Ascospore Dimorphism in *Sclerotinia trifoliorum* and Cultural Characters of Strains from Different-Sized Spores

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Portion of a Ph.D. thesis submitted to Tokyo University of Agriculture by the first author.

We thank Y. Kitani of Kihara Institute for Biological Research for useful advice, and I. Saito of Kitami Agricultural Experiment Station, Hokkaido, for contributing cultures.

Accepted for publication 28 October 1982.

ABSTRACT

Uhm, J. Y., and Fujii, H. 1983. Ascospore dimorphism in *Sclerotinia trifoliorum* and cultural characters of strains from different-sized spores. Phytopathology 73:565-569.

Among five isolates of *Sclerotinia trifoliorum* from different sources, all mature asci displayed ascospore dimorphism, with 4:4 segregation of large and small ascospores, arranged in six types of first and second meiotic division segregation. The second meiotic division frequency (mean = 20.8%) did not vary significantly among isolates. This indicated that dimorphism in spore size was regulated by the same allelic pair in all isolates. The strains derived from the large and small ascospores of an ascus were very similar in morphology, growth rate, and pathogenicity. Without exception, nonpaired cultures from single-ascosporic strains derived from small spores failed to produce apothecia, whereas those derived from large spores produced sporulating apothecia, although their asci again exhibited 4:4 segregation for spore size.

Sclerotinia trifoliorum Erikss., a plant pathogenic fungus on forage legumes, displays dimorphism in spore size, with a 4:4 segregation of large and small ascospores in each ascus (8). Although ascospore dimorphism is easily discernible in mature asci, it has seldom or never been referred to in taxonomic papers (7,11,12,15) dealing with measurements of asci and ascospores. To the authors' knowledge, Carr (3) first reported ascospore dimorphism in *S. trifoliorum*. He considered dimorphism not to be a character of the species as a whole, but the result of heterokaryosis between specific strains. A similar dimorphism was reported earlier in *S. trifoliorum* var. *fabae* Keay, and was also regarded as the manifestation of heterokaryosis (11). Only recently, ascospore dimorphism was established as a speciation criterion, since it was confirmed in all materials over a wide geographical range and was a specific character of *S. trifoliorum* not shared with other plant pathogenic *Sclerotinia* sp. (8). Recently, the authors also observed ascospore dimorphism in all materials collected from various localities in Japan.

However, the biological significance of ascospore dimorphism and possible cultural and biological difference between isolates derived from different-sized spores of a given ascus has not previously been determined. The present paper deals with the spore arrangements in the asci and their genetic interpretation, and with several cultural characters of isolates derived from different sized spores. Another paper (14) describes mating experiments.

MATERIALS AND METHODS

Isolates and cultural methods. Isolates were obtained from sclerotia from various localities in Japan and were identified according to Kohn's (8) delimitation of *S. trifoliorum*. Three isolates were from red clover, and two were from alfalfa and milk vetch, respectively. Two of the red clover isolates were contributed by I. Saito of Kitami Agricultural Experiment Station, Hokkaido, as single ascosporic strains.

Sclerotia collected in the field were surface sterilized, and the black rinds were removed aseptically and discarded. The medulla was plated on laboratory prepared potato-dextrose agar (PDA). Isolates were usually cultured for about 30 days at 20 C to obtain mature sclerotia.

Apothecial production. Sclerotia from ~30-day-old cultures were transferred to petri dishes containing polyurethane saturated with sterilized water (13) and kept in an incubator at 15 C with continuous fluorescent light (4,500 lux) to induce formation of apothecia.

Isolation of single-ascosporic strains. Asci and rosettes of asci were spread on one side of a 3% water agar plate on a microscope slide. Asci were dissected with a micromanipulator (Takashima Shoten, Japan), and ascospores 2, 4, 6, and 8 from the apex of the ascus were isolated. In some cases, whenever needed, a full set of eight ascospores was isolated. Ascospores were transferred to the opposite side of the plate at a distance apart from each other in the order of their original arrangement and allowed to germinate. After 12 hr, the germinated spores were transferred to PDA plates with the aid of a dissecting microscope. When colonies formed they were transferred to PDA slants and maintained at 5 C.

Each single-ascosporic strain was designated with the serial number of the ascus in the order of dissection, with each spore numbered according to position from the apex of the ascus, and with the symbol of the spore size: L for large spores and S for small.

Examination of ascospore arrangements. To prevent deformation and germination of the ascospores during examination, a mounting solution (4) was used to fix the material. A small bit of hymenium cut from the apothecium was put in a drop of mounting solution on a slide glass and tapped with the tip of a scalpel to suspend the individual asci in the solution. A coverslip was applied, and the specimen was examined at ×300.

Pathogenicity test. Two-month-old red clover plants grown in pots were inoculated by scattering dried inoculum (9) on the soil among the plants. They were covered with polyethylene bags and kept in a growth chamber at 20 C.

RESULTS

Patterns of ascospore arrangement. Patterns of ascospore arrangement in the asci from the five cultures of different sources were examined. All mature asci in which ascospore outlines were distinct showed 4:4 segregation of large and small ascospores. However, in immature asci in which the ascospores were relatively

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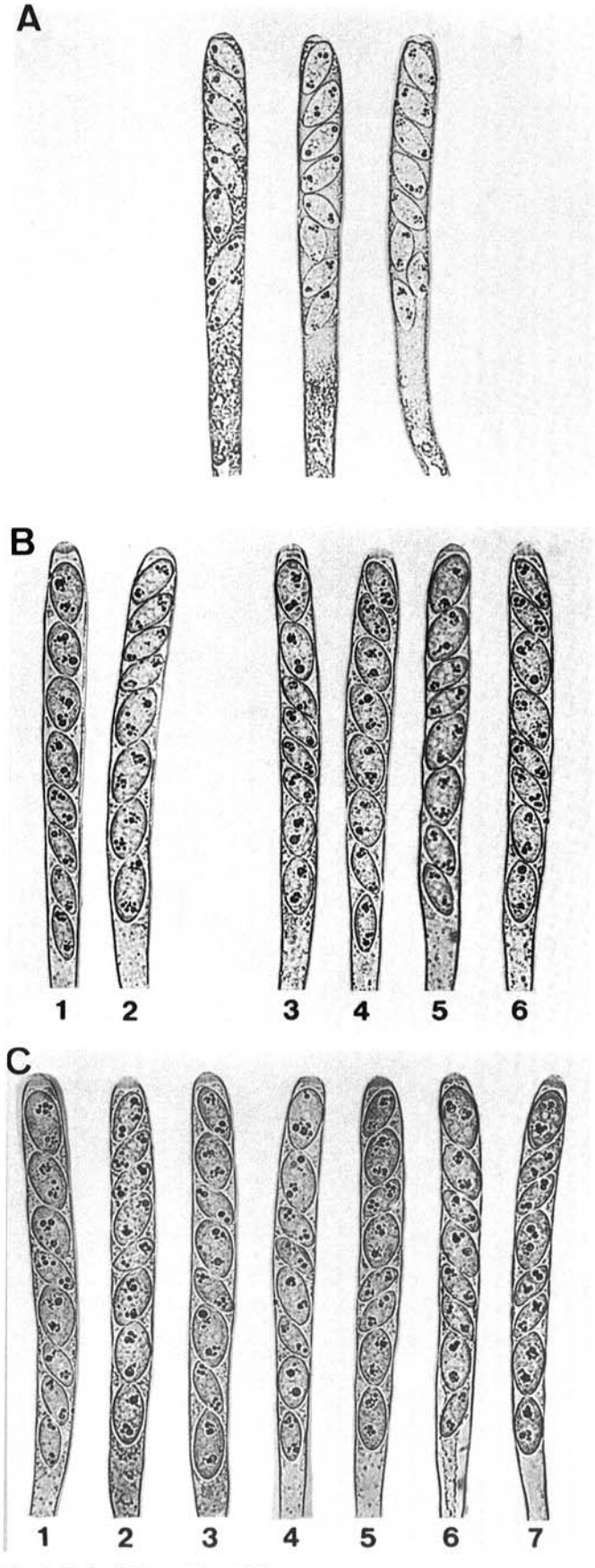

Fig. 1. Asci of *Sclerotinia trifoliorum*. **A,** Immature asci. Dimorphism in spore size is not discernible. **B,** Mature asci showing ascospore dimorphism. Large and small spores are arranged in six patterns of first- and second-division segregation. **C,** Asci with spore displacement showing partial disturbance in spore pairs of same sized spores. Note that the 4:4 segregation ratio of large and small ascospores is constant.

small with indistinct outlines, dimorphism was difficult to discern (Fig. 1A). The ascospore arrangements in most of the asci fell into six patterns of first and second meiotic division segregation (Fig. 1B). This is usual when the characters are controlled by a pair of alleles, as seen in the mating type segregation of *Neurospora crassa* (10).

In those six arrangement patterns, spores adjacent to each other in four spore pairs of the ascus were the same size; these were sister spores. However, in 3.7 to 12.3% of the asci the spore arrangements deviated from those six patterns by partial disturbance in pairing of the same sized spores, although the 4:4 segregation ratio was not disturbed (Fig. 1C).

According to the general scheme of ascosporogenesis in ordered tetrads, spores of the same genotypes are formed in pairs, since the postmeiotic division is mitotic. Considering the regularity and consistency of the segregation ratio, it was supposed that the disturbance in spore arrangements was due to spore displacement between one or two pairs of spores, which occurred after postmeiotic mitosis. Therefore, the spore arrangements of unusual asci could be adjusted by changing the spore position into one of the six usual patterns without making a difference in the genetic principle of spore size segregation. For example, in the first (left hand) ascus shown in Fig. 1C, if the fourth and fifth spores from the apex were reversed, the resulting spore arrangement became that of the first-division segregation pattern. In another example, in the third ascus shown in Fig. 1C, if the second and third, and the sixth and seventh spores were reversed, the segregation pattern showed an asymmetric second-division segregation.

When spore displacements occurred between adjacent spores, adjustment could be made only one way. However, in some arrangements such as shown in the seventh ascus of Fig. 1C, when spore displacement occurred between spores one spore apart, there were two possible ways of adjustment. In this particular case, the resulting arrangement is an asymmetric type of second-division segregation if the first and third spores are reversed, but becomes a symmetric type if the position of the second and fourth spores are reversed. However, since asci with such arrangements were very rare (less than 1%), they were omitted from the sample. The

TABLE 1. Ascospore displacements observed in linear arrangement of eight ascospores in asci of *Sclerotinia trifoliorum* isolate M35-1 and the arrangement after adjustment

Ascospore arrangements[a] before adjustment	Number of asci[b] observed	Ascospore arrangements after adjustment
L L L S L S S S	7	L L L L S S S S
S S S L S L L L	15	S S S S L L L L
L S L S S S L L	8	L L S S S S L L
L S L S L L S S	4	L L S S L L S S
L L S S L S L S	6	L L S S L L S S
S L S L S S L L	4	S S L L S S L L
S S L S L S L L	7	S S L L S S L L
L L S L S L S S	3	L L S S L L S S
S S L L L S L S	2	S S L L L L S S
S L S L L L S S	2	S S L L L L S S
S S L L S L S L	2	S S L L S S L L
L S L L S L S S	1	L L S S L L S S
L L S S S L S L	2	L L S S S S L L
L S L S S L S L	1	L L S S S S L L
L L S L S S L S	1	L L S S L L S S
L L S S L S S L[c]	1	L L S S L L S S
		L L S S S S L L
S L L S S S L L[c]	1	S S L L S S L L
		L L S S S S L L
S L L S L L S S[c]	1	L L S S L L S S
		S S L L L L S S

[a] L = large ascospore; S = small ascospore.
[b] Observed among 590 asci.
[c] Spore displacement occurred between spores of one spore apart and two ways of adjustment were possible, but these asci were omitted from the sample.

patterns of spore displacement observed in one culture, M35-1, and resulting spore arrangement after adjustment are shown in Table 1.

By using the methods described above, all asci with displaced spores were reclassified into one of the six patterns. The frequency of the six arrangement patterns in asci of the five original cultures is shown in Table 2.

It is noteworthy that the two single-ascosporic strains, R31 and R39 from red clover, also showed the 4:4 segregation for spore size. The segregation for spore size in asci of single ascosporic strains is very significant and will be discussed in detail later.

To acquire evidence for genetic control of ascospore dimorphism, the frequency of second-division segregation patterns was statistically analyzed by using the confidence limit curve (1). The frequency of second-division segregation in the five cultures did not differ significantly, the average being 20.8%.

Cultural morphology and growth rate of single-ascosporic strains. To study cultural morphology and growth rate of single-ascosporic strains derived from different sized spores, one set of tetrads from each of the five original cultures was isolated by the method described above. The spore arrangements in those asci are shown in Table 3. These sets of four single-ascosporic strains were used in various experiments throughout this and subsequent studies.

When the four ascospores from a single ascus, two large and two small, were placed on a water agar plate, germination took place almost simultaneously. However, the germ tubes from large ascospores elongated faster than those from the small ones (Fig. 2). When the germinated ascospores were transferred to PDA plates, the same slight difference was observed in lateral growth. However, when they were transferred once again to fresh medium as mycelial disks from the edge of these colonies, these differences were no longer detected. Therefore, it seemed that the difference in growth rate at the early stage was not an essential difference between the two isolates. The resulting cultures were so similar that it was impossible to discriminate between them (Fig. 3).

Pathogenicity. One pair of large and small single-spore cultures from each of the five tetrads shown in Table 3 was used for the pathogenicity test. Symptoms began to appear 3 days after inoculation as drooping leaves and water-soaked lesions on the petioles. As symptoms progressed, white mycelia became visible on the flaccid plants, and then mycelia covered the entire collapsed plants as they reached the final state of sclerotial formation. Considerable differences in virulence among the strains were seen, but those differences were not related to the size of the spore from which the strain was derived (Table 4).

TABLE 2. Frequency of the six patterns of spore arrangement in the asci of the five original cultures of *Sclerotinia trifoliorum*

Cultures	Frequency[a] of: LLSS[b]	SSLL	LSSL	SLLS	LSLS	SLSL	Total
A22	184	173	17	16	25	24	456
R31	201	231	32	16	33	30	543
M35-1	185	211	28	37	38	39	590
R38-3	257	286	24	16	37	34	654
R39	220	233	28	23	38	34	576

[a] Asci with spore displacement were included after adjustment and reclassification. Number of asci with spore displacement observed in each culture were: 17 in A22, 67 in R31, 65 in M35-1, 55 in R38-3, 59 in R39.
[b] L and S represent large and small ascospores, respectively.

TABLE 3. Description of single-ascosporic strains used in various experiments throughout this and successive studies

Original cultures	Host	Ascus[a]	Ascospore size[b] (from the apex of asci) 2[c]	4	6	8
A22	Alfalfa	1	L	S	L	S
R31	Red clover	8	L	L	S	S
M35-1	Milk vetch	14	L	L	S	S
R38-3	Red clover	67	L	S	L	S
R39	Red clover	85	L	L	S	S

[a] Serial number of dissected order.
[b] L and S represent large and small ascospores, respectively.
[c] 2, 4, 6, or 8 = ascospore isolated was second, fourth, sixth, or eighth from the apex of the ascus.

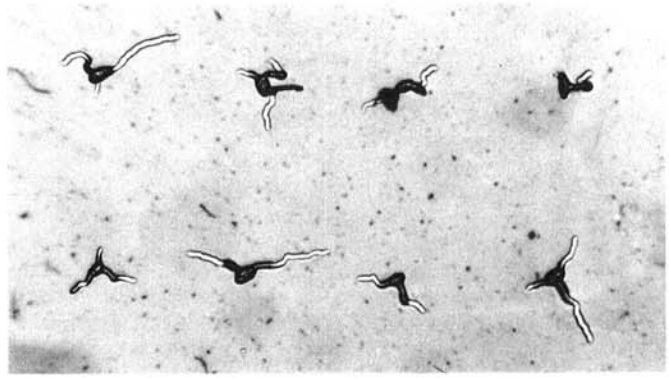

Fig. 2. Ascospore germination on water agar 12 hr after dissection of the asci. Germ tube elongation of large spores is slightly faster than that of small ones. First-division segregation pattern (top) and second-division segregation (bottom).

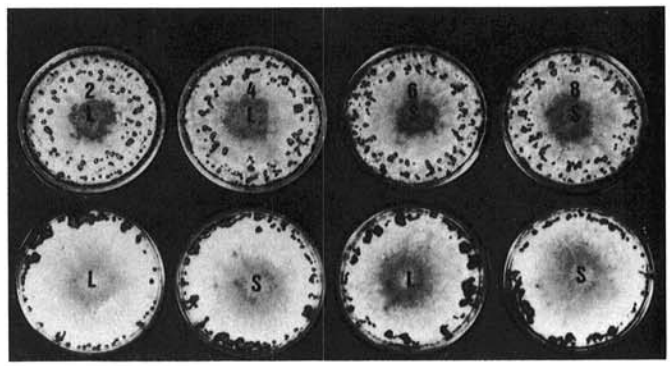

Fig. 3. Cultures of single-ascosporic strains derived from large (L) and small (S) ascospores on potato-dextrose agar at 20 C. Each tetrad from the same ascus is morphologically identical regardless of spore size.

TABLE 4. Pathogenicity on red clover of single-ascosporic strains of *Sclerotinia trifoliorum* derived from different sized spores

Strains[a]	Postinoculation symptoms[b] observed at: 3 days	4 days	5 days	6 days	7 days	8 days
1-2L	−	−	−	−	+	+
1-8S	+	++	++	+++	+++	+++
8-2L	−	−	−	+	+	++
8-8S	−	−	−	−	+	+
14-4L	−	−	−	+	+	++
14-6S	−	−	−	+	+	++
67-2L	−	+	+	++	++	+++
67-8S	−	−	−	+	+	++
85-2L	+	+	++	++	+++	+++
85-6S	+	+	++	++	+++	+++

[a] Each pair of strains was derived from one ascus. See Table 3 for origin of strains.
[b] − = No symptom; + = drooping of leaves and water-soaked lesion on the petiole; ++ = plants flaccid and white mycelia visible; +++ = sclerotia beginning to form.

Apothecial production. The five sets of tetrads shown in Table 3 were used in this experiment. Mature sclerotia from PDA cultures, 50 sclerotia per strain, were induced to form apothecia by the method previously described. A striking difference was found: all of the large-spore strains produced fertile apothecia within 2 mo, whereas the small-spore strains were all sterile. Sclerotia of the small-spore strains were examined for more than 1 yr thereafter; although no production of apothecia was observed, most of the sclerotia still produced hyphal growth when the black rinds were removed and the medullae were plated on PDA. Not a single exception was found in the tests to date of 114 single ascosporic strains isolated from 31 asci from various sources.

Segregation for spore size in asci of single large-spore strains. Since the spore size segregation was observed in asci of single-ascosporic strains, R31 and R39, in the previous examination, confirmation was needed to determine whether segregation was a general feature. The apothecia formed on single large spore strains mentioned in the previous section were utilized in this examination. In all mature asci, the 4:4 segregation of spore size was observed, and the patterns of spore arrangement were either of first- or second-division segregation with some asci showing spore displacements. As shown in Table 5, the frequency of each segregation type was essentially the same as that of the previous generation. The average frequency of second-division segregation (21.6%) did not deviate significantly from the previous examination. In addition, the frequency of the second-division segregations in each isolate did not differ significantly, as was seen in the previous examination.

In order to confirm the fertility or sterility of the single-ascosporic strains, 10 sets of tetrads, one from each isolate, were tested. All the single isolates derived from large spores were fertile, and those from small ones were sterile, as observed previously.

DISCUSSION

Carr (3) reported that ascospore dimorphism in *S. trifoliorum* was a result of heterokaryosis between two isolates from large and small ascospores, and therefore no segregation could be expected in the asci of homokaryotic cultures. Furthermore, he reported that in apothecia produced by heterokaryotic sclerotia, homozygous asci of large and small spores and heterozygous asci showing spore size segregation were present. Carr believed this fungus to be homothallic and interpreted segregation by spore size to be evidence of heterokaryosis. Results of the present study, however, confirmed that dimorphism in spore size was a constant feature; whenever an apothecium was formed, its asci showed segregation by spore size, regardless of whether the sclerotium was homokaryotic or heterokaryotic. Thus, Carr's observation was quite different from that of the present study.

The fact that patterns of spore arrangement fell into six principle types suggests that the spore size is determined by one gene. The six represent all possible ascus types of both first- and second-division segregation when two characters are controlled by a pair of alleles in a locus. In addition, the five original isolates did not differ significantly in second division segregation frequency. Second-division segregation is usually explained as a consequence of crossing over between the centromere and the locus for segregating allelic difference, and its frequency indicates the distance between the centromere and the allelic locus (5). Therefore, the constancy of second-division segregation frequency among the cultures suggests the presence of a common locus controlling spore size in all five isolates. Judging from these two findings, the spore size difference is obviously controlled by a single pair of alleles.

Spore size difference was strictly related to the fertility or sterility of single-ascosporic strains: single-ascosporic strains derived from large spores were invariably fertile, but those from small ones were completely sterile.

Even though homothallism has been reported in this fungus (2,3,6,7,11), it appeared that half of the single-ascosporic strains might have been sterile, since the small spores that gave rise to sterile cultures always segregated in a 1:1 ratio with large spores in each ascus. The presence of sterile strains among the offspring of single ascosporic strains was reported previously. When single strains were isolated randomly from the apothecia produced by single ascosporic strains, 10–30% of them were sterile (2), although the cause of the sterility was attributed to aberration during ascosporogenesis. Even though Björling (2) did not mention the relationship between sterility and spore size, those sterile strains might have been derived from the small ascospores.

The sterile strains, as demonstrated in this study, are the same as the fertile strains with respect to morphology, growth rates, and pathogenicity. Moreover, the initiation of such strains is under genetic control. In this respect, the sterility of a single small-spore strain may be regarded as due to self-incompatibility rather than to degeneration.

On the other hand, the single-ascosporic strains derived from large spores are fertile, but their asci again show the 4:4 segregation of large and small spores. If this fungus is homothallic, and therefore a clone after one generation of self-fertilization, it is impossible to explain the successive reappearance of small spores in the asci of single-ascosporic strains. Thus, further investigation of the mechanism of the self-fertility exhibited by single large-spore strains is needed, especially regarding the successive creation of the small-spore genotype as well as the sterility factor. These are discussed in a subsequent report (14).

TABLE 5. Frequency of six patterns of spore arrangement in the asci produced by single-ascosporic strains derived from large ascospores

Strains[a]	Frequency[b] of: LLSS[c]	SSLL	LSSL	SLLS	LSLS	SLSL	Total
1-2L	212	223	23	23	28	31	540
1-6L	208	200	18	21	25	32	504
8-2L	181	193	27	22	34	30	487
8-4L	183	206	23	18	36	38	504
14-2L	201	173	23	23	31	40	491
14-4L	187	208	28	28	32	31	514
67-2L	223	227	30	27	37	34	578
67-6L	220	241	23	26	38	31	579
85-2L	198	219	18	23	32	34	524
85-4L	203	238	27	25	36	37	566

[a] See Table 3 for origin of strains.
[b] Asci with spore displacement were included after adjustment and reclassification.
[c] L and S = large and small ascospores, respectively.

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