Factors Affecting Growth and Sporulation of *Ascochyta fabae* f. sp. *lentis*

WALTER J. KAISER, Research Plant Pathologist, and RICHARD M. HANNAN, Research Horticulturist, USDA-ARS, Western Regional Plant Introduction Station, Washington State University, Pullman 99164-6402; and JACK D. ROGERS, Professor, Department of Plant Pathology, Washington State University, Pullman 99164-6430

ABSTRACT

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Isolates of *Ascochyta fabae* f. sp. *lentis* from seed of lentil (*Lens culinaris*) collected from different countries varied greatly in growth, sporulation, colony appearance, and morphology. Optimum conidial production occurred on 5% lentil seedmeal agar and natural potato-dextrose agar (PDA), whereas optimum linear growth occurred on natural PDA or oatmeal agar. Optimum temperatures for linear growth and sporulation were 15–20 C and 15–25 C, respectively. Isolates did not sporulate at 30 C. In continuous light, sporulation of all isolates was three to 10 times greater than in darkness, but linear growth in continuous light was greater in only two of three isolates. Zonation (alternation of zones of mycelial and pycnidial production) did not occur in continuous light or continuous dark but was pronounced when some isolates were exposed to alternating light and dark. Conidia usually had one to three septa, but frequency distributions for septation number differed greatly among 13 isolates. The proportion of conidia with one septum ranged from 67 to 95%. Conidia with multiple septa were longer but not wider than conidia with one septum. Conidia with the same number of septa varied little in size, but multiseptate conidia were significantly longer than those with one septum. Conidiogenous cells, which were morphologically simple, lined the pycnidial cavity and proliferated percurrently. Conidia can originate and secede at the same level as, or at a higher or lower level than, the previous conidium. The pathogenicity of 24 isolates of *A. f. lentis* from 17 countries was tested on two commercial lentil cultivars. All isolates were pathogenic, producing necrotic lesions on leaves, petioles, and stems of inoculated plants.

Ascochyta blight, caused by *Ascochyta fabae* Speg. f. sp. *lentis* Gossen, Sheard, Beauchamp, & Morrall (= *A. lentis* Vassiljevsky) (2,3), is one of the most important foliar diseases of lentil (*Lens culinaris* Medik.) worldwide (11). The pathogen infects all aerial parts of the lentil plant, including seeds, and adversely affects plant growth, yield, and seed quality (10).

The lentil germ plasm collection of the U.S. Department of Agriculture is maintained at the Western Regional Plant Introduction Station in Pullman, Washington. There are presently 2,261 Plant Introduction (PI) accessions from 41 countries in the collection. Seeds are stored at 4 C and 30–35% RH. Periodically, lentil accessions are grown in a field near Pullman when seed supplies are depleted or germination decreases.

In 1986, *A. f. lentis* was reported from the original seeds of 46 PI lentil lines from 16 countries in the Pullman collection (6). The fungus was the most prevalent and potentially important seedborne pathogen isolated from seeds in this study. In another study, the blight fungus survived in infected lentil seeds after storage for 4 yr at temperatures from −196 C (liquid nitrogen) to 20 C, and storage at these different temperatures did not adversely affect survival or pathogenicity to lentil (7). The pathogen was isolated from seeds of several lentil PI accessions that had been in cold storage since 1948.

In our studies with *A. f. lentis*, we have observed that isolates of the fungus from different countries vary greatly in cultural characteristics (Fig. 1). The objectives of the present study were to clarify aspects of the morphology, cultural characteristics, and pathogenicity of isolates of the pathogen from several countries.

MATERIALS AND METHODS

Seeds of lentil were surface-disinfested in 0.25% NaOCl for 5 min, dried on paper towels, and plated on 2% water agar (WA) in 10-cm-diameter glass petri dishes. Dishes were incubated at 20–24 C under fluorescent lights (12-hr photoperiod, 77 $\mu E \cdot m^{-2} \cdot s^{-1}$). Hyphal tips of fungi growing from seeds were transferred to Difco potato-dextrose agar (PDA) in tube slants and dishes. Single-spore cultures of all isolates of *A. f. lentis* were prepared with conidia collected from oozing pycnidia on naturally infected seeds or from sporulating colonies on PDA. Spores were diluted in sterile distilled water and spread on 2% WA. Single-spore isolates were stored on PDA slants at 4 C in the dark.

To study the effects of culture medium, temperature, and light on linear growth

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and sporulation, three isolates of *A. f. lentis* that varied greatly in cultural characteristics were selected from 24 isolates from 17 countries. They were isolated from lentil seed of PI 174873 from India, PI 345631 from Russia, and PI 300254 from Syria.

Growth and sporulation of these isolates were observed on different Difco Bacto agar media, lentil seedmeal agar (LSMA) (50 g of lentil flour and 20 g of agar in 1 L of distilled water), and natural PDA. Natural PDA was prepared by steeping 250 g of freshly peeled potatoes in 1 L of distilled water at 60 C for 1 hr. The liquid was filtered through four layers of gauze, 20 g of dextrose and 20 g of agar were dissolved in it, and the medium was adjusted with distilled water to 1 L. Media were dispensed into glass petri dishes (20 ml of medium per 10-cm-diameter dish) and were inoculated with 3-mm-diameter plugs of mycelium-infested agar cut from the edge of an actively growing colony. Three replicate dishes per isolate on each medium were incubated at 20 C in the dark in a completely randomized design. The experiment was repeated twice. Final measurements of linear growth and sporulation were made after 13 days. Conidial production after 13 days was determined by cutting entire colonies into quarters and placing them into flasks with a measured volume of water. The flasks were placed on a shaker (100 rpm) for 15 min, and spores from three colonies per isolate per growth medium were counted individually in a hemacytometer. Analysis of variance (ANOVA) was performed on the pooled data from the two experiments, and they were compared further with Duncan's new multiple range test on means of colony diameter and sporulation per colony.

Effects of constant temperatures on linear growth and sporulation were determined for the Indian, Russian, and Syrian isolates on natural PDA in the dark. Linear growth and sporulation after 13 days were measured for six temperatures between 5 and 30 C following the procedure and experimental design outlined above. This experiment was conducted twice, and statistical comparisons were made based on the mean conidial counts and colony diameters for each temperature increment of 5 C.

Effects of light and dark on linear growth and sporulation of the three isolates were also tested on natural PDA at 20 C. Cultures were exposed to continuous light (87 $\mu E \cdot m^{-2} \cdot s^{-1}$) or incubated in continuous darkness. Linear growth and sporulation were measured and data from two experiments analyzed following the procedures outlined above, except that the experiment was terminated after 9 days. In a separate experiment to study the effect of light on zonation (alternation of zones of mycelial and pycnidial production), colonies were exposed to 12 hr of alternating light (87 $\mu E \cdot m^{-2} \cdot s^{-1}$) and dark.

Conidia from 13 isolates of *A. f. lentis* from different countries were measured. Isolates of the fungus were cultured on lentil seed agar (three dishes per isolate). Twenty autoclaved lentil seeds were covered with 20 ml of 2% WA in 10-cm-diameter petri dishes. A single conidium was placed in the center of each dish, which was incubated at 15 C in the dark. After 10 days, conidia were collected randomly from profusely sporulating areas of growth above lentil seeds. Conidial suspensions were stained in 1% cotton blue-lactophenol, and conidial length, width, and septation frequency were measured on 510–549 conidia. The resulting data were subjected to a simple

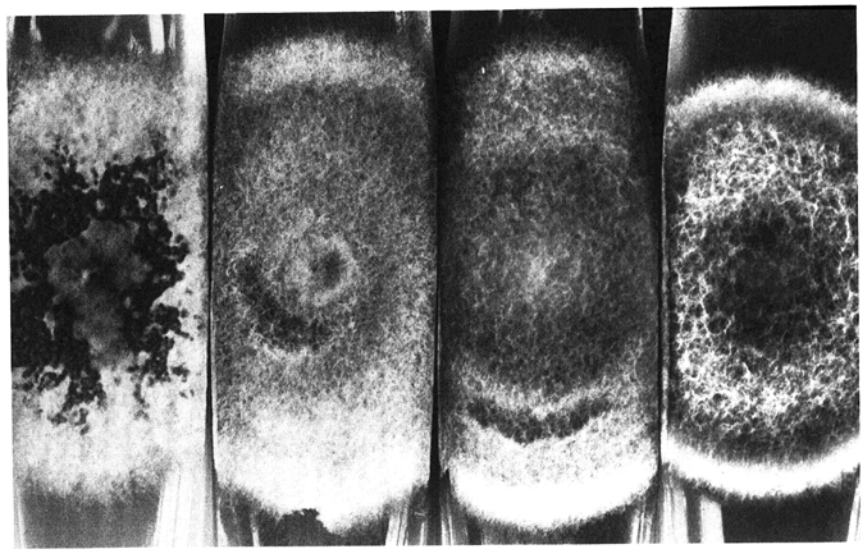


Fig. 1. Variation among single-spore isolates of *Ascochyta fabae* f. sp. *lentis* from (left to right) Morocco, Russia, Turkey, and Canada on potato-dextrose agar after 13 days at 20 C in the dark.

Table 1. Measurements of septation frequencies and dimensions of conidia of 13 isolates of *Ascochyta fabae* f. sp. *lentis*[w]

		Percent[x] and dimensions (μm)[y]								
		One septum			Two septa			Three septa		
Isolate	Origin	%	Length	Width	%	Length	Width	%	Length	Width
PI 299166	Chile	67	16.2	3.8	23	18.7	3.7	10	21.3	4.1
PI 471916	Canada	82	15.5	3.4	14	17.8	3.6	4	18.8	3.8
PI 438517	Turkey	84	14.1	3.4	11	17.9	3.7	5	19.5	3.7
PI 174873	India	90	15.1	3.9	9	17.9	3.8	1	20.0	3.8
PI 298924	Italy	91	14.7	3.7	8	17.5	3.9	1	20.3	4.1
PI 289076	Hungary	91	16.1	3.5	8	17.6	3.6	1	19.5	3.7
PI 300254	Syria	92	15.8	3.5	7	18.8	3.7	1	20.4	3.9
PI 374120	Morocco	92	15.2	3.4	6	17.2	3.6	2	19.8	4.0
PI 283608	Australia	93	14.4	3.2	6	18.6	3.7	1	20.6	4.1
PI 297779	Greece	94	14.3	3.5	5	17.7	3.9	1	18.8	3.9
PI 345631	Russia	94	15.5	3.7	5	18.0	3.6	1	20.2	3.8
PI 298644	Spain	95	15.3	3.5	4	17.1	3.7	1	19.6	3.9
PI 273644	Ethiopia	96	14.2	3.6	4	16.5	3.9		ND[z]	ND

[w] Data for length and width are means of 129–151 observations per colony with three colonies per isolate. Data for percent septations are means of 510–549 conidia per colony with three colonies per isolate. Fungal isolates were cultured on lentil seed agar at 15 C in the dark for 10 days.

[x] Differences among isolates for frequency percent within a given septation group were significant at $P = 0.05$.

[y] Differences among isolates for conidial dimensions within a given septation group were not significant according to F test.

[z] ND = no data; too few conidia with three septa were observed to make measurements.

Table 2. Effect of nutrient media on growth and sporulation of three isolates (PI 174873 from India, PI 300254 from Syria, and PI 345631 from Russia) of *Ascochyta fabae* f. sp. *lentis*[x]

Nutrient medium[y]	Concentration (%)	Mean colony diameter (mm)			Mean conidial production (conidia/ml × 10^6)		
		PI 174873	PI 300254	PI 345631	PI 174873	PI 300254	PI 345631
Potato-dextrose agar (natural)	...	71.3 a[z]	53.0 b	65.7 a	123.2 a	9.4 ab	1.8 cd
Oatmeal agar	7.2	68.3 b	55.3 b	63.3 b	39.4 b	20.8 ab	3.0 bc
Lima bean agar	2.3	64.7 c	49.3 c	60.0 c	4.6 d	10.4 ab	3.9 b
Potato-dextrose agar	3.9	63.3 c	42.3 d	60.3 c	46.5 b	16.3 ab	3.1 bc
Lentil seedmeal agar	5.0	61.3 c	42.0 d	49.0 f	19.5 c	24.5 a	6.8 a
Malt agar	4.5	55.0 e	52.0 b	57.0 d	13.3 c	7.7 ab	2.3 bcd
Cornmeal agar	1.7	49.3 f	31.3 e	53.7 e	2.3 d	2.3 b	1.6 cd
Czapek solution agar	4.9	40.0 g	33.0 e	49.3 f	1.5 d	1.0 b	1.3 d

[x]Cultures were incubated in glass petri dishes (10-cm-diameter) at 20 C in the dark for 13 days, then three colonies per isolate were measured. The experiment was repeated twice.
[y]Natural PDA was made from 250 g of potatoes, 2% dextrose, and 2% agar, and lentil seedmeal agar was made from 50 g of lentil flour and 2% agar. All other media were Difco products.
[z]Numbers in each column followed by the same letter are not significantly different according to Duncan's new multiple range test ($P = 0.05$).

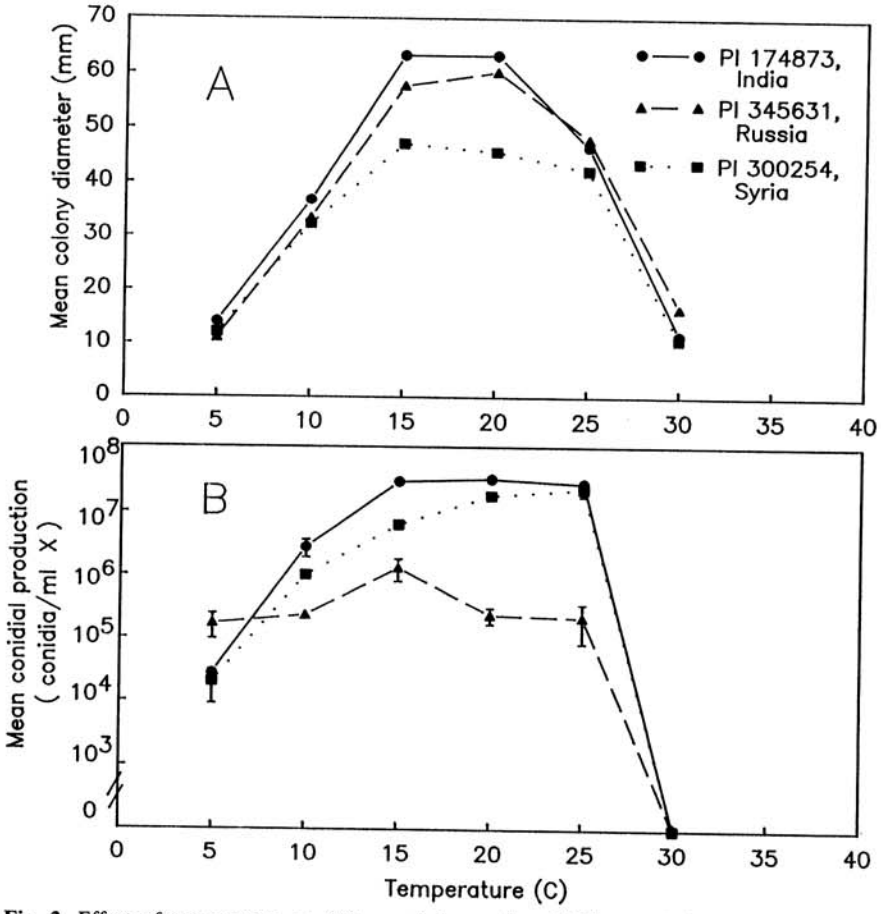


Fig. 2. Effect of temperature on **(A)** mycelial growth and **(B)** sporulation of three isolates of *Ascochyta fabae* f. sp. *lentis* on potato-dextrose agar after 13 days in the dark. Error bars represent mean values ± the standard error.

Table 3. Effect of light and dark on growth and sporulation of three isolates (PI 174873 from India, PI 300254 from Syria, and PI 345631 from Russia) of *Ascochyta fabae* f. sp. *lentis*[y]

Treatment	Mean colony diameter (mm)			Mean conidial production (conidia/ml × 10^6)		
	PI 174873	PI 300254	PI 345631	PI 174873	PI 300254	PI 345631
Light	48.4 a[z]	38.0 a	32.2 a	5.0 a	3.0 a	0.8 a
Dark	43.8 b	26.0 b	34.4 a	1.5 b	0.3 b	0.1 b

[y]Single-spore isolates were grown on natural potato-dextrose agar in glass petri dishes (10-cm-diameter) at 20 C in continuous light (87 $\mu E \cdot m^{-2} \cdot s^{-1}$) or continuous dark for 9 days. The experiment was repeated twice.
[z]Numbers in each column followed by the same letter are not significantly different according to ANOVA ($P = 0.05$).

ANOVA of the pooled means.

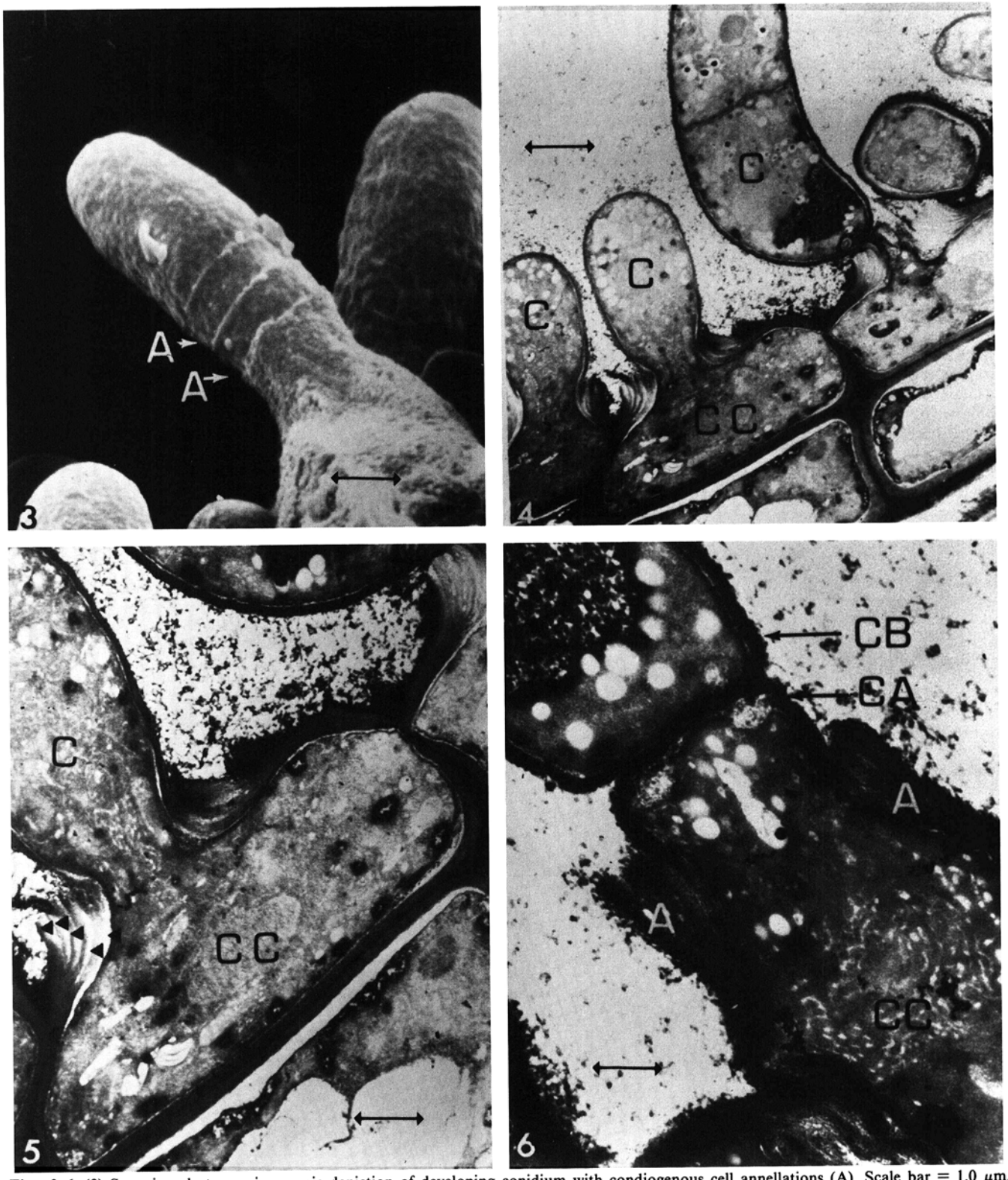
Conidia and conidiogenous material were prepared for scanning electron microscopy (13). Material for transmission electron microscopy was embedded in Spurr's resin, sectioned with a diamond knife, stained with barium permanganate, uranyl acetate, and Reynold's lead, and examined with a Hitachi H-300 transmission electron microscope (13).

The pathogenicity of 24 isolates of *A. f. lentis* was tested on two commercial lentil cultivars, Brewer (PI 477920) and Spanish Brown (PI 565081), in greenhouse inoculation studies. Included in the pathogenicity tests were the 13 isolates listed in Table 1, plus five isolates from the United States, two from Turkey, and one each from Australia, Brazil, Pakistan, and Macedonia. The isolates were cultured on natural PDA in petri dishes under fluorescent light (12-hr photoperiod, 77 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 10–14 days. Conidia were collected by flooding plates with sterile distilled water and gently scraping the colony surface with a bent glass rod. Conidial suspensions were adjusted to approximately 1×10^6 conidia per milliliter. The foliage of three to five 20-day-old plants was sprayed to runoff with conidial suspensions using a DeVilbiss atomizer. Control plants were sprayed with distilled water. These procedures were repeated twice. Plants were incubated in moist chambers for 96 hr, then on a greenhouse bench until the experiment was terminated. Pots were distributed in a completely randomized design in the moist chambers and on the greenhouse bench. The moist chambers consisted of a wooden frame (40 cm wide × 64 cm long × 56 cm high) that was covered on all sides but one with transparent plastic. The open end was placed in a metal tray filled with water to provide a saturated atmosphere. Temperatures in the greenhouse ranged from 18 to 22 C. Final disease readings were made 24 days after inoculation, at which time reisolations were made from surface-disinfected tissues (0.25% NaOCl for 5 min) plated on WA.

Five isolates of *A. f. lentis* used in this study were deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland. They were isolated from seed of lentil PI 174873 from India (ATCC 46980), PI 298644 from Spain (ATCC 46981), PI 345631 from Russia (ATCC 46982), PI 471916 from Canada (ATCC 46979), and PI 438517 from Turkey (ATCC 46987). One isolate, PI 468900 from Brazil, was deposited with the International Mycological Institute, Egham, England, as IMI 273933.

RESULTS

Effects of nutrient media on growth and sporulation. The three isolates of *A. f. lentis* from India, Russia, and Syria differed in linear growth, conidial production, and colony appearance on different nutrient media (Table 2). Maximum linear growth occurred on natural PDA for the Indian and Russian isolates and on oatmeal agar for the Syrian iso-



Figs. 3–6. (3) Scanning electron microscopic depiction of developing conidium with condiogenous cell annellations (A). Scale bar = 1.0 μm. **(4)** Transmission electron microscopic (TEM) depiction of conidiogenous cells (one labeled CC) producing conidia (C). Annellations are visible near the base of each conidium. Scale bar = 1.8 μm. **(5)** Magnified depiction of conidiogenous cell and conidium shown in Figure 4 (center). Conidiogenous cell (CC) bearing conidium (C). At least five annellations are visible (arrowheads). Scale bar = 0.9 μm. **(6)** TEM depiction of conidiogenesis. Base of conidium (CB) prior to secession from apex of underlying developing conidium (CA). Annellations (A) and conidiogenous cell (CC). Scale bar = 0.6 μm.

late. Conidial production was significantly greatest on natural PDA for the Indian isolate and on LSMA for the Russian isolate. However, there were few significant differences among media for the Syrian isolate. A comparison among isolates for conidial production revealed a greater difference between the Indian isolate on PDA and the other isolates. The Indian isolate had the fastest linear growth rate and highest spore production. Linear growth and conidial production were least in the Syrian and Russian isolates, respectively.

Effect of temperature on growth and sporulation. The optimum temperature range for linear growth of the three isolates on natural PDA in the dark was 15–20 C (Fig. 2A). Linear growth of isolate PI 300254 at 15 and 20 C differed significantly from that of the other isolates. The optimum temperature for conidial production of the three isolates was 15–25 C for the Indian isolate, 20–25 C for the Syrian isolate, and 15 C for the Russian isolate (Fig. 2B). At all temperatures except 30 C, there were significant differences among isolates in conidial production. At 30 C, linear growth declined markedly and the isolates did not sporulate.

Effect of light on growth and sporulation. Linear growth at 20 C on natural PDA was significantly greater in continuous light than in complete darkness for the isolates from India and Syria but not for the isolate from Russia (Table 3). Conidial production was 3.3–10.0 times greater for isolates in the light than in the dark. Zonation did not occur in any isolate of *A. f. lentis* incubated in continuous light or continuous dark. With a photoperiod, however, zonation was more pronounced in the Indian isolate than in the Russian and Syrian isolates. Zonation was also evident in other isolates of the fungus when they were exposed to 12 hr of alternating light and dark.

Conidial measurements. Frequency distributions for septation number differed significantly at $P = 0.05$ in 13 isolates of *A. f. lentis*. Isolates usually had conidia with one to three septa (Table 1). Fewer than 0.2% of the conidia of any isolate were one-celled or five-celled. Over all isolates, 67–96% of the conidia had one septum, 4–23% had two septa, and 1–10% had three septa. The isolate from Chile had the fewest conidia with one septum and the most conidia with two or three septa. The size of conidia varied with the number of septa (Table 1). The conidia with three septa were invariably longer than those with one or two septa but differed less in width. Within a septation group, there were no significant differences in the length and width of conidia among isolates.

Conidiogenesis. The conidiogenous cells of *A. f. lentis* were morphologically simple and lined the pycnidial cavity. Annellations were visible in scanning and transmission electron micrographs (Figs. 3–6) and indicated that condiogenous cells proliferated percurrently. Boerema and Bollen (1) noted that conidia originated and seceded at the same level as, or at a lower level than, the previous conidium. This is corroborated in Figures 4 and 5. Conidia also originated and seceded at a level above the previous conidium, as shown by the annellations in Figure 3.

Pathogenicity studies. Twenty-four isolates of *A. f. lentis* from 17 countries were included in the pathogenicity studies. All isolates were pathogenic on the two lentil cultivars, Brewer and Spanish Brown, producing necrotic lesions on stems, petioles, and leaflets. All isolates sporulated in the necrotic lesions on the foliage, and the fungus was reisolated from surface-disinfected tissues plated on WA.

DISCUSSION

Isolates of *A. f. lentis* from different countries varied greatly in growth rate, conidial production, and colony appearance. Under laboratory conditions, optimal linear growth and sporulation were dependent on the culture media, temperature, and light conditions. Sporulation of most isolates tested was usually higher on LSMA than on the other media. Recently, we observed that most isolates sporulated more abundantly on LSMA when 2% dextrose was added. This natural medium, which is easy to prepare, is used to produce large quantities of inoculum needed to screen lentil germ plasm for resistance to different isolates of the pathogen.

Temperature and light had profound effects on linear growth and sporulation of most isolates on PDA. The optimum temperature range for growth and sporulation was usually 15–25 C. At 30 C, linear growth decreased greatly and conidial production ceased. The fungus grew faster and produced more spores when exposed to continuous light. Greater sporulation in the presence of light has been reported for other *Ascochyta* species (5,9). Zonation was enhanced in some isolates of the fungus when exposed to alternating dark and light periods at 20 C. Zonation is common in other *Ascochyta* species (5,9).

Size and septation of conidia varied greatly among isolates of *A. f. lentis*. Within a septation group (e.g., one, two, or three), there were no significant differences in the length and width of conidia among the 13 isolates listed in Table 1. As would be expected, the more septa present, the longer the spore. Spore width, however, did not change appreciably when more septa were present. The isolate from Chile had fewer conidia with one septum and a higher percentage of conidia with two or three septa than any other isolate. More isolates from Chile need to be sampled to determine whether this phenomenon is particular to Chilean isolates.

Conidiogenesis in *A. f. lentis* is clearly annellidic, in our opinion. Our data support those of Boerema and Bollen (1), who separated *Ascochyta* from *Phoma* spp. primarily on the annellidic production of distoseptate conidia by the former vs. the phialidic production of nondistoseptate conidia by the latter. Punithalingam (12) disagreed with this interpretation, contending that conidiogenesis in both *Ascochyta* and *Phoma* is phialidic and that *Ascochyta* species do not produce distoseptate conidia. Sutton (14) tentatively accepted Punithalingam's interpretation, primarily because conidiogenesis in these organisms is difficult to determine with a light microscope, the instrument commonly used to make identifications. We have not examined conidiogenesis in other *Ascochyta* species and, moreover, have not investigated septum formation in *A. f. lentis*.

In the pathogenicity study, several isolates of *A. f. lentis* from the United States were compared with isolates from other countries. All isolates were obtained from infected seeds of commercial cultivars (U.S. isolates) or seeds from the Regional Plant Introduction Station at Pullman (isolates from other countries). Isolates did not lose their pathogenicity to lentil, even when seeds from which they were isolated had been stored at 4 C for over 30 yr. Preliminary observations indicate that isolates of *A. f. lentis* from the United States did not differ greatly in virulence from isolates from other countries. Additional information is needed concerning the pathogenic variability of isolates of *A. f. lentis*. In our studies, we used two lentil cultivars, Brewer and Spanish Brown, that are highly susceptible to Ascochyta blight. More revealing information on virulence of isolates might be produced by including lentil genotypes with more resistance to Ascochyta blight in future studies.

Little is known concerning the existence of pathotypes or strains of *A. f. lentis* on different lentil genotypes. In 1991, Jellis and Punithalingam (4) identified the teleomorph of *A. fabae*, a foliar pathogen of faba bean (*Vicia faba* L.), as *Didymella fabae* Jellis & Punith. In March 1992, the teleomorph of *A. f. lentis* was discovered on overwintered lentil debris from two fields near Genesee, Idaho (8). The teleomorph, a heterothallic *Didymella* sp., is currently being characterized. The teleomorph may contribute to the development of new pathotypes or strains of the fungus that may vary in virulence, morphology, or cultural characteristics.

ACKNOWLEDGMENTS

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