Basidiocarp Induction, Nuclear Condition, Variability, and Heterokaryon Incompatibility in *Athelia (Sclerotium) rolfsii*

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The authors thank J. Hall for preparation of the illustrations, L. Davis for making a number of single-spore isolations, and E. E. Butler and R. K. Webster for valuable discussions. We also thank S. L. Anagnostakis, N. L. Anderson, and A. D. M. Rayner for providing criticisms and suggestions of the manuscript.

Accepted for publication 13 April 1983.

ABSTRACT

Punja, Z. K., and Grogan, R. G. 1983. Basidiocarp induction, nuclear condition, variability, and heterokaryon incompatibility in *Athelia (Sclerotium) rolfsii*. Phytopathology 73:1273-1278.

One hundred single-basidiospore (S_1) strains were obtained from 10 field isolates of *Athelia rolfsii* from California that formed basidia on potato-dextrose agar containing 2% activated charcoal (C-PDA). They showed pronounced variability in growth rate, sclerotial production, and frequency of clamp formation, suggesting that the field isolates from which they were derived were either heterokaryotic or diploid. Of these 100 S_1 strains, 85 formed the basidial state in culture. An additional 62 field isolates of *A. rolfsii* from various hosts and geographical areas also varied in growth rate, sclerotial formation, and frequency of clamp formation; of these, 36 were previously induced to fruit on C-PDA. The hyphal tip cells of field isolates and S_1 strains were multinucleate, and discharged basidiospores contained two nuclei. Antagonism zones formed in pairings among sibling or non-sibling S_1 strains, but isolations made from the zone of interaction of 120 of these pairings yielded six morphologically distinct colonies, two of which were shown to be heterokaryotic by fruiting and progeny analysis. Formation of the antagonism zone in pairings between S_1 strains (heterokaryon incompatibility) is not a complete barrier to formation of heterokaryons.

Additional key words: heterokaryosis, teleomorph.

There are numerous papers describing aspects of the biology, ecology, and control of *Athelia rolfsii* (anamorph: *Sclerotium rolfsii*), but there is little information on the genetics or sexuality of this important plant pathogen. This lack of information may, in part, be attributed to a poor understanding of the factors influencing formation of the teleomorph and its significance in the life cycle. In some earlier studies in which isolates of *A. rolfsii* fruited in culture, the fungus was considered to be homothallic (9) or heterothallic (16). However, with the exception of Goto's observations (16) and an unpublished thesis by Lyle (24), the extent and significance of variability within and between isolates of *A. rolfsii* has not been recognized.

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We previously reported on factors influencing formation of the basidial state of *S. rolfsii* in culture (29,32); in this and in a concurrent study (31), we have attempted to develop critically needed information on aspects of the sexuality, and factors contributing to the variability of this plant pathogen. We present evidence in this paper indicating that field isolates of *A. rolfsii* are heterokaryotic or diploid. Single-basidiospore progeny of field isolates show distinct variability among siblings in many characters. A large number of these strains have been induced to fruit under laboratory conditions. From pairings of some of these presumed homokaryons, we have in two instances demonstrated the formation of heterokaryons. The nuclear condition of various stages in the development of *A. rolfsii* is described.

MATERIALS AND METHODS

Induction of fruiting and single-basidiospore isolations. *Field isolates.* Forty-six of 72 field isolates used in a previous (32) and in a concurrent (31) study were induced to form basidia on PDA containing 2% activated charcoal (C-PDA) (32). Ten single-basidiospore (S_1) strains were obtained from each of 10 field isolates from California (1003, 1059, 1126, 1130, 1132-2, 1132-6, 1132-8, 1134, 2672, and 2823). To make single-basidiospore isolations, basidiospore deposits on 1% water agar were obtained using the method described in a recent study (30). Following discharge, the dishes were incubated for 6–12 hr to allow the basidiospores to germinate. Well-separated spores were located under the dissecting microscope (×90) and transferred using a fine nichrome wire to PDA or malt agar slants. About 60% of the transfers yielded colonies after 10–14 days of incubation at 25 C.

Single-basidiospore strains. One hundred single-basidiospore (S_1) strains were tested for ability to fruit using a modification of the method described previously (32). Plastic petri dishes (100 × 15 mm) divided into quadrants by vertical partitions (quad-plates, American Scientific Products, Illinois 60085) were used: in one quadrant, about 12 ml of modified C-PDA (15 g Difco PDA, 8 g of Difco Noble agar, and 20 g of activated charcoal per liter of water) was poured; the remaining three quadrants received one-tenth-strength PDA (3.9 g Difco PDA and 12 g Noble agar per liter of water). A single 6-mm-diameter mycelial plug taken from the periphery of a 10-day-old PDA culture was placed on the C-PDA. Four dishes used for each strain were incubated at low (about 60–80 lux) light intensity and room temperature (24–28 C) for 4–5 wk. The experiment was repeated twice. From each of 10 randomly selected S_1 strains that formed basidia, 10 single-basidiospore cultures (S_2) were isolated.

Presence of clamp connections. The 72 field isolates and 100 S_1 and 100 S_2 strains were grown on cellophane over 1% water agar. Circular (about 50-mm-diameter) pieces of cellophane were boiled in distilled water for 30 min, rinsed, and placed over solidified water agar in 60 × 15-mm plastic petri dishes. A single 8-mm-diameter mycelial plug taken from a 7-day-old PDA culture was placed in each dish and the dishes were incubated for 24–36 hr at 27 C. The number of clamp connections on approximately 50 randomly selected hyphal tips was determined for each isolate by examining the dishes under the low power (×125) of a compound microscope.

Nuclear condition. The vegetative mycelium of 12 field isolates and five S_1 strains, various stages of hymenial development, and basidiospores were stained with acridine orange. Mycelium was taken from PDA or water agar cultures and hymenia were obtained from fruiting cultures on C-PDA. Basidiospores were stained directly from deposits made onto glass slides coated with a thin film of 1% water agar. The materials were dipped into a 400 μg/ml aqueous solution of acridine orange (Calbiochem, San Diego, CA 92112) for 10–20 sec, rinsed in distilled water for 5–10 sec, and examined under near-UV to blue light with a Zeiss fluorescence microscope equipped with a high-pressure mercury HBO 100 W energy source, BP450-490 exciter filter, FT 510 dichromatic splitter, and a LP520 barrier filter.

Formation of heterokaryons. Ten S_1 strains from each of three field isolates from California (1126, 1132-8, and 2672) were paired in all possible combinations on Snider and Raper's (37) migration complete (MC) medium using the method described in a concurrent study (31). One hundred and twenty pairings were selected; small (2 mm^2) pieces of agar were transferred from the zone of antagonism (31) to PDA and incubated for 7 days. From each colony that developed (H), a plug was transferred to a 100 × 15-mm petri dish of PDA or MC medium that also was inoculated with a plug from each of the two strains that contributed to the putative cross. The mycelial plugs were placed at the periphery of the dish (about 50 mm distance between any two adjacent plugs). Morphological characteristics, extent of sclerotial formation, and growth rate of each of the 120 H-colonies were compared to those of the S_1 strains that comprised the pairing.

RESULTS

Induction of fruiting. Of the 72 field isolates of *A. rolfsii*, 46 were induced to fruit on C-PDA (32). Eighty-five of the 100 S_1 strains also formed basidia on quad-plates. Hymenia usually developed as clumps or tufts of mycelium on the one-tenth-strength PDA but some strains also fruited in the quadrant containing C-PDA. In general, single-basidiospore strains formed basidia more readily than the field isolates. Many S_1 strains also formed sclerotia on one-tenth-strength PDA (Fig. 1). Hymenial tufts were readily apparent as white masses of mycelium (Fig. 2). Scanning electron microscopy of hymenia prepared as described previously (32) revealed the presence of immature basidia (Fig. 3) as well as mature basidia with sterigmata and basidiospores (Fig. 4).

Presence of clamp connections. All field isolates of *A. rolfsii* formed clamped hyphae, but the frequency of clamps on the terminal hyphae varied from 84 to 8%, depending on the isolate, with an average of 51%. Clamps were most apparent on the hyphal tip cells (Fig. 5 a,b). The 100 S_1 and S_2 strains also formed clamped hyphae (Fig. 6), but the frequency of clamp formation varied from 61% to less than 1%, with an average of 21%.

Nuclear staining. The hyphal tip cells of field isolates and S_1 strains contained 4–11 nuclei, with an average of 6–7 per cell (Figs. 7 and 8). In older cells, numbers of nuclei varied from two to five, with an average of three to four (Fig. 9). The following nuclear condition was suggested for successive stages of hymenial development: sub-hymenial hyphae were binucleate; young developing basidia were binucleate, and basidiospores on mature basidia or discharged onto water agar also were binucleate (Figs. 10–12). Upon germination, the basidiospores contained four nuclei and produced binucleate and eventually multinucleate germ tubes (Figs. 12 and 13). The nuclear condition of mature basidia and of young developing basidiospores was not determined.

Variation among field isolates and S_1 and S_2 strains. Field isolates grown on PDA varied in growth rate, numbers of sclerotia produced, and size of sclerotia (Fig. 14); all isolates, however, formed sclerotia. The S_1 strains also varied morphologically (Fig. 15); many produced few sclerotia and 28 out of 100 were asclerotial. These strains also differed from one another in radial growth rate, mycelial dry weight production, oxalic acid production, and virulence (*unpublished*). Some S_1 strains grew very slowly on PDA, and a few did not survive beyond two to three subcultures. The S_2 progeny from 10 selected S_1 strains displayed marked uniformity (Fig. 16). All progeny from a single S_1 strain were similar in characteristics such as radial growth rate, sclerotial production, and ability to form basidia, and also resembled their S_1 parent (Fig. 16).

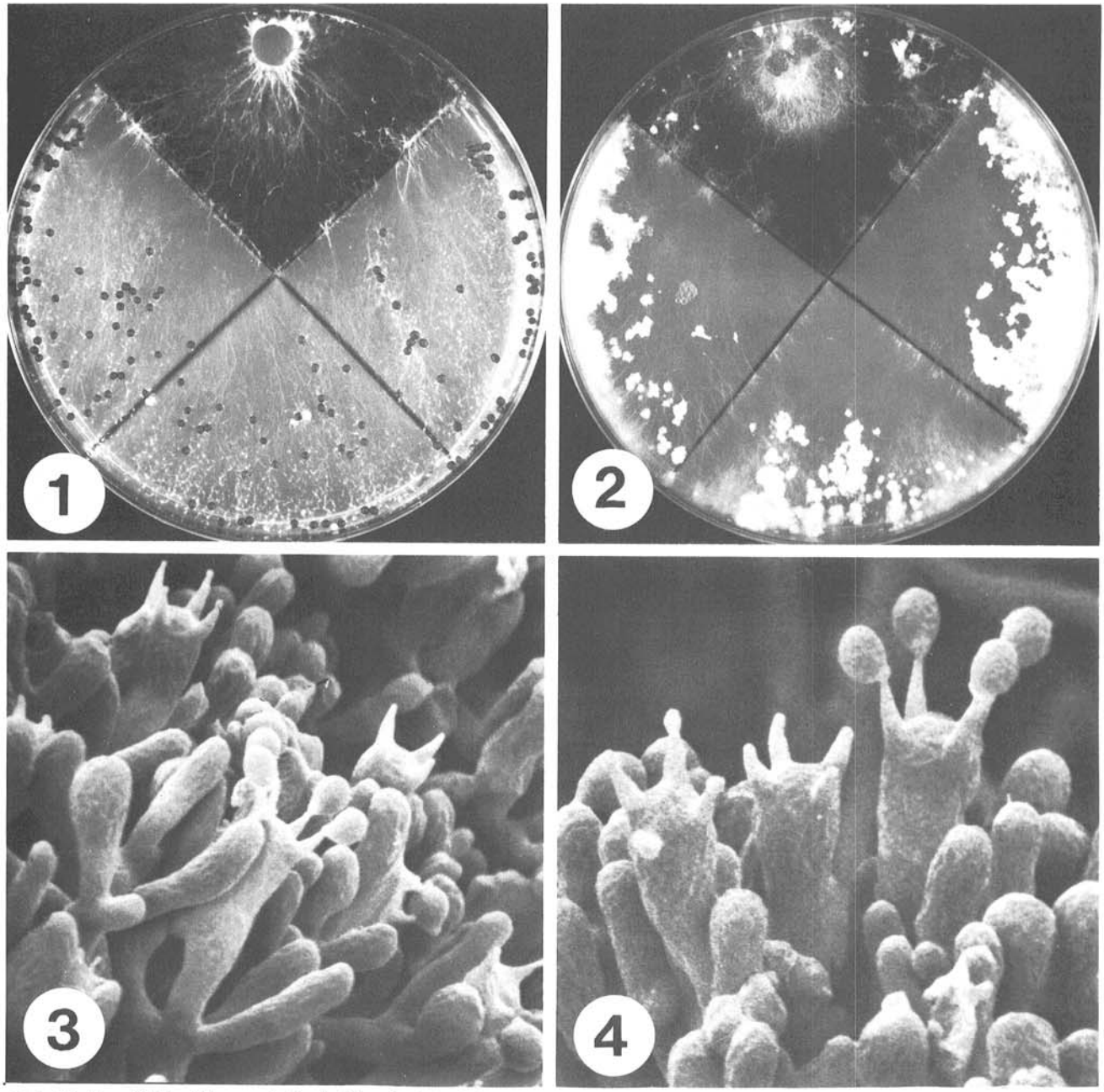
Formation of heterokaryons. Of 120 isolations made from the antagonism zone of selected S_1 pairings, 114 colonies resembled one or the other S_1 strain, suggesting that heterokaryons probably had not been formed. In the remaining six isolations, however, morphologically distinct H-colonies developed (Fig. 17). No H-colonies developed from merged mycelia in compatible pairings. The six H-colonies were antagonistic when paired to each of the parental S_1 strains. Hyphal-tip transfers were made, and two of the H-colonies were induced to fruit on quad-plates. Single-basidiospore strains from these H-isolates varied in morphological characteristics, suggesting that the H-isolates were probably heterokaryons. The extent of variation, however, generally did not

exceed that of the two parental S_1 strains and none resembled the initial H-isolate. In one cross, all single-basidiospore strains resembled only one of the S_1 parental strains.

DISCUSSION

The high frequency of basidial formation in S_1 strains of *A. rolfsii* suggests that monosporic isolates are capable of completing sexual reproduction. Our assessment of fruiting in these strains was based on the presence of hymenial fructifications in the petri dish. Although we have not examined critically whether viable or functional basidiospores were produced by all 85 S_1 strains, a random sample comprised of 15 of these strains have all produced functional basidiospores (*unpublished*). Variability in the extent to which single-spore progeny form hymenia in culture has been noted by others (16,24). In the present study, S_1 strains generally fruited more readily than their parents. Stretton et al (39) and Garza-Chapa and Anderson (15) noted a similar increase in the extent of hymenial formation in the progeny from certain field isolates of *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) and suggested that sterility factors may have been overcome with increased generations of selfing.

The S_1 strains of *A. rolfsii* displayed variability in many characteristics, suggesting that the field isolates from which they were derived were either diploid or heterokaryotic (7,8,27,41); these S_1 strains should be homokaryotic since four spores were observed on most basidia. In heterothallic Homobasidiomycetes, eg, *Coriolus versicolor* (35) and *Schizophyllum commune* (33,37), the absence of clamp connections on the mycelium of single-basidiospore strains is presumptive evidence of a monokaryotic condition (dikaryotic mycelia possess clamps, whereas monokaryotic mycelia do not). In others (eg, *Stereum*), clamps are produced on all mycelia (4). In *A. rolfsii*, clamp connections could not be used to distinguish parental isolates from S_1 strains as they

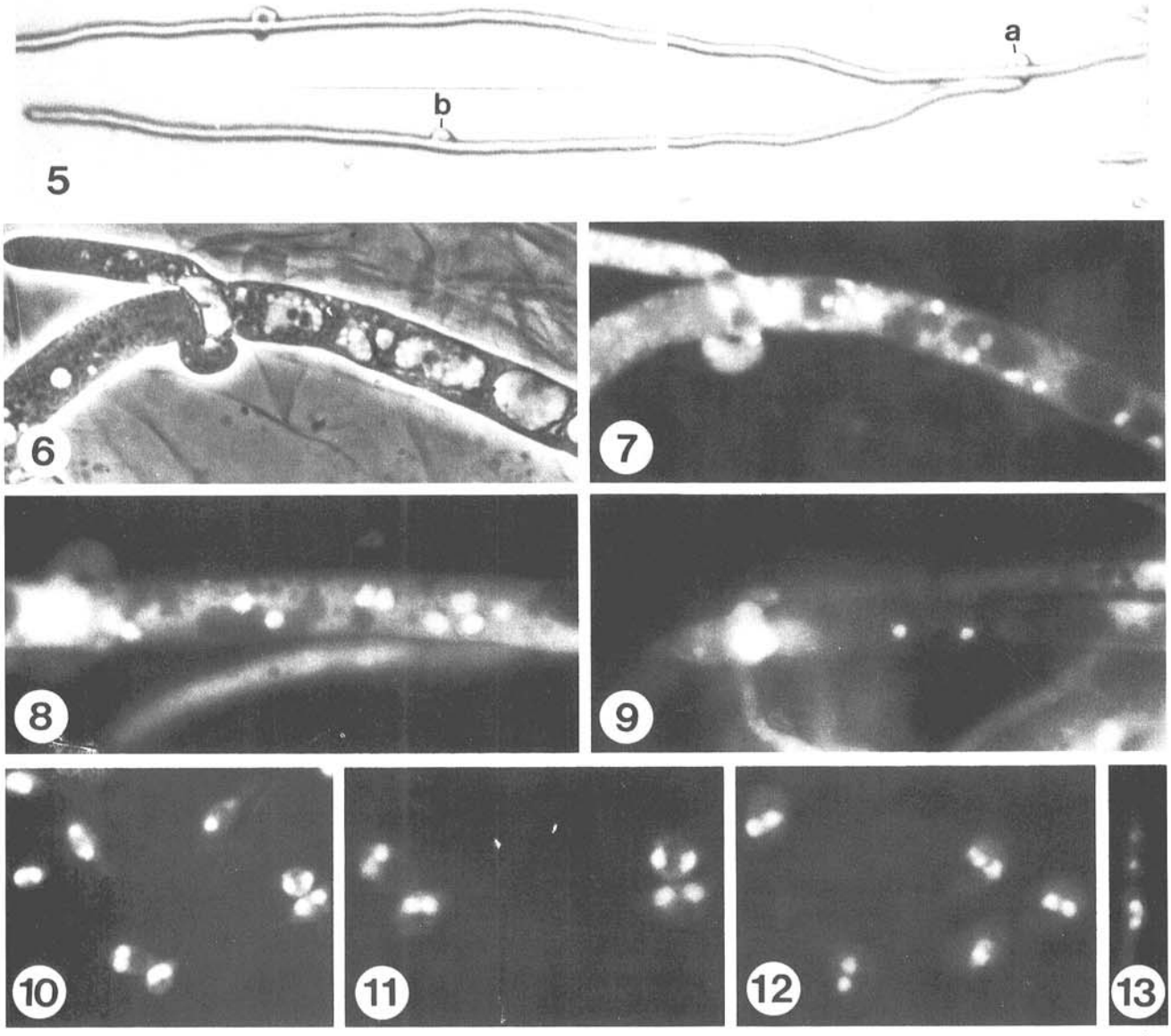


Figs. 1–4. *Athelia rolfsii* basidiocarp. **1,** Nonfruiting single-basidiospore (S_1) strain on quad-plate. Upper quadrant contains PDA with 2% activated charcoal; lower three quadrants contain one-tenth-strength PDA. Note sclerotial production in the latter quadrants. **2,** Fruiting S_1 strain on quad-plate; hymenial tufts are formed at the periphery of the dish on one-tenth-strength PDA. **3,** Scanning electron micrograph (SEM) of the hymenium of an S_1 strain, showing various stages of basidial development (×1,200) **4,** SEM, showing mature basidia of a field isolate (×1,600).

were formed on all mycelia. Fruiting and examination of the single-spore progeny for variation would be necessary to prove that these S_1 strains are indeed homokaryons. To date, we have confirmed the homokaryotic condition for 15 S_1 strains; the marked uniformity in the single-spore progeny (S_2) obtained from each of them suggested there was no further segregation for morphological markers (11,39,41).

The multinucleate condition of hyphal tip cells determined from acridine orange-stained materials (21,42) has been previously reported by others (16,19), although Tu et al (40) concluded that the vegetative cells of *A. rolfsii* were binucleate. Binucleate cells were observed in the present study only in mycelia taken from older portions of the colony, suggesting that the cells examined by Tu et al (40) may not have been hyphal tip cells. The binucleate condition of discharged basidiospores may have resulted from a mitotic division in the basidium prior to nuclear migration, or in the basidiospores. Alternatively, rare two- or three-spored basidia might also have produced binucleate heterokaryotic basidiospores (a situation analogous to amphithallism [22]), as was sometimes observed in *R. solani* AG-2 (12) and AG-4 (2). The percentage of heterokaryotic binucleate basidiospores of *A. rolfsii* produced from a given thallus is not known.

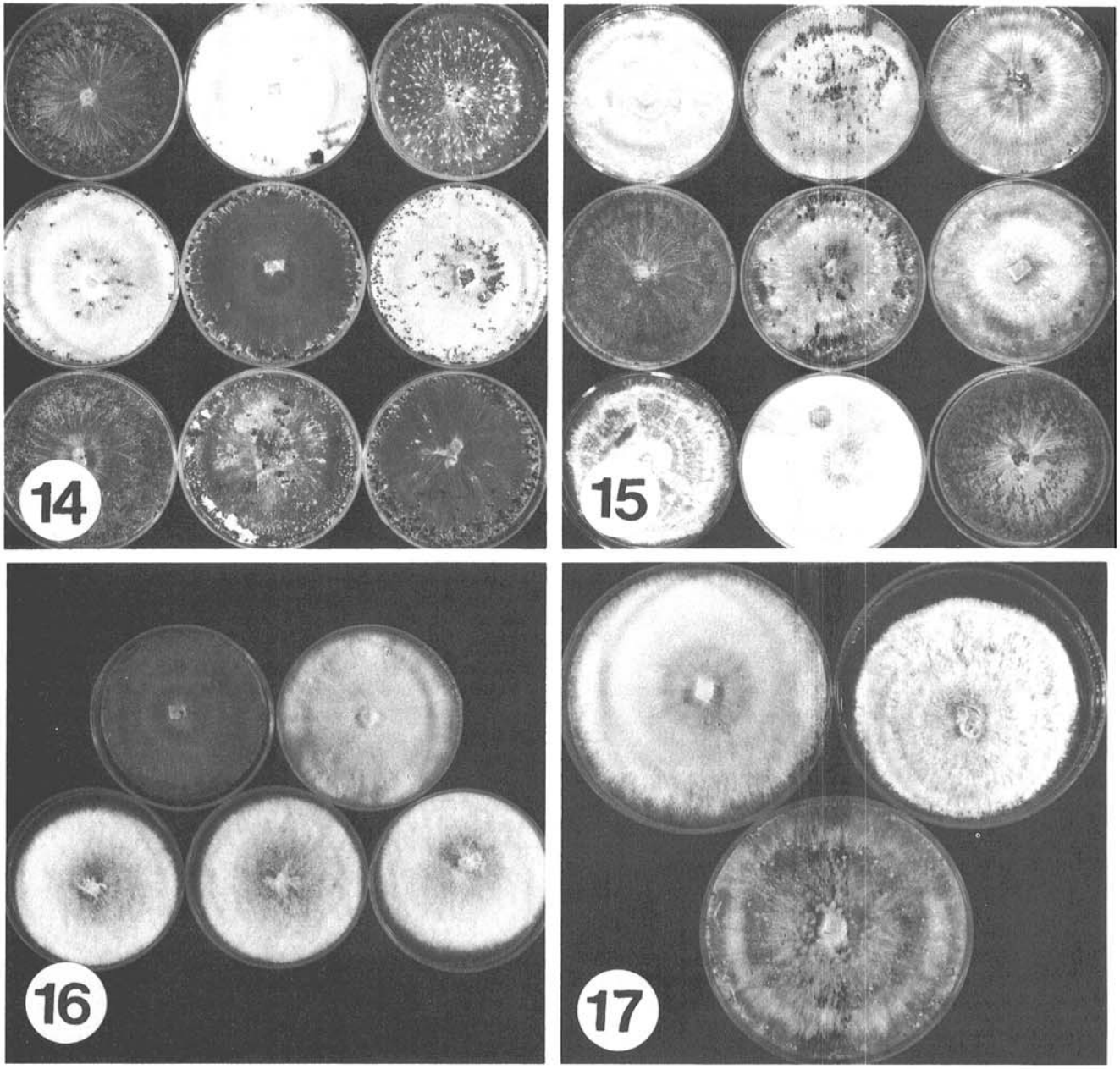
Given that a number of S_1 strains were homokaryons (our progeny analyses indicated that at least 15 strains were homokaryotic), we attempted to synthesize heterokaryons from random pairings of sibling and non-sibling S_1 strains. Heterokaryotic mycelia could not be as readily identified as the tuft mycelia in *R. solani* AG-1 and AG-4 (2,5) or those with clamps as in *S. commune* (33,37). Instead, evidence (putative) for heterokaryotic mycelia was based upon morphological differences in colony type, extent of sclerotial formation, growth rate (these being neutral characters) and the extent of variation among the progeny obtained from single spores (progeny analysis). When two asclerotial, slow-growing S_1 strains yielded a fast-growing sclerotial-forming colony, heterokaryon formation was suggested and eventually confirmed, where possible, by fruiting and progeny analysis. Pairings among sibling or non-sibling strains of *A. rolfsii* frequently developed antagonism zones, which are described in more detail elsewhere (31). This incompatibility resembles that reported in some basidiomycetes (11,18,25,38) and ascomycetes (heterokaryon incompatibility) (3,6,7,10,14,17,20). In many species in the homobasidiomycetes, the heterokaryon compatibility



Figs. 5–13. Clamp connections and nuclear condition of field isolates (FI) and single-basidiospore (S_1) strains of *Athelia rolfsii*. **5–6,** Clamp connections on the hyphae of FI growing on cellophane over water agar (×280), and an S_1 strain (×1,200), both phase-contrast. **7–13,** Hyphae and basidiospores stained with acridine orange and examined with near-UV to blue light. **7,** Same as in Fig. 6, showing multinucleate condition (×1,200). **8,** Multinucleate cell of FI (×1,200). **9,** Binucleate cell of FI (×1,140). **10–12,** Binucleate basidiospores (×730, ×1,180, and ×1,280). **13,** Germinating basidiospore with binucleate germ tube (×540).

factors usually also regulate nuclear migration and pairing, clamp formation, and formation of the sexual state (28,33). Thus, they are sexual factors (1,33) and are under homogenic control (33,34). Our observations suggest that genetic factors restricting heterokaryon formation exist in *A. rolfsii*; however, it is unclear at present how these factors relate to the sexual incompatibility factors of other basidiomycetes. The high frequency of fruiting of single-basidiospore strains of *A. rolfsii* and the presence of clamp connections on the mycelium of these strains could be taken as preliminary evidence for homothallism. However, there are reports of monokaryotic fruiting in basically heterothallic species (1,23,26); such fruiting could be the result of a failure of the sexual incompatibility system, the presence of a self-fertility allele, or may simply be a laboratory artifact phenomenon. These reports raise the possibility that a sexual incompatibility system may exist in *A. rolfsii*; further studies are required to clarify this.

The antagonistic reaction between S_1 strains of *A. rolfsii* results in hyphal death (31), but does not completely restrict heterokaryon formation. Stretton and Flentje (38) also demonstrated that the incompatible reaction that followed fusion of homokaryotic hyphae in *R. solani* AG-2 could lead to the formation of heterokaryons if the hyphae were macerated together to permit complete fusions, followed by transfer to a medium that permitted growth. This procedure yielded heterokaryons even in instances where macroscopic antagonism zones formed between homokaryons (38). In nature, the heterokaryotic or diploid condition of the mycelium of field isolates of *A. rolfsii* could conceivably arise from hyphal anastomoses and exchange of nuclei between two homokaryons, or possibly between a homokaryon and a heterokaryon. It may also, as others have pointed out, arise from mutation (8,11,13,27). The presence of a sexual system could also "override" the antagonistic reaction and permit the establishment of heterokaryons (36). Variability in *A. rolfsii* would thus be maintained via heterokaryosis, vegetative incompatibility (31), and formation of the sexual state.



Figs. 14–17. Variation among field isolates and single-basidiospore strains of *Athelia rolfsii* grown on potato-dextrose agar. **14,** Field isolates. **15,** Single-basidiospore progeny obtained from one field isolate. **16,** Field isolate (upper left), a single-basidiospore (S_1) strain from the field isolate (upper right), and single-basidiospore progeny obtained from fruiting of the S_1 strain (lower row). **17,** Two S_1 strains (upper row) and a heterokaryon obtained from pairing the two (lower dish).

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