Benomyl-Induced Somatic Segregation in Diploid *Armillaria mellea*

James. B. Anderson and Reza Yacoob

Department of Botany, University of Toronto, Erindale Campus, Mississauga, Ontario, Canada L5L 1C6.

This work was supported by an operating grant from the Natural Sciences and Engineering Council of Canada.
Accepted for publication 7 December 1983.

ABSTRACT

Anderson, J. B., and Yacoob, R. 1984. Benomyl-induced somatic segregation in diploid *Armillaria mellea*. Phytopathology 74:612-615.

Armillaria mellea is a bifactorially heterothallic fungus but, unlike other hymenomycetes, it has a persistently diploid vegetative phase in nature. Genetic analyses of *A. mellea* have been limited because this organism does not fruit reliably in culture. In order to induce somatic segregation of genetic markers, several diploid strains representing four of the intersterility groups or "biological species" of *A. mellea* in North America were treated with the fungicide benomyl. These diploids were heterozygous at the two mating-type loci and, in some cases, at nutritional loci as well. Somatic segregants of diploids were detected by testing 750 hyphal fragments derived from benomyl-treated material for auxotrophic phenotype and/or by visually selecting colonies that were morphologically different from the diploid progenitor. The mating types and auxotrophic requirements of each segregant were then determined. Although many of the segregants were homo- or hemizygous at each marker locus, some segregants were heterozygous at one locus or more, even when subcultured repeatedly in the absence of benomyl. Recombination was observed between all pairs of marker loci present in diploids synthesized in the laboratory. Because this protocol allowed recovery of numerous somatic segregants, parasexual genetic analysis in *A. mellea* can now proceed in the absence of in vitro fruiting.

Additional key words: basidiomycetes, fungal genetics, parasexuality.

Armillaria mellea (Vahl ex Fr.) Kummer is a destructive root pathogen of worldwide distribution. This fungus is bifactorially heterothallic, a pattern of sexuality common in the hymenomycetes, but it differs from other closely related fungi in that compatible pairings of haploid monokaryons yield diploid rather than dikaryotic mycelia (5,9,10,13). In addition, several intersterile populations or "biological species" of this fungus exist in Europe and North America (2,3,8). Mating within biological species is determined by bifactorial heterothallism; all matings of isolates from different biological species are sterile regardless of mating type.

Because fruiting bodies of *A. mellea* are produced rarely and unpredictably in the laboratory and because diploid strains are genetically stable during vegetative growth (4), genetic analyses have been limited. Recently, a preliminary screening of several agents for genetic effects showed that benomyl induces somatic segregation of markers in diploids of *A. mellea* (1). In that study, all of the diploids were synthesized in the laboratory from matings of sibling haploids derived from a single fruiting body of North American Biological Species I (2,3). Thus, the universality of the effect of benomyl on diploids of *A. mellea* was unknown. Furthermore, relatively few somatic segregants were recovered by testing large numbers of benomyl-treated progagules for mutant phenotype. To test the universality of the genetic effect of benomyl in *A. mellea*, we examined both laboratory-synthesized and naturally occurring diploids representing several of the biological species of *A. mellea*. In addition, two methods for detecting somatic segregants were employed in the present study: a visual screening procedure, based on a morphological difference between segregant and diploids, as well as the screening procedure used earlier.

MATERIALS AND METHODS

Media, growth conditions, and the origins of diploid strains 3.1 and 1.7 (Tables 1 and 2) were described previously (1). Diploid strains 3.1 and 1.7 were synthesized from compatible matings of auxotrophic strains that were members of North American Biological Species I. The other diploid strains were isolated from the caps of *A. mellea* mushrooms from the following sources: strain 19, Biological Species III, growing on sugar maple, Underhill, VT; strain 35, Biological Species II, host undetermined, Camel's Hump, VT; strains 200 and 201, Biological Species VII, host undetermined, Mississauga, Ont.

To induce somatic segregation, diploid strains were inoculated on malt extract medium (1.25%) containing 25 μg of benomyl per liter, which was added directly to the medium after autoclaving. After 1 mo of incubation at room temperature, five colonies of each strain were macerated for 2 min at high speed in a Waring blender. The macerate was then vacuum-filtered through nylon mesh (10-μm openings) into a side-arm flask. This filtration removed all but mostly one- and two-celled hyphal fragments. The filtrate was diluted, plated on malt extract medium, and incubated until colonies appeared. Somatic segregants were detected in two ways: by testing 750 colonies individually for prototrophy or auxotrophy (provided the original diploid strain was heterozygous for auxotrophic markers) and/or by selecting fluffy colonies appearing against a background of more numerous crustose colonies at a dilution of ~5–50 colonies per 10-cm-diameter petri dish. The rationale for the latter selection is that diploids heterozygous at the *A* and *B* mating-type loci produce flat, crustose cultures, whereas strains homo- or hemizygous at the *A* and *B* loci (Fig. 1) produce fluffy colonies (4). Finally, the mating types and auxotrophic requirements of each segregant were determined as described previously (1).

Because the ploidy of the segregants was unknown, the genotypes of the segregants can be inferred from the observed phenotypes as follows. The mating-type alleles are codominant, and therefore heterozygosity at these loci is readily distinguishable from homo- or hemizygosity by the mating reactions of a segregant paired with haploid tester strains of mating types *A1B1*, *A2B2*, *A1B2*, and *A2B1* (1). Incompatible pairings retain a fluffy appearance, whereas compatible pairings become flat and crustose (13). For example, a segregant heterozygous at the *A* locus and homo- or hemizygous for *B1* would be compatible with the *A1B2* and *A2B2* testers (Fig. 2). Similarly, a segregant heterozygous at both *A* and *B* would be compatible with the *A1B1*, *A2B2*, *A1B2*, and *A2B1* testers. In the case of the nutritional loci, the mutant alleles are recessive to the wild type. Therefore, the mutant phenotype could be homo- or hemizygous but not heterozygous at a nutritional marker locus, and the wild-type phenotype could be either homo- or hemizygous wild type or heterozygous.

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RESULTS

Screening 750 randomly selected colonies from each of diploid strains 3.1 and 1.7 yielded 33 and 87 auxotrophic segregants, respectively (Table 1). Some of the segregants of diploid 1.7 were heterozygous for one, the other, or both mating-type loci (Table 1).

TABLE 1. Auxotrophic segregants recovered after treatment of diploid strains of *Armillaria mellea* with benomyl at 25 μg/L of medium

	Phenotypes of segregants	
No. of segregants isolated[a]	Nutritional requirement[b]	Mating specificity[c]
Strain 1.7[d,e]		
6	Pyr	*A*1, *B*1
12	Met, Pyr	*A*1, *B*1
6	Pyr	*A*2, *B*2
1	Met, Pyr	*A*1, *B*2
1	Met	*A*1, *B*2
1	Pyr	*A*1, *B*2
12	Pyr	*A*2, *B*1
10	Pyr	*A*1/*A*2, *B*1
1	Met, Pyr	*A*1/*A*2, *B*1
5	Pyr	*A*1/*A*2, *B*2
4	Met	*A*1, *B*1/*B*2
3	Met, Pyr	*A*1, *B*1/*B*2
2	Pyr	*A*2, *B*1/*B*2
12	Pyr	*A*1/*A*2, *B*1/*B*2
10	Pyr	?, ?[f]
1	Met, Pyr	?, ?
Strain 3.1[g]		
21	Ade	*A*1, *B*2
7	Ade, Arg	*A*2, *B*1
1	Ade	*A*1, *B*2
2	Arg	*A*2, *B*2
2	Ade	?, ?

[a] A total of 750 colonies were screened for auxotrophic requirement.
[b] Ade = adenine, Arg = arginine, Pyr = pyridoxine, and Met = methionine.
[c] Assayed by pairing segregant strains with haploid testers of mating types *A*1*B*1, *A*2*B*2, *A*1*B*2, and *A*2*B*1 and scoring each pairing for compatibility or incompatibility.
[d] Diploid genotype: *A*1/*A*2 *B*2/*B*1 *rs*-2−/*rs*-2+ *pyr*+/*pyr*−.
[e] Identifying characters preceding a diagonal indicate genotype of one haploid mate; those following a diagonal indicate that genotype of the other mate used to construct the diploid. *A* and *B* = mating-type loci; *rs*-2− = auxtotrophic requirement for reduced sulfur such as supplied by methionine, complementation group 2; *pyr*− = auxotrophic for pyridoxine; *ade*− = auxotrophic requirement for adenine; *arg*− = auxotrophic requirement for arginine.
[f] ? = Mating reactions not clear.
[g] Diploid genotype: *A*1/*A*2 *B*1/*B*2 *ade*−/*ade*+ *arg*+/*arg*−.

These segregants remained heterozygous at the mating-type loci even after subculture on malt extract medium without benomyl. Other segregants of diploid strain 1.7, as well as all segregants of diploid strain 3.1, were either hemi- or homozygous at each of the mating-type loci. Recombinants were observed for each pair of marker loci (nutritional and mating-type) with respect to the original haploid strains used to construct the diploid (Table 1) with the sole exception of the *arg* and *A* loci. (Note that in Table 2 recombinants with respect to *arg* and *A* were recovered.) Some of the reciprocal classes of recombinants, however, were not recovered. The colony morphology of segregants (not indicated in Table 1) was consistent with the observations of earlier studies

TABLE 2. Fluffy segregants recovered after treatment of diploid strains of *Armillaria mellea* with benomyl at 25 μg/L of medium

	Phenotypes of segregants	
No. of fluffy segregrants isolated[a]	Nutritional requirements[b]	Mating specificity[c]
Strain 1.7[d,e]		
1	None	*A*2, *B*2
1	Pyr	*A*2, *B*2
1	None	*A*2, *B*1
1	Pyr	*A*2, *B*1
3	None	*A*2, *B*1/*B*2
1	Pyr	*A*2, *B*1/*B*2
1	Pyr	?, ?[f]
Strain 3.1[g]		
7	None	*A*1, *B*2
3	Ade	*A*1, *B*2
11	None	*A*2, *B*2
1	None	*A*2, *B*1/*B*2

[a] Fluffy colonies are produced by all strains homo- or hemizygous at the *A* and *B* loci, by all strains homo- or hemizygous at *A* and heterozygous at *B* and by some (but not all) strains heterozygous at *A* and homo- or hemizygous at *B*.
[b] Ade = adenine, Pyr = pyridoxine.
[c] Assayed by pairing segregant strains with haploid testers of mating types *A*1*B*1, *A*2*B*2, *A*1*B*2, and *A*2*B*1 and scoring each for compatibility or incompatibility.
[d] Diploid genotype: *A*1/*A*2 *B*2/*B*1 *rs*-2−/*rs*-2+ *pyr*+/*pyr*−.
[e] Identifying characters preceding a diagonal indicate genotype of one haploid mate; those following diagonal indicate the genotype of the other mate used to construct the diploid. *A* and *B* = mating-type loci; *rs*-2− = auxotrophic requirement for reduced sulfur such as supplied by methionine, complementation group 2; *pyr*− = auxotrophic for pyridoxine; *ade*− = auxotrophic requirement for adenine; *arg*− = auxotrophic requirement for arginine.
[f] ? = Mating reactions not clear.
[g] Diploid genotype: *A*1/*A*2 *B*1/*B*2 *ade*−/*ade*+ *arg*+/*arg*−.

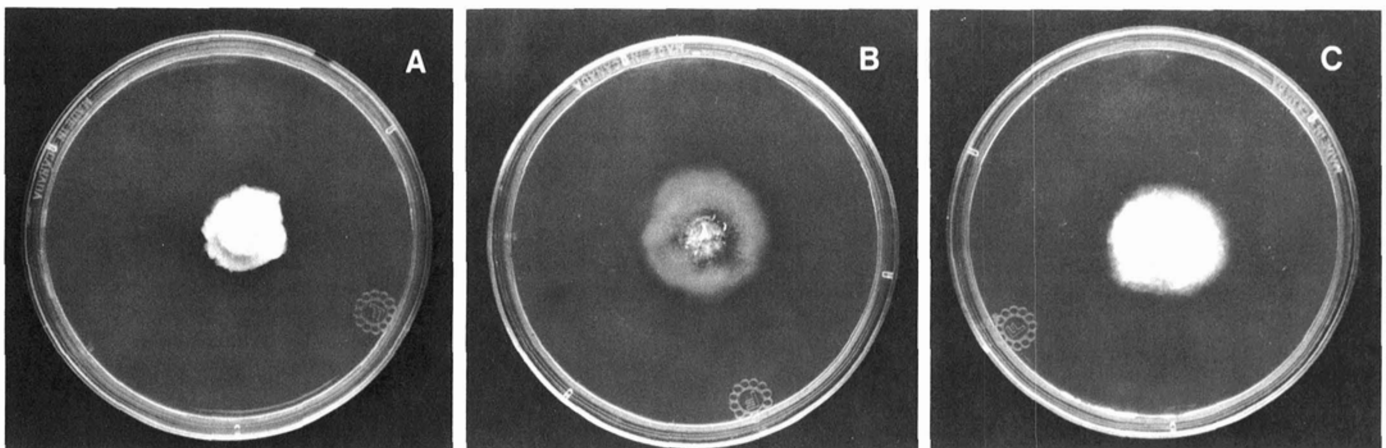

Fig. 1. Colony morphology of haploid, diploid, and segregant strains of *Armillaria mellea*. **A,** Haploid strain. **B,** Diploid strain heterozygous at the *A* and *B* mating-type loci. **C,** Segregant strain homo- or hemizygous at the *A* and *B* loci. Note that the haploid and segregant strains have a fluffy appearance, whereas the diploid strain has a flat, pigmented appearance.

(1,4). In the earlier and present studies (Tables 1 and 2), all of the segregants heterozygous at both of the mating-type loci produced colonies that were crustose. Also, all segregants heterozygous at neither mating-type locus, or the *B* locus only, produced colonies that were fluffy. Finally, some of the segregants heterozygous at the *A* and not the *B* locus produced fluffy colonies, but others produced crustose colonies.

Visual selection of fluffy colonies allowed recovery of somatic segregants from each of the six diploid strains treated with benomyl (Tables 2 and 3). No attempt was made to count the total number of colonies screened to recover these segregants, because determination of colony morphology is often difficult when colonies are small. Many colonies initially thought to be fluffy turned out to be flat after transfer to a separate dish, and conversely, many fluffy colonies may have been missed in the initial screening. From diploid strains 3.1 and 1.7, 22 and nine fluffy segregants, respectively, were recovered (Table 2). Twenty-four of the segregants from diploid strains 3.1 and 1.7 were prototrophic and only seven were auxotrophic. Five of the segregants were heterozygous for the *B* mating-type locus, but all of the others were homo- or hemizygous for both the *A* and the *B* loci. Segregants of various mating specificities were also recovered from diploid strains 19, 35, 200, and 201 after treatment with benomyl (Table 3).

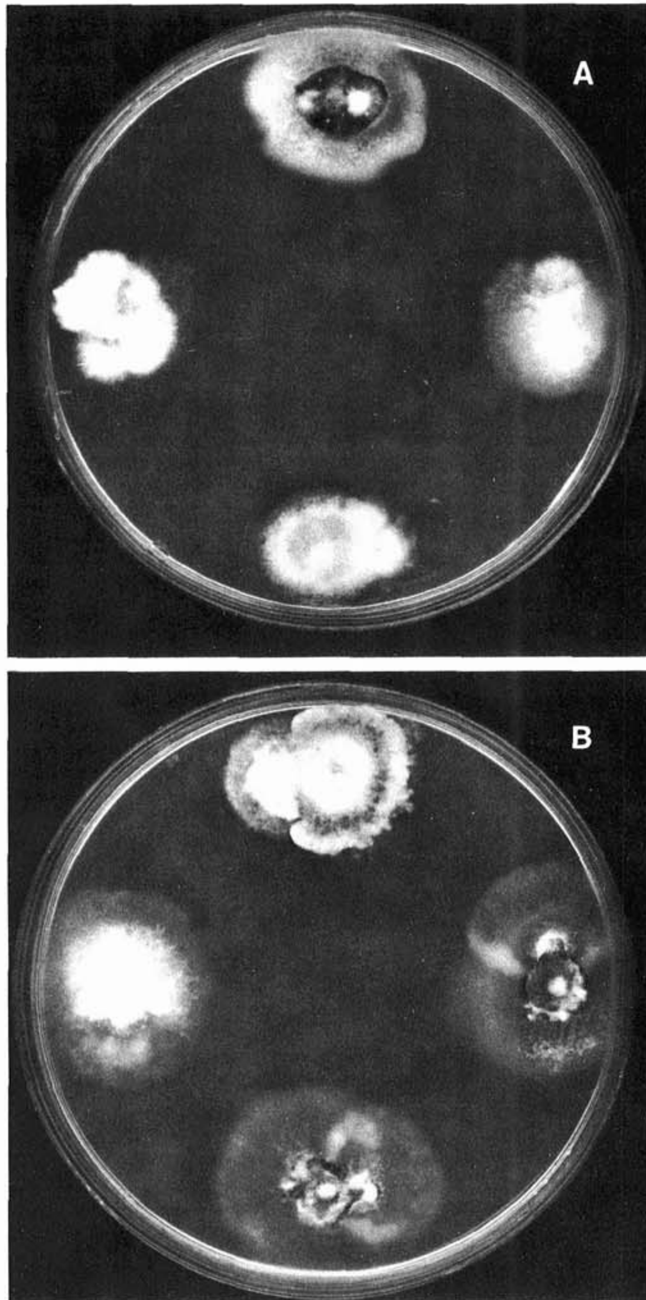

Fig. 2. Pairings of segregant strains of *Armillaria mellea* with haploid testers of mating types (clockwise from the top) *A1B1*, *A2B2*, *A1B2*, *A2B1*. **A,** Segregant strain was homo- or hemizygous for *A2* and *B2*. **B,** Segregant strain was heterozygous at *A* and homo- or hemizygous for *B1*. Compatible pairings have a flat, pigmented appearance, whereas incompatible pairings have a fluffy appearance.

DISCUSSION

The primary genetic effect of benomyl on diploids of higher fungi is abnormal segregation of whole chromosomes at mitosis (6,7,14). The probable reason for this genetic effect is that benomyl binds to the β subunit of tubulin (12) and interferes with microtubule function (11). It is likely that the observed segregation in diploids of *A. mellea* treated with benomyl occurs by a similar mechanism, but in the absence of known linked markers, events that affect whole chromosomes cannot be distinguished from mitotic crossing over or chromosome breakage. Because many of the segregants showed segregation at all four marker loci, however, mitotic crossing over or chromosome breakage would be extremely unlikely; these events tend to occur independently, and the probability of obtaining many such segregants, each showing multiple crossover or breakage events, is low. On the other hand, methyl benzimidazole-2-yl-carbamate, the active breakdown product of benomyl, causes multiple loss of whole chromosomes at a frequency much higher than the product of the probabilities for the loss of individual chromosomes (14).

If the assumption that benomyl causes abnormal segregation of whole chromosomes in diploids of *A. mellea* is correct, then a number of possibilities follow. First, the strains showing segregation at all four marker loci may be haploid or nearly haploid. Second, strains showing heterozygosity at some loci, but not others, may be aneuploid. We are currently measuring nuclear DNA content in order to determine whether segregants contain the same or less DNA than the parent diploid. Third, the four markers in diploid 3.1 and in 1.7 are probably unlinked, since recombinants with respect to each pair of marker loci were recovered. No recombination would occur between linked loci if the segregations were indeed the result of whole-chromosome events.

Although many different classes of segregants were recovered after treatment of diploid strains with benomyl, one limitation of this technique for genetic work is that each segregant is not necessarily derived from an independent segregational event. Clonal propagation of segregants undoubtedly occurs during benomyl treatment and the numbers of segregants in each phenotypic class might reflect different growth rates rather than numbers of actual segregational events. The clonal effect and differential growth rates might explain, for example, the ratio of *pyr−* to *pyr+* segregants (82:5) in Table 1 or the fact that many more prototrophic than auxotrophic segregants were recovered by the visual selection procedure (Table 2). It has been evident in this

TABLE 3. Fluffy segregants recovered after treatment of diploid strains representing different biological species of *Armillaria mellea* with benomyl

Biological species[a]	No. of segregants	Mating specificity[b]
VII (200)	1	*A2*, *B2*
	4	*A1*, *B1*
VII (201)	5	?[c]
II (35)	9	*A2*, *B2*
	1	*A1*, *B1*
	1	*A1*, *B1*/*B2*
III (19)	2	*A1*, *B1*

[a] Strains in Tables 1 and 2 represent biological species I.
[b] The *A* and *B* mating type designations of strains representing different biological species are not equivalent. The designation of one mating-type locus as *A* and the other as *B* is arbitrary.
[c] Mating reactions with testers not clear. These segregants did produce clear-cut compatible reactions with other monosporous isolates representing biological species VII.

and previous work with *A. mellea* that growth rates vary considerably from strain to strain (*unpublished*).

Benomyl-induced somatic segregation was evident in all isolates tested, including representatives of North American Biological Species I, II, III, and VII. It is therefore likely that somatic segregation can be induced in any diploid strain of *A. mellea* whether naturally occurring or synthesized in the laboratory. The technique might be readily applied to parasexual genetic analysis in *A. mellea* and might be useful in the manipulation of induced genetic markers or in determining the mating-type alleles carried by isolates of vegetative material from the field. The induction of somatic segregation in diploid strains of *A. mellea* by benomyl is potentially important for future studies because reliable laboratory fruiting has not been achieved.

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