Hyphal Interactions and Antagonism Among Field Isolates and Single-Basidiospore Strains of *Athelia (Sclerotium) rolfsii*

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ABSTRACT

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Seventy-two field isolates of *Athelia rolfsii* from 19 hosts and 15 geographical areas were paired in culture in all possible combinations. On the basis of antagonism zones (also called barrage zones) that developed in 86.5% of these pairings, the isolates were assigned to 25 interaction groups (i-groups). Within an i-group, all of the isolates grew together when paired and the hyphae intermingled with little or no cell death. In pairings between members from any two groups, antagonism zones developed; this was accompanied by the plasmolytic killing of hyphal cells. Isolates within a given i-group varied in morphological characteristics and frequently were isolated from different hosts or geographical areas. Antagonism zones also developed in 80–92% of sibling pairings and in 86% of non-sibling pairings among 50 single-basidiospore (S_1) strains obtained from five parent field isolates from California that belonged to i-group 1. Similar antagonism zones developed in all pairings between the S_1 strains and these field isolates. Formation of antagonism zones in sibling and non-sibling pairings was not significantly influenced by media or by different incubation temperatures. The implications of these findings are discussed relative to similar observations reported for wood-decaying basidiomycetes.

Additional key words: aversion, cytoplasmic incompatibility, heterogenic incompatibility, heterokaryon incompatibility, vegetative isolation, vegetative incompatibility.

The soilborne plant pathogenic fungus *Athelia rolfsii* (anamorph: *Sclerotium rolfsii*) represents a heterogeneous collection of isolates which characteristically form small spherical (0.5 to 2–3 mm in diameter) tan to dark-brown sclerotia with a rind, cortex, and medulla. Young colonies usually are white, the hyphae have clamp connections at some septa and frequently coalesce to form strands, and the hyphal tip cells are multinucleate. Isolates from different hosts and geographical areas vary considerably in some of these characteristics. The extent to which heterokaryosis and formation of the sexual state contribute to this variability have been reported elsewhere (22).

There are some reports suggesting that differences among isolates of *A. rolfsii* in virulence (7,8,29), morphological characteristics (14), and ability to form the basidial state (14) indicate the existence of strains of the fungus. However, there is no evidence that these isolates may be separable into groups or subpopulations based upon other criteria, such as the extent to which hyphal anastomoses occur, or the extent to which vegetative (somatic) incompatibility or antagonism (2,5,9,14,26) is manifested. A few workers (8,20,29) have reported formation of antagonism zones (also called aversion, barrage, or demarcation zones) when field isolates of *A. rolfsii* from different areas were paired in culture, suggesting some form of incompatibility. However, the significance of this phenomenon, perhaps as a mechanism whereby anastomoses and exchange of cytoplasm or genetic material between individuals may be reduced, has never been investigated. In addition, there have been no studies to establish the degree of compatibility or relatedness between field isolates from different hosts or geographical areas and among single-basidiospore strains. Thus, the existence of groups or subpopulations within the species has never been documented.

In this paper, we describe the occurrence and extent of antagonism zone formation between the mycelia of 72 field isolates of *A. rolfsii* from different hosts and geographical areas, and among 50 single-basidiospore strains from five of these field isolates from California. On the basis of the antagonistic reaction between field isolates, we have identified 25 interaction groups. Although the nature of the antagonism has not been determined, it could play a significant role in delimiting subpopulations or individuals of *A. rolfsii*, between which exchange of cytoplasm may be restricted. Thus, vegetative clones similar to those reported in many basidiomycetous fungi (6,12,26) may exist in nature. Preliminary results from this study have been reported (21).

MATERIALS AND METHODS

Isolates. The hosts of origin and areas of isolation of the 72 field isolates of *A. rolfsii* used in this and in a concurrent study (22) have been described elsewhere (23). All isolates were hyphal-tip clones, and were maintained on potato-dextrose agar (PDA) slants at room temperature (24–28 C) with ~1,200 lux light intensity (provided by cool-white fluorescent lights) during a 14-hr/day photoperiod. Fifty single-basidiospore strains previously obtained from five of these field isolates (22) also were tested. For use in subsequent studies, mycelial plugs of each isolate were transferred to 100 × 15-mm glass petri dishes containing about 25 ml of PDA and incubated for 7–10 days.

Hyphal interactions among field isolates. The isolates were paired with themselves and with each other in all possible combinations (2,628 pairings) on Snider and Raper's migration complete (MC) medium (27). For each pairing, 8-mm-diameter mycelial plugs from PDA cultures were placed about 40 mm apart in 60 × 15-mm petri dishes and these were incubated at 27–30 C and in the dark. The dishes were examined macroscopically after 5–7 days for presence or absence of an antagonism zone in the region of mycelial interaction between the two isolates in each dish. The intensity of antagonism was rated for each pairing as follows: 0 = no antagonism, mycelia and sclerotia of the two isolates intermingling; 1 = slight aversion (antagonism zone about 1 mm wide); 2 = moderate aversion (zone 2–4 mm wide); 3 = strong aversion (zone 4–8 mm wide).

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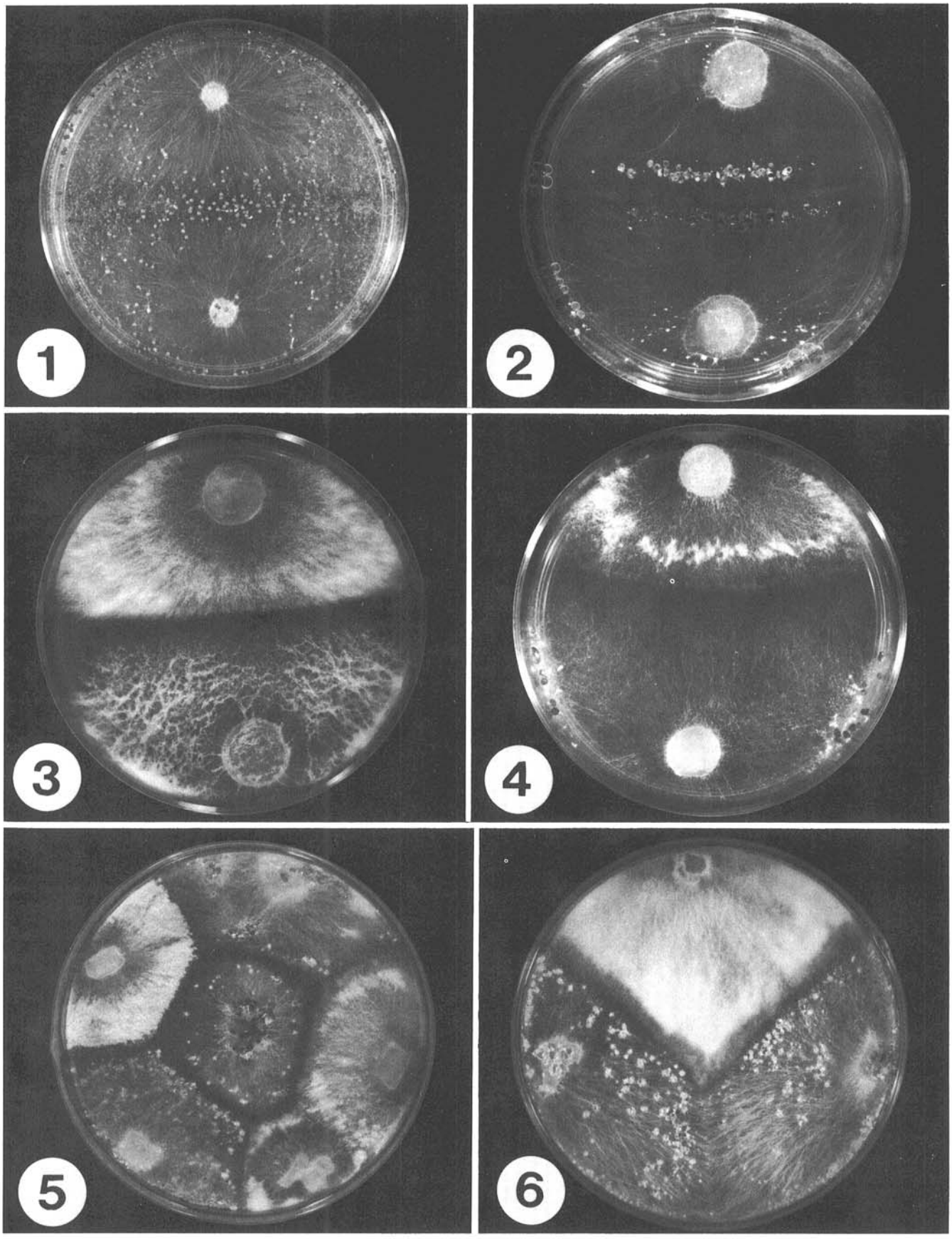


Figs. 1–6. Hyphal interactions and antagonism among field isolates and single-basidiospore strains of *Athelia (Sclerotium) rolfsii* on MC agar. **1,** Compatible reaction between two field isolates, showing intermingling of mycelia and sclerotia. **2,** Incompatible reaction between two field isolates, with development of an antagonism zone and two lines of sclerotia. **3,** Antagonistic reaction between two sibling single-basidiospore (S_1) strains; the intensity of the antagonism zone corresponds to a scale reading of two. **4,** An antagonistic reaction as in Fig. 3 corresponding to a scale reading of three. **5,** Antagonism zone formation between the parent isolate (center) and five S_1 strains. Also note antagonism zone formation between certain of the sibling strains. **6,** Antagonism zone formation between an S_1 strain (top) and two field isolates (bottom); the field isolates are compatible.

Hyphal interactions among single-basidiospore strains. Pairings were made on MC medium as described above between S_1 strains from the same parent (sibling pairings) and between strains from different parents (non-sibling pairings) (1,225 pairings). Pairings also were made between the S_1 strains and the five parent isolates (250 pairings). An additional 225 sibling pairings were made among 50 S_2 single-basidiospore strains previously obtained from five S_1 strains (22). All pairings were rated after 5–7 days based on the intensity of antagonism (0–3 scale) as described above.

Influence of temperature and media. Fifty sibling or non-sibling pairings between selected S_1 strains in which antagonism ranged from slight (=1) to strong (=3) were made on MC medium. Two replicate dishes of each pairing were incubated at temperatures ranging from 18 to 33 C, at 3 C increments. To determine the influence of media on antagonism zone formation, these 50 pairings were also made on each of the following media and incubated at 27–30 C: MC medium containing 1 or 2% (w/v) activated charcoal (Darco® G-60; Matheson, Coleman, and Bell, Norwood, OH 45212), PDA (Difco Laboratories, Detroit, MI 48232), PDA containing 1 or 2% activated charcoal, Joham's agar (17), 1% Bacto water agar (Difco), 3% malt agar (Difco), and PDA containing 0.1% yeast extract (Difco). The intensity of antagonism (0–3 scale) was rated for each pairing after 5–7 days.

Microscopic observations. Twenty selected pairings, 10 between field isolates and 10 between S_1 strains (representing a range of antagonism from 0 to 3) were made on cellophane over water agar (22) as described above. Observations were made periodically at time intervals ranging from 36 hr to 6 days by mounting and examining the dishes directly under the compound microscope (×125). The occurrence of hyphal anastomoses within and between hyphae of the isolates in each pairing and the extent of cell death in compatible (no antagonism zone) and incompatible (antagonism zone formed) pairings were noted.

RESULTS

Hyphal interactions among field isolates. When field isolates were paired, the mycelia either intermingled and sclerotia were formed throughout the plate (Fig. 1), or an antagonism zone developed, which appeared as a cleared area in the region of contact of the mycelia. In some pairings, a line of sclerotia formed on either side of this zone (Fig. 2). When mycelial plugs of the same isolate were placed on a dish, the hyphae intermingled and there was no antagonism zone (Fig. 1). Of 2,556 pairings (the pairings of isolates with themselves are not included), 86.5% developed antagonism zones.

On the assumption that field isolates that did not show antagonism when paired were somatically compatible and presumably similar genetically (1,4,9,24,26), the 72 isolates of *A. rolfsii* were assigned to 25 interaction groups (Table 1). Within a group, the paired isolates grew together and no antagonism zones developed; between isolates from any two groups, antagonism zones always formed. Forty-two isolates of *A. rolfsii* from California were placed in 10 interaction groups (i-groups) (Table 1). Thirty-six of these isolates were in four i-groups, and the remaining six each belonged to a different i-group. Twenty isolates from annual bluegrass-bentgrass golf greens in California belonged to i-groups 1 and 3 (Table 2). Although several isolates from a specific host or geographical area belonged to the same i-group, eg, i-group 5 was comprised of all of the isolates from Louisiana and i-group 8 contained three out of the four isolates from Maryland (Table 1), members of an i-group frequently were unrelated, eg, i-groups 3 and 8 were comprised of isolates from various geographical areas. Furthermore, there was no correlation between the intensity of antagonism (0–3 scale) and the geographical origin of the isolates. In pairings between isolates from the same area, or between isolates from different areas, a range of antagonism (from mild to strong) was observed.

Hyphal interactions among single-basidiospore strains. *Sibling and non-sibling pairings.* Antagonism zones ranging in intensity from 1 to 3 (Figs. 3 and 4) developed in 80–92% of the sibling pairings and in 86% of the non-sibling pairings. In either case, the data did not conform to a pattern that could be interpreted as indicating that antagonism zone formation was under unifactorial or bifactorial control. When S_1 strains were paired with the parental or with non-parental isolates, antagonism zones developed in all instances (Figs. 5 and 6).

Sibling pairings among S_2 strains. Antagonism zones did not develop in any of these pairings, or in pairings between the S_2 strains and their parent S_1 strain.

Influence of temperature and media. Antagonism between S_1 strains was most pronounced at 27–30 C. However, zones

TABLE 1. Assignment of 72 field isolates of *Athelia* (*Sclerotium*) *rolfsii* into 25 interaction groups based upon the development of antagonism zones[a]

Interaction group[b]	Area of isolation	Number of isolates from each location
1	California	23
	North Carolina	1
2	California	7
	Texas	1
3	California	2
	Florida	1
	Georgia	1
4	California	4
5	Louisiana	3
6	California	1
	Washington	2
7	North Carolina	2
8	California	1
	Alabama	1
	Georgia	2
	Maryland	3
9, 10, 11, 12	California	1 each
13	Arkansas	1
14, 15	Georgia	1 each
16	Kansas	1
17	Maryland	1
18, 19, 20	Australia	1 each
21	Chile	1
22,23	Israel	1 each
24, 25	ATCC	1 each

[a] Presence or absence of antagonism zones on MC medium was rated after 5–7 days of incubation at 27–30 C and in the dark.
[b] Interaction group numbers were assigned arbitrarily. Within a group, all isolates grew together when paired and antagonism zones did not develop; between isolates from any two groups, antagonism zones formed.

TABLE 2. Assignment of 42 field isolates of *Athelia* (*Sclerotium*) *rolfsii* from California into 10 interaction groups based upon the development of antagonism zones[a]

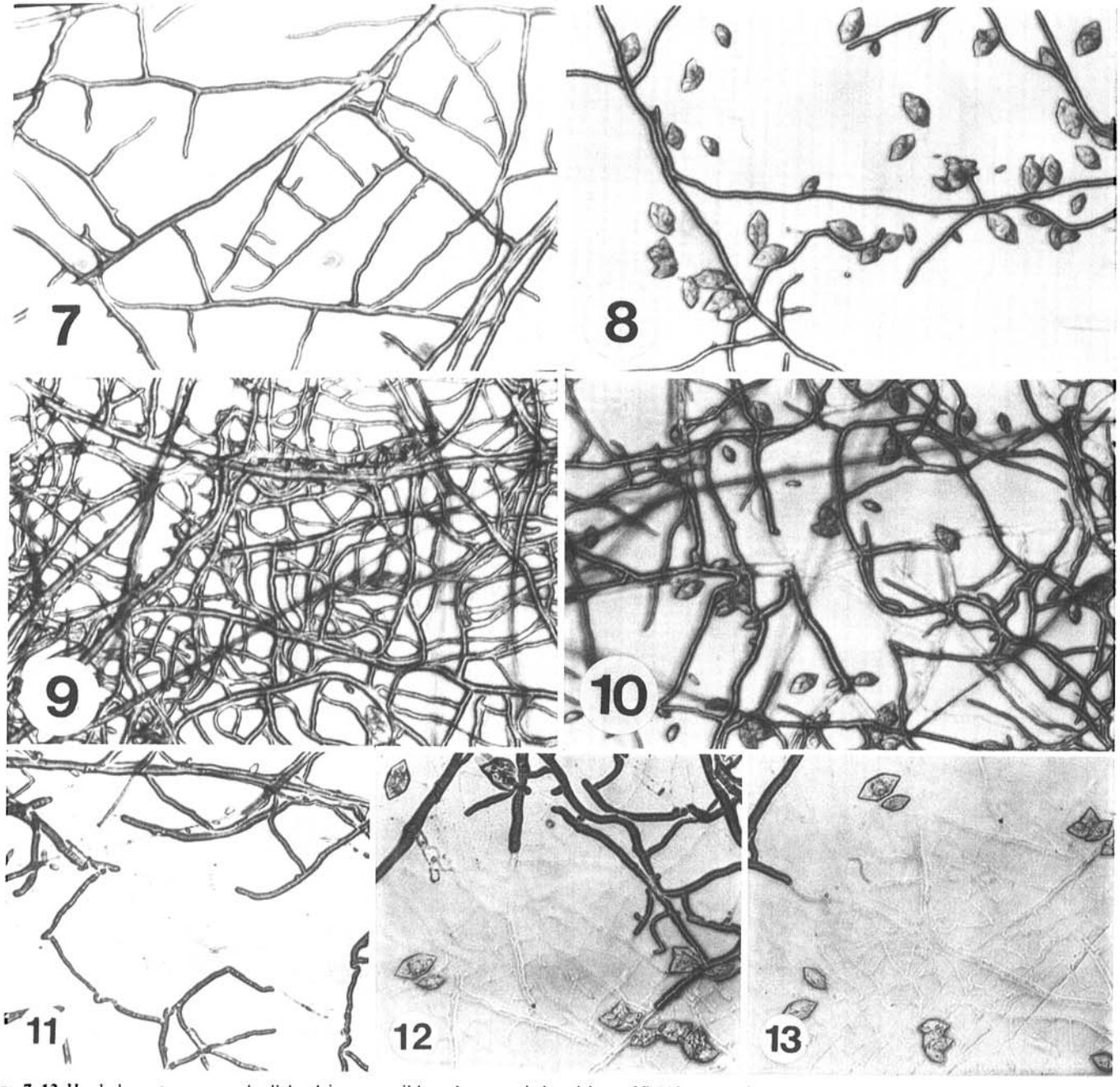
Interaction group	Host of origin	Number of isolates from each host
1	Annual bluegrass-bentgrass	18
	Bean	1
	Onion	1
	Sugar beet	2
	Sunflower	1
2	Bean	5
	Garlic	1
	Tomato	1
3	Annual bluegrass-bentgrass	2
4	Bean	1
	Sugar beet	3
6	Creeping bugleweed	1
8	Apple	1
9	Sugar beet	1
10	Apple	1
11	Creeping bugleweed	1
12	Cantaloupe	1

[a] Presence or absence of antagonism zones on MC medium was rated after 5–7 days of incubation at 27–30 C and in the dark.
[b] Interaction group numbers are the same as those used in Table 1.

developed at all temperatures between 18 and 33 C. The intensity of antagonism at these temperatures, when compared to the rating obtained at 27 C, was variable, but in no case was a reading of 0 (no antagonism) observed. When pairings were made on different media, the intensity of antagonism also varied. It was most pronounced on MC medium, PDA, and PDA containing 1% yeast extract, and was less discernible on the other media after 5–7 days of incubation. However, with prolonged incubation (up to 28 days), antagonism zones formed in all pairings on these latter media.

Microscopic observations. When an isolate was paired with itself, or in pairings between two isolates in which an antagonism zone did not develop, the hyphae intermingled and grew together. Hyphal tips made contact about 36 hr after inoculation but seldom anastomosed; instead the hyphae grew over each other, and by 72 hr had intermingled. Subsequently, however, lateral branches that served as anastomosis bridges were observed between and within the hyphae of the two isolates (Fig. 7). Crystals (presumed of oxalate [24]) were abundant (Fig. 8). By 96 hr, the individual mycelia could not be discerned (Fig. 9).

In pairings between isolates in which an antagonism zone formed, the mycelia initially grew in the same manner as described above. However, about 96 hr after inoculation, disruption of hyphal cells became apparent, leading to a thinning-out of the mycelium in the zone of interaction (Fig. 10). Various stages of hyphal disruption and lysis were observed (Figs. 11 and 12) and in some instances this resulted in complete death of all the hyphae (Fig. 13), leaving plasmolyzed cells or "ghosts." Where antagonism on MC medium was strong, all hyphae in the zone of contact and hyphae some distance away were disrupted.



Figs. 7–13. Hyphal anastomoses and cell death in compatible and antagonistic pairings of field isolates of *Athelia (Sclerotium) rolfsii*. All photomicrographs are of hyphae growing on cellophane over water agar. **7,** Self-anastomoses between hyphae of a field isolate, showing lateral anastomosis bridges. **8,** Crystals (presumed of oxalate) formed on cellophane over water agar. **9,** A compatible reaction, showing merging and anastomoses of the hyphae of the two isolates with no cell death. **10–13,** Antagonistic reactions between two isolates showing hyphae at various time intervals and stages of cell death. **10,** At 72 hr. **11 and 12,** At 96 hr. **13,** At 120 hr, complete killing.

DISCUSSION

The 72 field isolates of *A. rolfsii* used in this study represent a heterogeneous collection from different hosts and geographical areas, and there were obvious morphological differences among them. Since these differences can be influenced by media and environment, they do not constitute dependable criteria for grouping. We therefore have attempted to group these isolates based on whether or not the hyphae are compatible when paired. In all pairings (compatible and incompatible), anastomoses were observed to some extent between the hyphae of the two isolates. However, where antagonism was noted, the hyphae in the zone of contact were lysed. On the basis of this antagonistic reaction, which other workers have suggested denotes the presence of genetically distinct mycelia (1,4,9,25,26), interaction groups were established. Members within a group varied in morphological characteristics and similar-appearing isolates frequently belonged to different i-groups.

There are numerous examples of the existence of isolated groups (or clones) of individuals, or subpopulations, within a fungal species (2,4,6,13,26), and members of different groups show antagonism or vegetative incompatibility when paired. The genetic or cytoplasmic dissimilarities between members of different i-groups of *A. rolfsii* may have resulted from the isolation and adaptation of different isolates of the pathogen to different ecological niches or selection pressures. Thus, when genetically distinct mycelia from one area do not interact with mycelia from a different area, cytoplasmic exchange may be restricted. Within a given geographical area, the number of i-groups appears to be limited. Undoubtedly, as more isolates become available for testing, the number of i-groups in any given area will increase.

In other fungi, antagonism zone formation also has been observed in pairings among sibling homokaryons (heterokaryon incompatibility) (10,15,19), between single-basidiospore strains and their parent (1,10,15), and among field isolates (1,2,4,13,15,25). Hyphae in the zone of contact in many of these pairings were killed (2,4,10,11,19). Our observations indicate that the antagonistic reactions in *A. rolfsii* may be similar to those reported by others. However, the relationship between antagonism zones formed in pairings between heterokaryotic (or diploid) field isolates of *A. rolfsii* and between homokaryotic single-basidiospore strains has not been established. In addition, it is not clear at present whether antagonism zone formation between S_1 strains is related to sexual incompatibility of other basidiomycetes.

The mechanism of antagonism or incompatibility is not known. Although in *A. rolfsii* anastomosis may precede death of hyphae in incompatible pairings between field isolates or between S_1 strains, the role of a diffusible toxin or enzyme cannot be excluded in view of the low frequency of anastomoses in some incompatible pairings. The antagonistic reaction between S_1 strains was not overcome by making pairings on different media or incubation at various temperatures, or by placing mycelial plugs of the strains close together (about 1 cm apart) and incubating for periods of up to 2 mo (*unpublished*). Apparently, however, the killing of hyphae in some of these pairings does not completely prevent the exchange of cytoplasm or genetic material between the antagonistic S_1 strains (22). Anagnostakis and Day (3) also reported that barrage formation between field strains of *Endothia parasitica* from different antagonism groups did not prevent the exchange of hypovirulence factors in every instance. The extent to which formation of the antagonism zone prevents nuclear and cytoplasmic exchange between heterokaryotic field isolates of *A. rolfsii* belonging to different i-groups, or between parent and single-basidiospore isolates, needs to be investigated.

Vegetative or somatic incompatibility has been suggested by some to be a mechanism that prevents the buildup of other "exploitive" or different nuclei in the mycelium (16), or as a mechanism that may protect fungi from viruses and other cytoplasmic determinants (5). The basis for incompatibility in the Myxomycetes has been discussed by Ling and Clark (18). In the literature pertaining to the basidiomycetes, there are reports that vegetative incompatibility or antagonism among field isolates can be used to distinguish genetically distinct mycelia; for example, clones or strains may be established within a fungal species, between which individuals show antagonism zones when paired, suggesting that cytoplasmic exchange may be restricted (4,6,12,26). Formation of these antagonism zones also may be used to define the extent of spread of a clone in a given area (4,6,15). These observations have profound implications when related to studies on the establishment of fungal populations and distribution in nature (26,28).

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