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Cytology and genetics of sexual incompatibility in *Didymella rabiei*

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Abstract: Mating crosses in all possible combinations between 15 monoascosporic isolates of *Didymella rabiei* (Ascomycotina, Dothideales), derived from diseased chickpea stems collected at a single location in Genesee, Idaho, were prepared on sterile chickpea stem pieces incubated on moist sterile filter papers in glass petri dishes for 5–6 weeks at 10 C or in nylon mesh bags placed on the soil surface outdoors to overwinter for 6 months. The discharge of large numbers of viable ascospores from mature pseudothecia forming on the chickpea stems was indication of successful matings. Pairings between sympatric isolates demonstrated that the fungus is heterothallic with a unifactorial (bipolar) homogenic mating incompatibility system. Papazian test pairings between tester strains and mating types from three locations in Idaho and Washington indicated the absence of complete interfertility between allopatric populations and provided strong evidence that the fungus is biallelic, lacking multiple alleles at the single mating locus. The nuclear content of somatic and reproductive cells at all phases of the life cycle was examined using Giemsa stain. Ascospores and conidia were commonly multinucleate and appeared to undergo multiple mitotic divisions prior to germination. Somatic hyphae derived from ascospores and conidia were predominately uninucleate. The importance of sexual reproduction, sexual incompatibility, and the teleomorph in the disease cycle is discussed relative to environmental factors required for their occurrence and implications for disease control.

Key Words: Ascochyta blight, chickpea, mating system, nuclear cytology, population genetics

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INTRODUCTION

Didymella rabiei (Kovachevski) Arx, the teleomorph of *Ascochyta rabiei* (Pass.) Labrousse, is a serious fungal pathogen of chickpea (*Cicer arietinum* L.) in many regions of the world (Nene and Reddy, 1987; Saxena and Singh, 1984). Chickpea is a major leguminous crop of countries in certain parts of southern Europe, Asia, the Middle East, and the northwestern United States. Ascochyta blight, among the most important diseases of chickpeas, can cause devastating damage and high economic losses when cool, wet weather occurs during the growing season. For example, the annual economic losses in the Palouse region of eastern Washington and northern Idaho alone may exceed U.S. $1 million (Kaiser and Muehlbauer, 1988). The disease, characterized by the formation of necrotic lesions on all aboveground plant parts at any stage of crop development, can lead to blight and eventually death of the plant in severe cases. Overwintering pseudothecia and pycnidia on previous-season crop residues produce primary inoculum in the spring that initiates isolated infection foci within the new crop. Pycnidia then form in large numbers on necrotic tissue, producing conidia for secondary infections when suitable wet conditions occur. The asexual phase repeatedly causes secondary disease cycles throughout the growing season. The number of cycles generally determines the rate and severity of disease development and spread.

The teleomorph was first discovered by Kovachevski (1936) on overwintering chickpea debris in Bulgaria and was subsequently reported on chickpea residues in Russia (Gorlenko and Bushkova, 1958), Greece (Zachos et al., 1963), Hungary (Kövics et al., 1986), Spain (Jiménez-Díaz et al., 1987), Syria (Haware, 1987), and the United States (Kaiser and Hannan, 1987). The anamorph *A. rabiei* was first recorded in North America in Saskatchewan, Canada (Morrall and McKenzie, 1974). The fungus, an exotic pathogen in North America, was presumably introduced in seeds of infested chickpea lines imported to assess the potential of chickpeas as a new leguminous crop for western Canada. The asexual stage was independently reported to have appeared in 1983 in Washington state (Kaiser and Muehlbauer, 1984) in the U.S. Pacific Northwest and in Ontario, Canada (Tu and Hall, 1984). The anamorph was found extensively in two Idaho counties a

year later (Derie et al., 1985). These unrelated reports collectively indicate that outbreaks of chickpea blight in North America resulted from plantings of *D. rabiei*-infested chickpea seed originating from Turkey, India, Bulgaria, Syria, and possibly other countries in Europe and Asia. The seed transmissibility of the pathogen (Halfon-Meiri, 1970; Kaiser and Hannan, 1988) and its ability to rapidly produce large amounts of inoculum are the most probable reasons why Ascochyta blight has become so widely distributed in areas throughout the world where chickpea is cultivated.

The transfer of some *Mycosphaerella* species, including *Mycosphaerella rabiei* Kovachevski, to *Didymella* by Müller and von Arx (1962), was supported previously by Corbaz (1956) and subsequently maintained by von Arx (1987). This transfer to *Didymella* is based on the larger ascomata of members in this taxon, the parallel arrangement of nonfasciculate asci, the presence of pseudoparaphyses, the broader unequal two-celled ascospores that are strongly constricted at the septum, and the different anamorph (*Ascochyta*) from those now associated with *Mycosphaerella*. Trapero-Casas and Kaiser (1992b) recently suggested that future references to the teleomorph should utilize the name *D. rabiei*, instead of *M. rabiei*, which is prevalent in the literature. A transfer of the anamorph from *Ascochyta* to *Phoma* also has been proposed (Khune and Kapoor, 1980), but this was not widely accepted. Descriptions of the anamorph were provided by Punithalingam and Holliday (1972a). More recent taxonomic reviews of the genus *Didymella* were presented by Corlett (1981), Holm (1975), and Sivanesan (1984).

The discovery of the importance of the teleomorph in long-distance dissemination, increasing genetic diversity in the pathogen population, and as overwintering inoculum has increased the need to determine the genetic factors controlling its formation and development. Prior studies have suggested that the fungus is heterothallic since two separate thalli were required for successful sexual reproduction (Trapero-Casas and Kaiser, 1992b). However, the genetic mechanisms controlling sexual incompatibility were unknown. An elucidation of the mechanism was essential to further understand the factors involved in the epidemiology and survival of this pathogen.

The objectives of this study were to determine the genetic system controlling sexual incompatibility, to investigate intercompatibility between allopatric isolates to assess whether multiple alleles exist for the factor(s) controlling mating incompatibility, and to examine the nuclear cytology of somatic and reproductive cells throughout the life history of this fungus. A preliminary report of this work has been published (Wilson and Kaiser, 1994).

MATERIALS AND METHODS

Isolation and culture.—Chickpea crop residues, consisting of stems and pods bearing lesions with pseudothecia and pycnidia of *D. rabiei*, were collected from three Ascochyta blight-infested fields in the Palouse region of eastern Washington and northern Idaho. The designations for these collections and their sources were as follows: WJK-GE 115 ID from a private farm near Genesee in Latah Co., Idaho; WJK-SP 204 WA from a field near Spangle in Spokane Co., Washington; and WJK-WW 306 WA from a farm near Walla Walla in Walla Walla Co., Washington. The specimens were collected in April 1990 when conditions were favorable for maturation of ascospores within pseudothecia that began development on chickpea debris the previous fall. Stems were cut into 6–12 cm segments, air-dried, and maintained at 4 C and 30–40% relative humidity until use. Representative specimens from the WJK-GE 115 ID collection, containing both anamorphic and teleomorphic stages of the pathogen, were deposited at the Washington State University Mycological Herbarium (WSP) in Pullman under accession number WSP 69540.

Isolates were obtained from chickpea stem sections 1.0–1.5 cm long with mature pseudothecia. Stem sections were split in half longitudinally and affixed to 1-cm^2 blocks of 2% water agar (WA) that were attached to the inner surface of a petri dish lid. The lid was inverted over the dish bottom which contained WA. Ascospores began discharging onto the water agar within 2 h after incubation at 21–23 C. Ascospore germination began within 6–10 h after discharge. Germinating ascospores were cut out and transferred individually to either Difco potato-dextrose agar (PDA) or chickpea seed meal-dextrose agar (CDA) medium. CDA consisted of 5% chickpea flour, 2% dextrose, and 2% agar (Trapero-Casas and Kaiser, 1992a). Fifteen single-ascospore isolates (GE 1–15) were selected from the WJK-GE 115 ID collection for pairings of sympatric isolates. In addition, eight single-conidiospore isolates of *A. rabiei*, designated (SP 1- SP 4) and (WW 2, 3, 5 and 6), were obtained from the WJK-SP 204 WA and WJK-WW 306 WA collections, respectively, for pairings of allopatric isolates. Monoconidial isolates were obtained by flooding a separate WA dish with 2 ml of a conidial suspension of each isolate prepared from oozing pycnidia, incubating for 24 h at 21–23 C, and transferring single germinating conidia individually to PDA or CDA. Cultures from single ascospores and conidia were incubated at 21–23 C under fluorescent lights with 12-h photoperiod. Subcultures on PDA and CDA slants, maintained at 4 C in the dark, were used for short-term storage of isolates.

Autoclaved chickpea stem segments were used for long-term storage. Sterile stem segments 5–6 cm long were incubated in a heavy spore suspension of each isolate for 30 min and then plated on WA at 21–23 C with a 12-h photoperiod for 2 wk. Colonized stem segments were dried in sterile petri dishes for 5 days and stored at −18 C. Cultures (ATCC 76501 and ATCC 76502) of representative mating types of *D. rabiei,* referred to as MAT1-1 and MAT1-2, respectively, were deposited with the American Type Culture Collection.

Mating system and intercompatibility studies.—Fifteen sympatric, monoascosporic isolates of *D. rabiei* derived from Genesee, Idaho collection WJK-GE 115 ID were paired in all possible combinations on blight-free chickpea stems. Stem pieces were collected from a chickpea field in Central Ferry, Washington, where no Ascochyta blight was found. The stem pieces were sectioned into 6 to 8-cm-long segments, sterilized with propylene oxide (Hansen and Snyder, 1947), and placed into 2.2 × 17.5-cm test tubes containing conidial suspensions (1.0 - 6.9 × 10^6 conidia/ml) prepared from either a single isolate (serving as controls) or mixtures of two isolates for each possible pairing combination. The stem segments, 4–6 per tube, were soaked in the conidial suspensions for 30 min, drained on sterile paper towels for 30 min, and placed on 10 sterile wet filter papers in 10-cm glass petri dishes. The filter papers were wetted with 15 ml of sterile distilled water prior to and during incubation as needed to maintain high humidity. Dishes were incubated for 24 h at 21–23 C and then at 10 C for 5–6 wk. The experiment was repeated with inoculated chickpea stem segments placed in 10-cm-square, 0.5-mm-mesh nylon bags stapled closed and placed on the soil surface at least 4 m apart in a field (Observatory Hill) near Pullman, Washington, in October 1990 to overwinter for 6 months. The nylon bags were covered with a large mesh screen to prevent dispersal of the bags by wind. The stem segments were removed from the bags in early April, washed free of any dirt particles and debris, and dried on clean paper towels. Small pieces from each segment were cultured on CDA to determine if they were colonized by other organisms in addition to *D. rabiei.* Each stem segment sampled also was examined under a stereoscope at 50× to distinguish between pycnidia and pseudothecia of *D. rabiei* using characters described previously (Trapero-Casas and Kaiser, 1992b). Macroscopic observations of mycelial growth on stem sections from compatible and incompatible pairings were noted for comparison. Stem segments with mature pseudothecia were affixed to the lids of 2% WA plates using methods described previously to discharge ascospores. Compatible pairings (+) between sympatric isolates were recorded only when large numbers of viable ascospores were discharged from mature pseudothecia. Incompatible pairings (−) were indicated when few or no viable ascospores were discharged.

Papazian test pairings (see Papazian, 1951) between 12 allopatric isolates, four isolates from each of the three collection sites (Genesee, Idaho; Spangle and Walla Walla, Washington), were prepared to determine the intercompatibility between isolates from separate geographical localities and to determine whether multiple alleles exist at the mating locus controlling sexual incompatibility. Four tester strains (GE- 1, 2 = A_1, and GE- 6, 8 = A_2) were selected from collection WJK-GE 115 ID and paired in all possible combinations with four isolates (SP 1- SP 4) from collection WJK-SP 205 WA and four isolates (WW 2, 3, 5, and 6) from collection WJK-WW 306 WA. Pairings were scored as either compatible or incompatible as in pairings of sympatric isolates.

Cytological studies.—The nuclear content of somatic and reproductive cells at all phases of the life cycle was examined using nuclear staining. Ascospores and conidia taken before and after germination along with hyphae derived from these spores were prepared on WA using methods described previously for single spore isolations. Spores were cut out on blocks of the WA, inverted onto slides, and flooded with filtered 0.025% Haupt's adhesive-fixative prepared in 4% formalin solution (Bissing, 1974). Reproductive structures within immature and mature pseudothecia, including ascogenous hyphae, pseudoparaphyses, asci initials, and mature asci with ascospores, were teased from ruptured ascomata on glass slides with the aid of a stereoscope (90×) and flooded with Haupt's adhesive-fixative. The slides were heated overnight on a slide warmer at 40–45 C. The specimens were subsequently stained using a permanent nuclear staining protocol with Giemsa stain (Wilson, 1992). The filtered Giemsa stock solution consisted of 0.76% (w/v) Giemsa stain prepared in 1:1, absolute methanol-glycerol solution and stored at 4 C in the dark. Numbers of nuclei in cells of each structure were recorded with observations of nuclear migration, nuclear volume, and septa formation to deduce the nuclear condition throughout the life cycle. Certain structures such as asci and ascospores were stained for comparison with 0.07% aqueous aniline blue containing 12% lactic acid and 14% glycerol to demonstrate some key taxonomic features and to determine the effects of the Giemsa protocol on the shapes and sizes of these structures.

	A_1									A_2				
	1	2	3	5	7	9	11	12	13	6	8	10	14	15
1	\	−	−	−	−	−	−	−	−	+	+	+	+	+
2	−	\	−	−	−	−	−	−	−	+	+	+	+	+
3	−	−	\	−	−	−	−	−	−	+	+	+	+	+
5	−	−	−	\	−	−	−	−	−	+	+	+	+	+
7	−	−	−	−	\	−	−	−	−	+	+	+	+	+
9	−	−	−	−	−	\	−	−	−	+	+	+	+	+
11	−	−	−	−	−	−	\	−	−	+	+	+	+	+
12	−	−	−	−	−	−	−	\	−	+	+	+	+	+
13	−	−	−	−	−	−	−	−	\	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	\	−	−	−	−
8	+	+	+	+	+	+	+	+	+	−	\	−	−	−
10	+	+	+	+	+	+	+	+	+	−	−	\	−	−
14	+	+	+	+	+	+	+	+	+	−	−	−	\	−
15	+	+	+	+	+	+	+	+	+	−	−	−	−	\

FIG. 1. Mating results for pairings between 14 monoascosporic isolates of *Didymella rabiei* from Genesee, Idaho collection WJK-GE 115 ID. Compatible pairings (+) indicate successful matings resulting in the formation of large numbers of viable ascospores that were discharged from pseudothecia. Incompatible pairings (−) indicate that few or no ascospores were discharged.

		GE				SP				WW			
		1	2	6	8	1	2	3	4	2	3	5	6
A_1	1	\	−	+	+	+	−	+	+	+	+	+	+
	2	−	\	+	+	+	−	+	+	+	+	+	−
A_2	6	+	+	\	−	−	+	−	−	−	−	−	+
	8	+	+	−	\	−	+	−	−	−	−	−	+
A_2	1	+	+	−	−	\	+	−	−	−	−	−	+
A_1	2	−	−	+	+	+	\	+	+	+	+	+	+
A_2	3	+	+	−	−	−	+	\	−	−	−	−	−
	4	+	+	−	−	−	+	−	\	−	−	−	−
A_2	2	+	+	−	−	−	+	−	−	\	−	−	−
	3	+	+	−	−	−	+	−	−	−	\	−	−
	5	+	+	−	−	−	+	−	−	−	−	\	−
$A_?$	6	+	−	+	+	+	+	−	−	−	−	−	\

FIG. 2. Mating results for pairings between allopatric isolates of *Didymella rabiei* from Genesee, Idaho (GE), Spangle, Washington (SP), and Walla Walla, Washington (WW). Tester strain genotypes are: A_1 = GE- 1, 2; A_2 = GE- 6, 8. Compatible pairings, (+); incompatible pairings, (−).

RESULTS

Mating system and intercompatibility studies.—Mating crosses in all possible combinations between 15 sympatric isolates of *D. rabiei* from collection WJK-GE 115 ID at Genesee, Idaho indicated that 14 of the 15 isolates belonged to only one of two mating types, A_1 or A_2 (FIG. 1). The genotype designations of mating types that could be assigned among the 15 isolates derived from the Genesee, Idaho (WJK-GE 115 ID) collection are as follows: A_1 = 1, 2, 3, 5, 7, 9, 11, 12, 13; A_2 = 6, 8, 10, 14, 15. Data derived from pairings on chickpea stems in vitro were in agreement with data obtained from pairings on chickpea stems that overwintered in the field. Isolations from chickpea stem segments on CDA indicated that they were almost exclusively colonized by *D. rabiei*. Control inoculations with inoculum derived from single ascospores showed that all 15 monoascosporic isolates were self-sterile with only pycnidia forming on chickpea stems. Similarly, all pairings between isolates of the same mating type were incompatible and negative for production of abundant viable ascospores within pseudothecia.

Compatible pairings generally produced large numbers of ascospores that germinated normally to produce viable cultures. However, some crosses that were considered negative resulted in the production of small numbers of ascospores that were discharged from apparently typical pseudothecia, but which formed few asci within. No macroscopic interactions were observed in mycelia forming on chickpea stems that could be used to distinguish compatible from incompatible crosses. The results of these pairings were consistent with the mating pattern typical of a unifactorial (bipolar) mating incompatibility system. However, isolate GE-4 was aberrant and did not conform to this pattern. GE-4 was compatible with four of nine isolates from mating type A_1 (GE- 7, 11, 12, 13) and two of six isolates from mating type A_2 (GE- 6, 10). Consequently, the mating-type allelomorph of this isolate could not be determined.

Intercompatibility pairing tests between allopatric isolates, representing mating types from three geographically separated populations of *D. rabiei* in Washington and Idaho, indicated that isolates from these three locations shared identical alleles at the mating incompatibility locus (FIG. 2). Complete intercompatibility was not observed in pairings between tester strains or representative mating types from any two locations. This provides strong evidence that *D. rabiei* does not possess multiple alleles at the single mating locus. However, a few pairings with aberrant isolate WW-6 gave inconsistent and unexpected results. Iso-

TABLE I. Allelomorphs of mating types for isolates of *Didymella rabiei* from Genesee, Idaho, Spangle, Washington, and Walla Walla, Washington

Mating type allelomorphs	Isolates		
	Genesee (GE)	Spangle (SP)	Walla Walla (WW)[a]
A_1	1, 2	2	—
A_2	6, 8	1, 3, 4	2, 3, 5

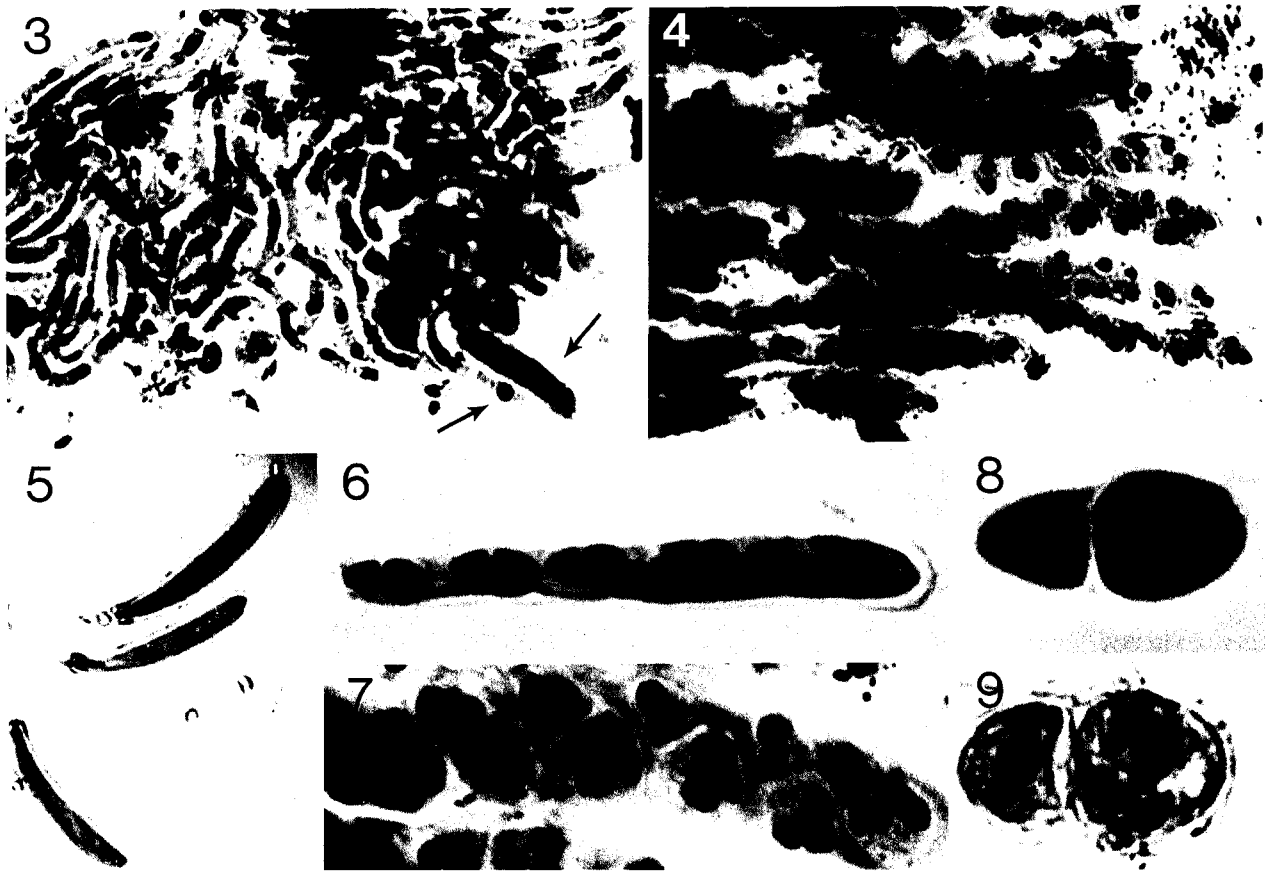
[a] Isolate WW-6 was aberrant since it was compatible with tester strains GE-1 and SP-2 (A_1); and with GE-6, 8, and SP-1 (A_2).

late WW-6 was compatible with two isolates from mating type A_1 (GE- 1 and SP- 2) and three isolates from mating type A_2 (GE- 6, 8 and SP- 1). Isolate WW-6 also was incompatible with the other three isolates (WW- 2, 3, 5) from the same location. This conflicting information precluded designation of an allelomorph for the mating locus in this isolate. Genotypes that were designated for mating types of the remaining isolates are provided in TABLE I. The majority of the data from the intercompatibility crosses was consistent with a unifactorial incompatibility system.

Cytological studies.—The nuclear cytology of events leading up to meiosis, asci formation, and ascospore production in *D. rabiei* were typical of those observed in other ascomycetes. An abundance of nuclei were observed in ascogenous hyphae within the hymenium. Ascogenous hyphae gave rise to ascomal initials that often apparently contained a single diploid nucleus based on nuclear volume (FIG. 3). This was followed by the formation of multiple, much smaller nuclei presumably after meiosis. Asci were produced in a parallel arrangement perpendicular to the hymenium (FIG. 4). Asci became partially elongated prior to meiosis (FIG. 5). Mature pseudothecia were uniformly devoid of pseudoparaphyses, which tended to be evanescent during early ascomal formation. Eight ascospores normally were produced within each papillate, bitunicate ascus (FIG. 6). The unevenly two-celled ascospores were often multinucleate (FIG. 7). The majority of ascospores had one or two nuclei per cell immediately after formation, but tended to become increasingly multinucleate with predominantly two to four nuclei per cell as the germination process approached. This is consistent with the hypothesis that multiple mitoses occur prior to germination. The larger of the two cells generally contained more nuclei than the smaller cell in mature ascospores just before germination. The smaller cells typically contained one to two nuclei compared with three to four in the larger cells. The relative frequency of ascospore cells containing specified numbers of nuclei (one to four) is presented in TABLE II. Discharged ascospores stained with aniline blue demonstrated the constriction at the septum, clear cell walls, and somewhat more pointed cells (FIG. 8) than were observed with Giemsa-stained ascospores (FIG. 9). The effects of dehydration and osmotic changes during the Giemsa staining procedure appeared to cause the cells of ascospores to assume a more rounded shape compared to prestained cells and cells stained with aniline blue under aqueous conditions.

The nuclear events during developmental stages of ascospore germination are shown in photomicrographic sequence (FIGS. 10–15). Multinucleate ascospores (FIG. 10) initiated the germination process with germ tube formation (FIG. 11) presumably following multiple mitoses. One to several nuclei migrated through the germ tube, began mitotic divisions, and initiated control of septa formation (FIGS. 12, 13). One to several nuclei often remained in the spore. Some ascospores simultaneously germinated from both cells giving rise to two germ tubes (FIG. 14). The two cells of ascospores usually had equal or nearly equal numbers of nuclei per cell prior to germination when simultaneous germination followed at equal rates from both cells. Other ascospores germinated at uneven rates from the two cells with one germ tube developing much more rapidly than the other (FIG. 15). When uneven rates of germination were observed from the two cells, germ tubes generally tended to elongate much more rapidly from the larger cell of the ascospore that contained larger numbers of nuclei per cell than the smaller ascospore cell which contained fewer nuclei prior to germination. The cells resulting from septa formation in the germ tube were sometimes binucleate, although the uninucleate condition predominated. Some cells close to the growing tip of the elongating germ tube, as well as subsequently formed hyphal tips, contained three or more nuclei prior to septa formation. However, hyphal cells were increasingly uninucleate with distance from the spore and as new septa were laid down. Consequently, somatic hyphae derived from ascospores were predominantly uninucleate, except near hyphal tips immediately following mitoses, but before septa formation.

The patterns of nuclear divisions, nuclear migrations, and septa formation observed in sequential developmental stages (FIGS. 16–20) of conidiospore germination were very similar to those of ascospores. The one- and two-celled conidia contained predominantly uninucleate and binucleate cells prior to germination (TABLE II), although three and four nuclei were observed in some conidia. Conidia were mostly binucleate immediately prior to germination (FIG. 16) and during germ tube formation following presumptive mitoses (FIG. 17). Some nuclei migrated into the germ



FIGS. 3–9. *Didymella rabiei.* Nuclear staining and morphology of sexual reproductive structures of *D. rabiei.* 3. Ascogenous hyphae in hymenium of mature pseudothecium with ascomal initials (arrows) containing large diploid nucleus and small multiple nuclei. × 3590. 4. Mature parallel asci with multinucleate ascospores. × 2350. 5. Immature bitunicate asci prior to meiosis. × 2470. 6. Papillate, bitunicate ascus containing eight two-celled ascospores. × 2010. 7. Multinucleate ascospores within single ascus. × 8820. 8. Discharged two-celled ascospore constricted at the septum. × 5980. 9. Single multinucleate discharged ascospore. × 5920. FIGS. 6, 8 structures stained with aqueous aniline blue; all others stained with Giemsa stain.

tube, while others remained in the spore (FIG. 18). Most conidia appeared to germinate from only one of the two cells in two-celled conidia and from only one end of the spore in one-celled conidia. The multiple germ tubes characteristic of ascospores were rare in conidia. Germ tubes produced both binucleate and multinucleate cells (FIG. 19), but uninucleate cells were most abundant. Higher numbers of nuclei were concentrated near the growing tip of the germ tube (FIG. 20). Hyphae derived from conidia consistently produced more uninucleate cells than in germ tubes, but binucleate cells were occasionally found as in hyphae from ascospores. Hyphae from conidia also were increasingly uninucleate with distance from the spore, except near hyphal tips, consistent with observations of hyphae from ascospores.

TABLE II. Numbers of nuclei found in ascospores, conidia, germ tubes, and hyphae of *Didymella rabiei*

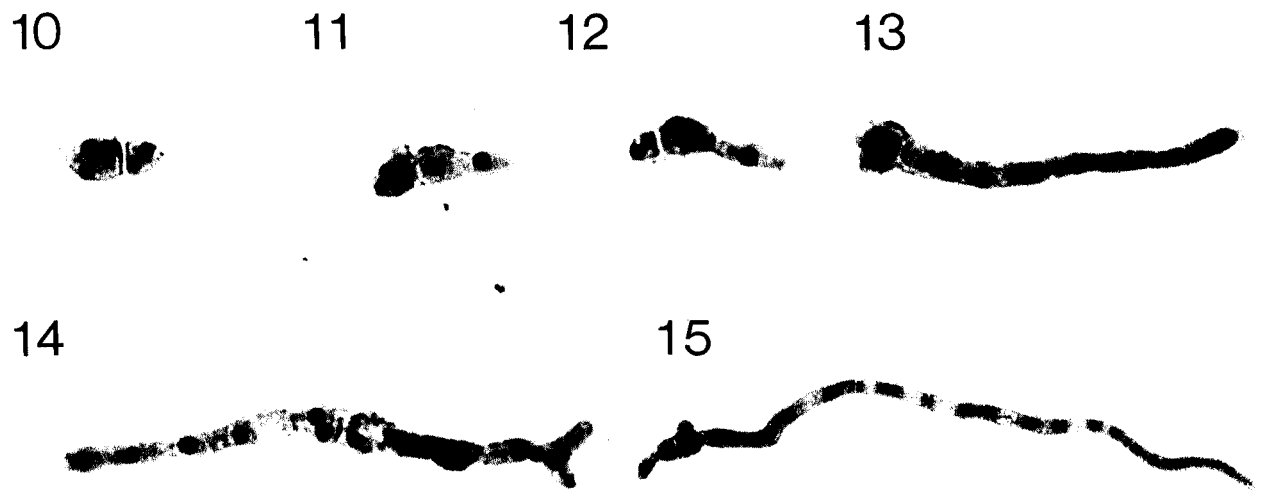
	No. of structures with indicated no. of nuclei per cell[b]							
	Ascospores				Conidia			
Structure[a]	1	2	3	4	1	2	3	4
Spore	44	109	46	47	144	201	38	11
Germ tube	146	77	24	9	126	76	9	7
Hyphae	186	16	0	0	191	9	0	0

[a] Nuclei in spores, germ tubes and hyphae derived from each respective spore type.

[b] Numbers of structures with one to four nuclei in each cell (observations on a per cell basis).

DISCUSSION

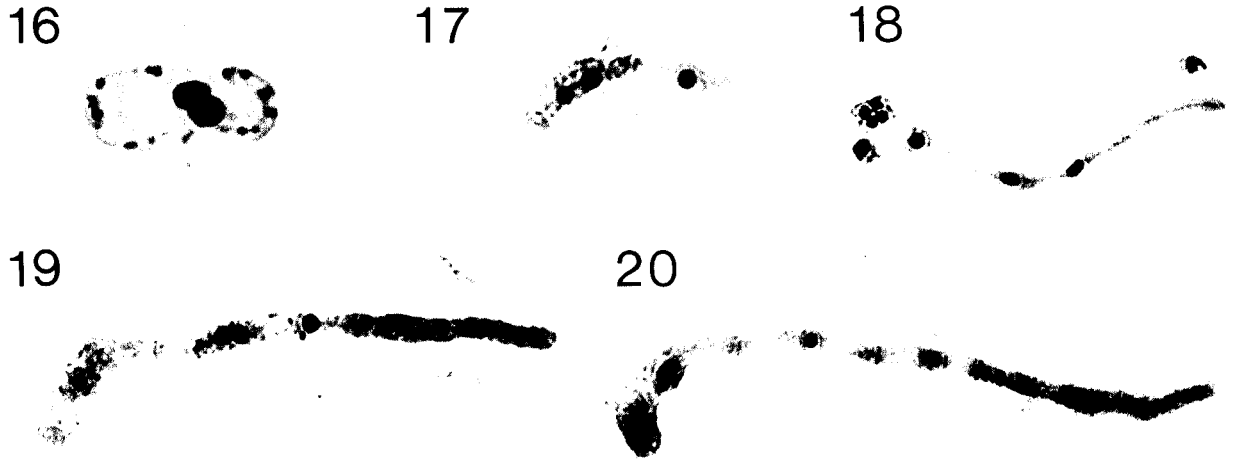
Mating crosses in all possible combinations between 15 sympatric, monosporous isolates of *D. rabiei* from Genesee, Idaho demonstrated that the fungus is heterothallic with sexual incompatibility controlled by a unifactorial (bipolar) homogenic mating system. This



FIGS. 10–15. *Didymella rabiei.* Nuclear cytology of ascospores at sequential stages of the germination process. 10. Multinucleate ascospore prior to germination. × 2600. 11. Ascospore initiating germ tube formation following multiple mitoses. × 2690. 12. Migration of nucleus into germ tube. × 2530. 13. Uninucleate cells within germ tube. × 2360. 14. Ascospore germinating from both cells with uninucleate and multinucleate germ tubes. × 2630. 15. Ascospore germinating at both ends with binucleate and multinucleate cells of germ tube. × 1440. All structures stained with Giemsa stain.

pattern of sexuality is consistent with those of most other heterothallic ascomycetes (see Esser, 1971), but contrasts with homothallism that appears to predominate in other *Didymella* species (Chiu and Walker, 1949; Corbaz, 1956; Punithalingam and Holliday, 1972b; Walker and Baker, 1983). These results confirm the preliminary results reported by Trapero-Casas and Kaiser (1992b) who concluded that *D. rabiei* is heterothallic.

Some crosses that appeared partially compatible based on the production and discharge of small numbers of ascospores were considered incompatible because this result occurred randomly and sporadically with no ostensible genetic basis for regulation. Pseudothecia forming occasionally in these incompatible crosses developed few asci. Since only pairings that resulted in the production of large numbers of viable ascospores were considered indicative of truly com-



FIGS. 16–20. *Didymella rabiei.* Nuclear cytology of conidia at sequential stages of the germination process. 16. Binucleate, single-celled conidium prior to germination. × 6920. 17. Binucleate conidium at early stages of germination with uninucleate cell of germ tube. × 2580. 18. Multinucleate conidium adjacent to binucleate conidium with elongating, uninucleate cells of germ tube. × 2540. 19. Two-celled conidium with binucleate and multinucleate cells of germ tube. × 2540. 20. Two-celled conidium with uninucleate cells of germ tube. × 2300. All structures stained with Giemsa stain.

patible crosses, determining compatibility necessarily required discharge and quantification of viable ascospores produced from each pairing. Unfortunately, no visible macroscopic interactions were observed that could distinguish between compatible and incompatible pairings. Fertile crosses with aberrant isolates GE-4 and WW-6, exhibiting compatibility with strains of both mating types, were attributed to: 1) accidental inoculations of chickpea stem segments with mixtures of mycelia carrying different mating types, 2) mating-type switching (see Perkins, 1987), or possibly, 3) one or more intrafactor mutation(s) or recombinations at the "A" mating incompatibility locus (see Kniep, 1923; Raper, 1966).

Interfertility tests using Papazian test pairings between tester strains and mating types of *D. rabiei* from three geographically separated localities in Idaho and Washington provided incompatibility results that were very similar to those found in crosses of sympatric isolates with only two mating types identified. The absence of complete interfertility between these three allopatric populations indicates that the fungus is biallelic, lacking multiple alleles at the single mating incompatibility locus. These results are supported by results of similar crosses, exhibiting incomplete interfertility, between tester strains from Idaho (USA) and mating types from Spain, Syria, and Turkey (Trapero-Casas and Kaiser, 1992b). The absence of multiple alleles at the mating locus for *D. rabiei* is consistent with observations of other unifactorial ascomycetes, which appear to universally lack multiple allelomorphs, in contrast to unifactorial basidiomycetes (Raper, 1966). If multiple alleles had existed, they could have been useful in tracing the origins (source countries) of inoculum that was imported into the Pacific Northwest. The known sources of *A. rabiei*-infested chickpea seed that were believed to have given rise to Ascochyta blight outbreaks in the USA and Canada suggest that the imported inoculum probably originated from either a Middle Eastern or south Asian country where the fungus is endemic. Nevertheless, the precise origin(s) could be determined more reliably and with greater certainty using other genetic techniques, such as RFLP analysis and RAPD markers.

The patterns of mitotic nuclear divisions prior to germination, nuclear migrations into germ tubes, and septa formation following germination of ascospores and conidia were similar. Both ascospores and conidia were commonly multinucleate immediately prior to germination. This appeared to occur through multiple mitotic divisions that increased the numbers of nuclei within the spores as germination approached. These presumptive multiple mitoses may serve to provide sufficient numbers of additional nuclei to control cellular functions for all of the new cells rapidly forming after germination, facilitating rapid development of a hyphal network. Control of the numbers of nuclei available for this purpose seem to be regulated by the maintenance of some nuclei within the spore as germination progressed and through septa formation. The need for more nuclear control during the germination process is also supported by observations that numbers of nuclei are correlated with cellular volume. Higher numbers of nuclei often were observed in spore cells containing larger volume. For example, the larger of the two cells of ascospores often had four nuclei compared with two nuclei in the smaller cell. There also tended to be different rates of germination from the two unequally sized cells of ascospores. Germ tubes usually formed more rapidly from the larger cell containing more nuclei prior to germination than from the smaller cell with fewer nuclei. Conidiospores usually only germinated from one cell even when two-celled.

Some of the multiple nuclei migrated into the germ tube following germination of ascospores and conidia to produce primarily uninucleate and binucleate cells with a multinucleate condition close to the hyphal tips. However, this multinucleate condition gradually disappeared in hyphae with increasing distance from the spore as regulation of septa formation began maintaining the uninucleate condition. Consequently, somatic hyphae derived from ascospores and conidia were predominately uninucleate, and presumably haploid, since the haploid condition generally is expected to predominate in a heterothallic species with unifactorial mating incompatibility, although multinucleate somatic cells are found in many ascomycetes.

The dehydration steps in the Giemsa staining procedure appeared to have caused slight changes in the shape and sizes of *D. rabiei* spores on permanent slides. One should be aware of this potential osmotic effect to avoid possible errors that could occur if these slides were to be used for taxonomic or descriptive purposes. These applications of permanent Giemsa slides are not recommended.

Ascochyta blight usually spreads within chickpea fields by rain-splashed conidia from infection foci created by primary inoculum during intermittent wet spring weather. Until recently, pycnidiospores were considered the only primary inoculum (Nene and Reddy, 1987). The potential importance of ascospores as primary inoculum was first recognized in the Palouse region when the teleomorph, which occurred throughout the region in 1986, appeared to be responsible for establishing new infection foci in chickpea fields and contributing significantly to the epidemiology of Ascochyta blight (Kaiser and Hannan, 1987). Observations in subsequent years have shown that the sexual stage consistently develops and discharges ascospores

from pseudothecia on overwintered chickpea debris during wet spring months following cool, moist winters.

Elucidation of the environmental conditions required for formation of the teleomorph was essential for understanding and establishing the potential importance of the sexual state. Previous work established that high moisture availability was the most essential factor for saprophytic growth and induction of pseudothecial development of *D. rabiei* on infested chickpea debris, but cool temperatures at 5–10 C for extended periods of time (5–7 months) were required for maturation of most pseudothecia and ascospores (Trapero-Casas and Kaiser, 1992b). Other well-known phytopathogenic *Didymella* species, including *D. bryoniae, D. ligulicola,* and *D. pinodes,* do not have these requirements (Chiu and Walker, 1949; Punithalingam and Holliday, 1972b; Walker and Baker, 1983). Light was not required for development of pseudothecia in vitro and had little influence on the quantity of ascospores discharged (Trapero-Casas and Kaiser, 1987). However, certain growth factors present in chickpea debris were required for induction and maturation of the teleomorph of *D. rabiei,* since pseudothecia could not be induced to form solely on nutrient agar media in petri dishes (Trapero-Casas and Kaiser, 1987). All of these conditions are often present during the wet fall and cool winter months common to the Palouse region of the Pacific Northwest. The requirements of specific environmental conditions for formation of the teleomorph may explain why the sexual stage rarely occurs in many drier chickpea-growing regions of the world (Kaiser, 1973; Nene and Reddy, 1987), and why the teleomorph may be important only in regions of the world where chickpea residues between crops are exposed to cool moist climates. Most field reports from other countries have indicated that mature pseudothecia formed only on overwintered chickpea debris (Gorlenko and Bushkova, 1958; Haware, 1987; Jiménez-Díaz et al., 1987; Kovachevski, 1936; Kövics et al., 1986). Zachos et al. (1963) reported that mature pseudothecia remained functional, discharging ascospores for 1 yr on chickpea debris in a region of Greece with lower rainfall and milder winter conditions. Ascospores were discharged from pseudothecia only during the spring in the Palouse (Trapero-Casas and Kaiser, 1992b).

The identification of two mating types in two of three fields examined in this study and the regular occurrence of conditions required for formation of the teleomorph suggest that the sexual stage could play a significant role in the disease cycle in the Palouse region of Washington and Idaho. The presence of the sexual state could greatly increase the difficulty of controlling the disease in several ways. First, the extensive development of pseudothecial initials on chickpea crop residues remaining on the soil surface after harvest, during cool wet fall and winter months, followed by the production and release of large numbers of ascospores from mature pseudothecia in the spring provides an abundance of airborne ascospores that may serve as primary inoculum for establishing new infection foci in the succeeding chickpea crop. Ascospores also may provide an additional means of long-distance spread to other fields over 8 km from blighted source fields (Kaiser and Muehlbauer, 1988). The problem of primary inoculum forming on overwintering crop residue is exacerbated by the recent use of minimum tillage practices by many farmers in the Palouse to conserve moisture and reduce soil erosion. Previously, farmers practiced deep ploughing which buried infested chickpea debris remaining on the soil surface, causing the fungus to lose viability in 2–3 months (Trapero-Casas and Kaiser, 1987). Furthermore, sexual reproduction increases the possibility that the pathogen will develop new pathotypes, through genetic recombinations, that may overcome host resistance or have increased resistance to fungicides. Unifactorial sexual incompatibility may further contribute to the generation of new pathotypes in the field by promoting the union of compatible mating types with different virulence factors to form new unique combinations of pathogenic genotypes.

Control measures that take into account the presence of the sexual stage should include the use of deep ploughing or field burning, crop rotation, or holding the land in fallow for 1 yr to prevent carryover of inoculum in chickpea residues to the next crop. Since new infection foci may arise from inoculum in infested seed, an integrated approach to disease control also should incorporate other cultural practices, such as planting with pathogen-free seed and utilizing resistant cultivars to reduce the accretion of inoculum potential.

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