A second inoculum of each isolate served as the control and was placed at the outer edge of the Petri plate on the same side as the tester of the isolate. Pairings were incubated for 3 wk at 24 C at which time they were rated as described in Experiment 3 and the incompatibility system determined.

Data based on the decision rules (absence or presence of clamp connections and crustose or fluffy colonies) for determining incompatibility were compared.

RESULTS

Experiment 1. —Several single-basidiospore isolates were obtained from each of 30 collections of *Armillaria*. These represented four biological species based on pairings with known tester strain: six collections each of *A. ostoyae* (NABS I) and *A. calvescens* (NABS III), two of A. *sinapina* (NABS V) and sixteen of *A. gallica* (NABS VII) (TABLE I).

Clamp connections were observed on basidiospore dilution plates for all *Armillaria* isolates (TABLE I). Clamp connections were observed at 2 wk of incubation for 19 of the 30 basidiospore dilution sets and were found in the remaining 11 sets at 8 wk. No relationship was evident between or among species regarding incubation times required for clamp connection development. Clamp connections were more prevalent in areas with distorted or swollen hyphae than in other areas. We interpreted these distorted hyphae to be incipient crustose hyphal mat cells that presumably lead to the development of the "crustose" colony morphology.

Experiment 2. —Based on the occurrence of clamp

TABLE II

CLAMP CONNECTION OCCURRENCE RESULTING FROM PAIRINGS OF BASIDIOSPORES FROM *ARMILLARIA GALLICA* AND *A. OSTOYAE*

Species	Isolate pairings	Number of spore pairings	Number (%) of spore pairings with clamps
A. gallica	**MA-1 x MA-1**[a]	31	9 (29)
A. gallica	**ME-2 x ME-2**[a]	35	8 (22.9)
A. ostoyae	**PR-8 x PR-8**[a]	11	4 (36.4)
A. ostoyae	**PR-10 x PK-10**[a]	17	5 (29.4)
A. gallica	**MA-1 x ME-2**[b]	14	14 (100)
A. ostoyae	**PR-8 x PR-10**[b]	34	34 (100)
A. gallica x *A. ostoyae*	**MA-I. x PR-10**[c]	14	0 (0)

[a] Basidiospores from a single basidioma.
[b] Basidiospores from two basidiomata of the same species.
[c] Basidiospores from basidiomata of two different species.

connection formation, the results of intrabasidioma basidiospore pairings indicated a range of 23% to 36% compatibililty, approximating that expected for a bifactorial mating system (TABLE II). Clamp connections varied from relatively frequent within *A. ostoyae* (PR-8, PR-10) to infrequent within *A. gallica* (MA-1, ME-2). As in the mass basidiospore dilutions, single-basidiospore pairings producing clamp connections were usually associated with distorted and swollen hyphae, and these were normally absent if clamp connections were not found. Clamp connections were usually found in the confrontation zone between pairings. In pairings with clamp connections, the frequency of clamp connection formation decreased with increasing distance from the confrontation zone and were usually absent from margins.

Clamp connections were present in all pairings of germinated basidiospores from different basidiomata of the same species; no clamp connections were found in interspecific basidiospore pairings between *A. gallica* (MA-1) and *A. ostoyae* (PR-10) (TABLE II).

Experiment 3. — Clampconnections were present in all pairings of haploid margin-hyphae isolates between two collections of the same species in both *A. gallica* and *A. ostoyae.* As in the pairings of germinated basidiospores, clamps were associated with swollen, ampullate, and otherwise distorted hyphae.

Experiment 4. — Intrabasidioma pairings of *A. gallica* and *A. ostoyae* yielded a bifactorial mating pattern using either the presence-absence of clamp connections or crustose-fluffy colony-morphology as decision rules. Both decision rules yielded near-identical results. One discordant observation was recorded in the *A. gallica* intrabasidioma pairings; clamp connections were predicted to occur in this pairing but were not observed.

The lack of crustose colony morphology in two pairings of compatible incompatibility types were noted in the *A. gallica* pairings. Two other *A. gallica* pairings expected to produce a positive reaction possessed uninterpretable colony morphology.

The *A. ostoyae* intrabasidioma pairings observed for clamp connections and colony morphology possessed one discrepancy. With both techniques, the same putative compatible reaction was rated as incompatible based on a lack of clamp connections or crustose colony morphology. Furthermore, three additional pairings possessed uninterpretable culture morphology reactions.

DISCUSSION

The occurrence of clamp connections, a morphological response of mycelia involved in a compatible pairing, is an additional precise means for assessing identity of *Armillaria* NABS and determining incompatibility systems. Clamp connections were produced reliably *in vitro* in compatible matings for the *Armillaria* NABS investigated. Although we did not further pursue the occurrence of clamp connections in *A. calvescens* and *A. sinapina,* we presume that because basidiospore dilution plates possessed clamp connections (Experiment 1), haploid isolates of

these two species would perform in a similar manner in the kind of experiments performed with A. ostoyae and *A. gallica.*

Although culture morphology of haploid isolates has been considered the most useful criterion for routine NABS identification, the occurrence of clamp connections in the interaction zone of haploid margin-hyphae pairings provides a precise means of determining compatibility. In our experience, the use of basidiospores for this purpose is reliable but the protocol is cumbersome. In addition, we have encountered considerable difficulty in maintaining viable basidiospores for extended periods under a variety of storage conditions. Even though pairings of margin-hyphae can be examined for clamp connections at 5 to 11 days, the amount of time involved in the actual examination of pairings by microscopy may exceed the time required for reading colony morphology from a conventional pairing. However, the use of clamp connections could still be implemented as an adjunct test in those pairings where colony morphologies are not typical for a compatible or incompatible response.

The spacing of inocula (germinated basidiospores or margin-hyphae) in pairings was critical to the production of clamp connections. Our protocol prescribes 1-to 3-mm spacing. At distances greater than 3 mm (e.g., 5, 10, or 15 mm), the occurrence of clamp connections becomes less predictable, or they are absent. We believe that, with wider spacing, clamp connection formation is inhibited by accumulation of secreted metabolites or an adverse pH shift.

Also worthy of mention is the occurrence of swollen, distorted, ampullate, and closely septate hyphae in many compatible pairings. Although we presume that these structures are precursors of the appressed hyphal mat that imparts a crustose character to colony morphology, their reliability as indicators of compatibility remains to be elucidated.

ACKNOWLEDGMENTS

We thank Drs. J. Anderson, J. Bruhn, and C. G. Shaw III for their helpful comments and discussions. Ms. R. Rentmeester is acknowledged for her capable technical assistance throughout the study.

LITERATURE CITED

Anderson, J. B. 1982. Bifactorial herterothallism and vegetative diploidy in *Clitocybe tabescens. Mycologia* **74:** 911-916.

———. 1986. Biological species of *ArmiIlaria* in North America: redesignation of groups IV and VIII and enumeration of voucher strains for other groups. *Mycologia* **78:** 837–839.

———, **K. Korhonen, and R. C. Ullrich.** 1980. Relationships between European and North American biological species of *Armillaria mellea. Exp. Mycol.* **4**: 87–95.

———, and R. C. Ullrich. 1979. Biological species of *Armillaria mellea* in North America. *Mycologia* **71:** 402–414.

Banik, M. T., M. J. Larsen, and H. H. Burdsall, Jr. 1990. The formation *of* clamp connections between compatible basidiospore germlings of species of *Armillaria. Mycol. Soc. Amer. Newslett.* **41** (1): 4.

Hintikka, V. 1973. A note on the polarity of *Armillariella mellea. Karstenia* **13:** 32–39.

Korhonen, K., and V. Hintikka. 1974. Cytological evidence for somatic diploidization in dikaryotic cells of *Armillariella* mellea. Arch. *Microbiol.* **95:** 187–192.

Rayner, A. D. M., and L. Boddy. 1988. *Fungal decomposition of wood.* Vol. VI. John Wiley and Sons, Chichester, United Kingdon. 587 pp.

Ullrich, R. C., and J. B. Anderson. 1988. *Armillaria mellea,* cause of rots in woody species. *Advances Pl. Pathol.* **6:** 491–499.

Accepted for publication November 27, 1991