Influence of Environment and Culture Media on Spore Morphology of Alternaria alternata

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ABSTRACT

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Conidia of *Alternaria alternata* formed in natural habitats usually are larger, have longer beaks, and are more uniform in size than those produced in vitro on common agar media. The size and morphology of conidia produced in vitro were influenced both by the composition of the substrates (e.g., various agar media or pieces of tomato stems) and environmental conditions. Lowering the relative humidity during incubation by removal of the lids from petri-plate cultures for at least 6 hr daily increased the size of matured conidia. Conidia as large as those collected from stem cankers on tomato in the field, but with somewhat shorter beaks, were formed on synthetic media when 2-day-old cultures were exposed to continuous drying by removal of the petri dish lids. Reductions in the water potential of agar media by vapor equilibration or by additions of osmotica before inoculation also increased the size of conidia formed in culture, as did the exposure of cultures to lower temperatures during formation and maturation of conidia. Size and morphology of conidia were not significantly affected by exposure of agar cultures to either white fluorescent or ultraviolet light, to various levels of O_2 and CO_2 or to various pH values between 4 and 8. The number of spores produced was not affected by light but was depressed by CO_2 concentrations greater than 1.3%. These results indicate that only the conidia formed in nature are fully typical of *A. alternata*, but near-typical conidia can be produced in vitro by choice of media and culture conditions.

Conidia of *Alternaria alternata* (Fr.) Kiesler f. sp. *lycopersici* (the causal organism of tomato stem canker) collected from tomato plants (*Lycopersicon esculentum* Mill.) in the field, were significantly larger and more uniform in morphology than those obtained from in vitro culture (2). In preliminary studies, we noted that the morphology of conidia produced in vitro was greatly influenced by environmental factors such as temperature, relative humidity (RH), and composition of the culture media. Although the influence of environmental factors on morphology of various fungi has been reported (1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18. 19, 20, 21), its importance has not been emphasized in most taxonomic studies of *A. alternata*. In view of the reliance on conidial morphology for description and identification of *Alternaria* spp. (12, 14, 17), this study was done to determine more precisely the influence of defined environmental factors and culture media on the morphology of *A. alternata* conidia.

MATERIALS AND METHODS

Isolates.—Three pathogenic isolates of *Alternaria alternata* f. sp. *lycopersici*, isolated from tomato stem canker (2), and three saprophytic isolates of *A. alternata* collected from rotting ripe tomato fruits in a field near Davis, California, were used in all experiments. The isolates were maintained on 1.7% Difco cornmeal agar (CMA) and pathogenicity was tested as described previously (2). To determine the effect of various factors on the morphology of conidia produced under different environments, petri dishes containing 20 ml of various media were inoculated with 5-mm-diameter plugs taken from the edge of young cultures. To determine the relative numbers of conidia produced, four disks, 1 cm in diameter, were cut with a cork borer at 1 and 2 cm from the center of the colony. Conidia were washed and brushed off the disks with a rigid transfer needle into 5 ml of water and the number of conidia in the resulting suspension was estimated with a hemocytometer. Length and width of the conidia and the length of beaks of at least 100 conidia from each isolate were measured on projected photomicrograph images that were matched with a micrometer image. All of the experiments were repeated at least twice, with at least four replications. Unless stated otherwise, the temperature, intensity of cool-white fluorescent light, and ambient RH were 25 C, 813 lux, and 20 - 30%, respectively.

Effect of light.—Cultures were grown on CMA and exposed to fluorescent light (813 lux) for 2, 4, 8, 12, 16, 18, and 24 hr daily for 6 days. Another set of cultures, after incubation for 48 hr, were exposed to ultraviolet light (250 or 375 nm) for 10, 15, and 20 min and then were kept under 12 hr of fluorescent light daily for 3 additional days before determinations were made of size and morphology of the conidia. Comparable cultures were grown in continuous darkness.

Effect of temperature.—Cultures on CMA were incubated at various temperatures between 6 and 33 C in the dark for 8 days and then were examined for conidial morphology.

Effect of O_2 and CO_2 concentrations.—Petri dishes

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containing CMA made in 0.05 M citrate buffer (pH 5.7) were inoculated and placed inside 5-liter jars at 24-25 C in the dark. To promote gas exchange, the lids of the petri dishes were placed in a slightly lifted position. With the exception of inlet and outlet tubes, the jars were closed. Each jar initially was flushed with 50 liters of nitrogen (N_2) for 30 min, and then was connected to a system of manometers and capillary tubes which provided 10 liters/hr of N_2 gas containing the desired concentrations of O_2 and CO_2. The percentages of O_2, CO_2, and N_2 of gas samples taken from inlet and outlet tubes were determined with a gas chromatograph at the start and conclusion of each experiment. The following concentrations of O_2 and CO_2 were used: (i) 0.02% CO_2 with 0.07, 1.84, 3.2, 7.0, 16.3, and 21% O_2; (ii) 15% O_2 with 0.006, 0.03, 1.28, 3.51, 6.48, 12.0, 16.1, and 21.2% CO_2; (iii) the following combination percentages of CO_2 and O_2, respectively: 0.006/21.2, 0.03/21.0, 1.61/18.3, 4.4/16.6, 7.9/11.3, 12.7/7.2, 16.6/3.8, and 20.3/0. Cultures were exposed to the above gas concentrations for 9-10 days in the dark before examination.

Effect of substrate composition.—To determine the effect of composition of culture media on morphology of conidia, the following media were used: CMA, potato-dextrose agar (PDA), acidified PDA (APDA), V8 agar [20% (v/v) V8 juice, 0.25% $CaCl_2$, and 1.5% Bacto agar], glucose-nitrate agar (1.5% glucose, 1% $NaNO_3$, and 1.5% Bacto agar in 0.05 M phosphate buffer), and yeast-peptone agar (YMPG) (0.5% yeast extract, 0.5% meat extract, 0.5% Difco proteose peptone, and 1% Bacto agar). The carbon to nitrogen (C/N) ratio of glucose-nitrate agar also was varied by adjusting the quantities of glucose (0.2-4.0%) and sodium nitrate (0.1-0.9%). The molar C/N ratios used were 0.6, 1.4, 2.3, 4.9, 14.6, 19.8, 43.7, and 113.0. In some experiments, CMA was adjusted with 0.05 M citrate buffer at pH 4.0, 5.1, and 6.1 and with phosphate buffer at pH 7.1 and 8.0.

The isolates also were cultured on pieces of stem (5 × 8 mm) from the stem canker-susceptible tomato cultivar EP7 (2). Fresh or air-dried stem pieces, nonsterilized or sterilized by autoclaving for 15 min or by exposure to propylene oxide gas for 24 hr, were placed on moist, sterile Whatman filter papers in petri dishes. They were inoculated with about 1×10^3 conidia in 1 ml of water and incubated at 25 C for 7 days.

Effect of low humidity and water potential.—Petri plates containing 0-, 1-, 2-, 3-, 4-, and 5-day-old colonies on CMA or APDA were placed in a Microvoid® (Air Control, Inc., Narberth, PA 19072) hood and their lids were removed for various periods of time (0, 2, 4, 6, 8, 12, and 24 hr) on 6 consecutive days. Filtered air at 10-20% RH was forced continuously through the hood with a fan. The temperatures of the exposed agar surfaces were between 19.1 and 20.1 C (about 4 C lower than ambient air) as determined with fine-wire thermocouples. Two 15-mm-diameter wells cut in the agar at the outer edge of some plates were periodically filled with sterile distilled water to prevent excessive drying of the agar. The water

TABLE 1. Effect of substrate on the dimensions of conidia and conidiophores produced by *Alternaria alternata* f. sp. *lycopersici* at 25 C

Measurements (μm)[a]	Media			
	YMPG[b]	Cornmeal agar	Fresh pieces[c] of tomato stem in vitro	Tomato stems in the field
Spore length:				
Mean	11.2	19.8	30.9	32.3
Range	8-15	10-30	19-47	18-50
S.D.[d]	1.9	1.6	1.7	2.8
Spore width:				
Mean	10.0	9.5	10.9	12.4
Range	6-12	7-13	7-15	7-18
S.D.	2.2	1.6	1.9	2.8
Beak length:				
Mean	0.6	2.0	3.4	6.8
Range	0-2	1-4	1-6	2-20
S.D.	0.8	1.3	1.8	6.0
Percent of spores with beaks	13	62	76	72
Conidiophore length:				
Range	2-26	2-40	6-59	3-62

[a]Each figure (except those in the last column on the right) is the average of measurements of about 600 spores of three pathogenic and three saprophytic isolates (all six isolates responded similarly). Each figure in the last column on the right is the average of measurements made on about 300 spores of *A. alternata* f. sp. *lycopersici* collected from stem cankers in the field in Ventura County, California.

[b]A medium made of 0.5% yeast extract, 0.5% meat extract, 0.5% Difco protease peptone, and 1% Bacto agar.

[c]Fresh nonsterilized stem pieces (5 × 80 mm) of cultivar EP7 of tomato were inoculated with 1.0 ml of a spore suspension containing 10^4 spores per ml from three saprophytic and three pathogenic isolates of *A. alternata*.

[d]Standard deviation.

potential of sample disks from the media was determined periodically with a thermocouple psychrometer. Inoculated pieces of tomato stems also were placed in the microvoid chamber, and exposed to a low humidity each day by lifting and closing lids of the petri dishes for various periods of time.

Effect of water potential.—The influence of water potential on sporulation was examined further by adjusting CMA to various water potentials before inoculation with *A. alternata*. The water content and potential of 5 ml of CMA in small (55-mm diameter) petri dishes were allowed to equilibrate with the water potential of $CaCl_2$ solutions by vapor exchange: this was done by placing the petri dishes in 2-liter jars containing 250 ml of $CaCl_2$ solutions with water potentials between −1,132 and −14 bars (15) for 18 days prior to inoculation. The jars were submerged in a large tub of water at room temperature (25 C) which minimized fluctuations and gradients in temperature. After inoculation, the petri plates were incubated for 7 days in the same jars in which they had equilibrated. The effect of osmotically-induced water potential on conidia morphology was determined in cultures grown in closed petri dishes on CMA containing appropriate amounts of sucrose, KCl or a salt mixture (NaCl, KCl, and Na_2SO_4) as specified by Robinson and Stokes (15) and Scott (16).

RESULTS

Effect of light.—Light was not required for sporulation, and neither the size, shape, nor the numbers of conidia formed by any of the isolates was altered significantly by the various light regimes that were tested.

Effect of temperature.—All isolates grew on CMA at temperatures in the 6-33 C range, but none grew on that medium at 36 C. Colonies grown at 27 C had the largest diameters. There was, however, a negative correlation between incubation temperature and the size of the conidia formed (Fig. 1, 2). The size of the conidia produced in cultures grown at 6 and 9 C were even larger (Fig. 2) than those collected from stem cankers in the field (average 32×12 μm). Conidial initiation was observed on CMA cultures after 3 and 5 days of incubation at 25 and 12 C, respectively. To determine the effect of duration of exposure to low temperature on the size of conidia, inoculated CMA plates were incubated at 25 C for different periods of time before transfer to 12 C for 6 additional days. Likewise, cultures were incubated at 12 C for different periods of time before transfer to 25 C. The results of these experiments indicated that size of conidia was increased only by exposure to low temperatures during the early stages of conidia formation. The largest conidia were formed within 5 to 7 days on cultures that were transferred to 12 C not later than 12 hr after inoculation at room temperature (25 C).

Effect of CO_2 and O_2.—The diameter of the colonies and conidia measurements were not affected significantly by increased CO_2 concentrations up to 12%, with or without a concomitant decrease in O_2. Conidial measurements and colony diameter also were not influenced by decreased O_2 concentrations to as low as 0.7% when the CO_2 level was maintained at 0.03%. However, the number of conidia was decreased in CO_2 levels above 1.28% with both high and low O_2 concentrations. The fungus grew very little and did not sporulate in an atmosphere devoid of O_2.

Effect of substrate composition.—All isolates produced larger conidia when grown on pieces of tomato stem in vitro than when grown on YMPG or CMA, and conidia formed on pieces of stem in vitro were nearly as large as those collected in the field (Table 1). However, the

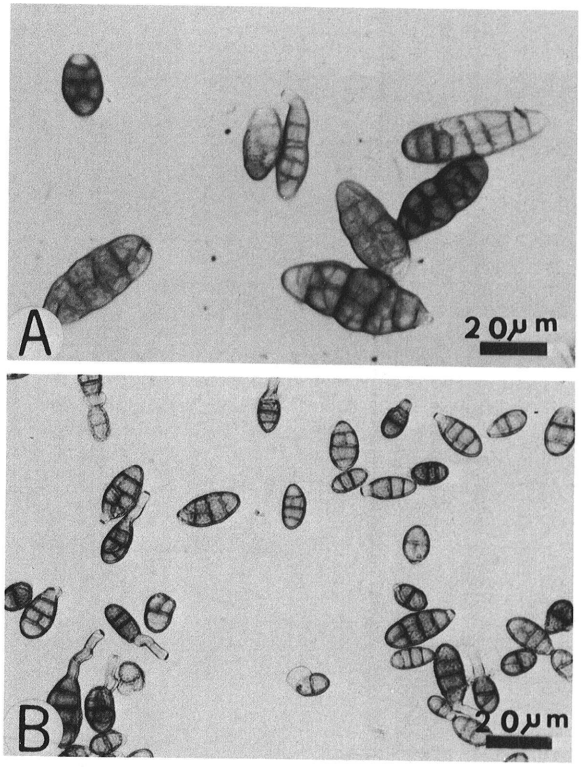

Fig. 1-(A, B). Conidia of *Alternaria alternata* f. sp. *lycopersici* produced on cornmeal agar incubated in the dark for 7 days at **A)** 6 C and **B)** 33 C.

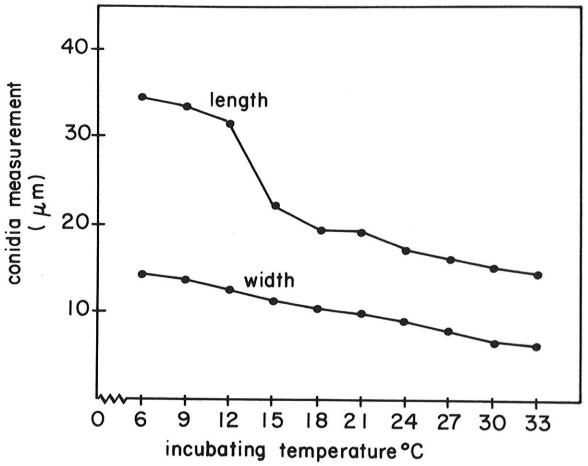

Fig. 2. Effect of incubation temperature on conidia size of *Alternaria alternata* grown on cornmeal agar in the dark. Each point represents an average of about 600 measurements made from conidia of three pathogenic and three saprophytic isolates.

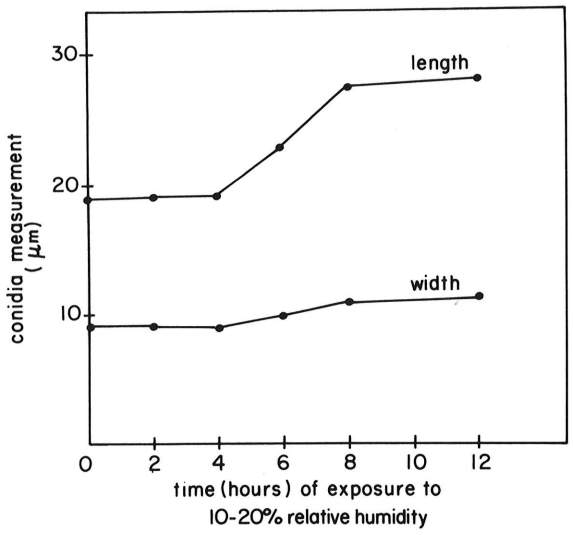

Fig. 3. Effect of alternating high and low or constant high humidity on size of conidia of *Alternaria alternata* grown on cornmeal agar at 25 C and 12 hr of light daily (813 lux). Cornmeal agar cultures (48-hr-old) were exposed for different times (hours) to air of approximately 10-20% RH daily for 4 consecutive days by removal of the culture plate lids. Each point is the average of about 600 measurements made from conidia of three pathogenic and three saprophytic isolates.

beaks of the conidia formed on stem pieces in vitro were only slightly longer than of those grown on CMA, and were only about half as long as those collected from stem cankers in the field (Table 1). There were no significant differences in the dimensions of conidia produced in vitro on fresh, sterilized, or nonsterilized tomato stems. The length-to-width ratio of conidia from cultures grown on CMA or PDA was about two, whereas that of conidia produced on YMPG was close to unity. The size and morphology of the conidia also were affected by the C/N ratio. For example, the length-to-width ratio of conidia progressively decreased from 2.23 to 1.44 as the C/N ratio was increased from 1:4 to 113:0. Conidial morphology and sizes were not affected by the pH of the media. Although the fungus grew and sporulated readily at all pH values tested (4.0, 5.1, 6.1, 7.1, and 8.0), the optimum pH for both growth and sporulation was about 7.1. All isolates responded similarly to the different pH values.

Effect of humidity and water potential.—The responses of all isolates to humidity were similar. Larger conidia were formed in petri dish cultures from which lids were removed for 6 hr or more daily during the first 5 days of incubation than in petri dish cultures with the lids left in place (Fig. 3). However, conidia as large as those collected from stem cankers in the field were produced on CMA or PDA only if 36- to 48-hr-old cultures were subjected to continual drying by removal of the petri plate lids (Fig. 4). The length of beaks on conidia produced on

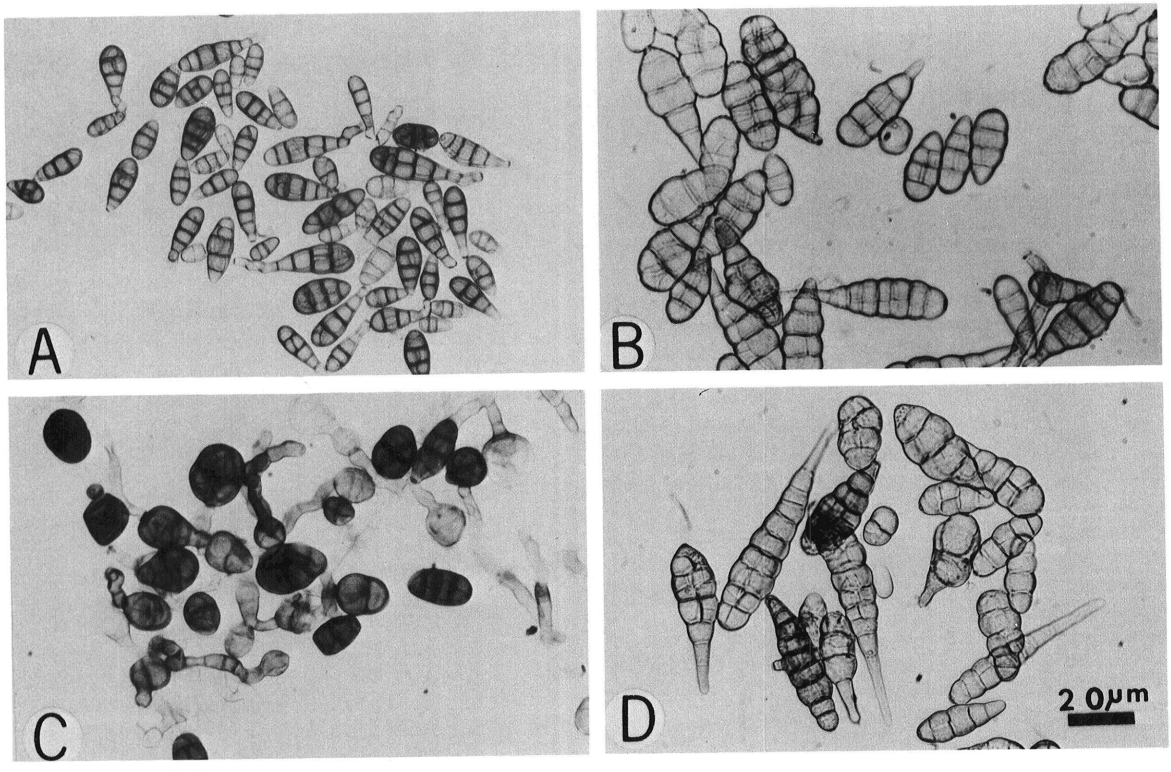

Fig. 4-(A to D). Differences in size and morphology of conidia of *Alternaria alternata* f. sp. *lycopersici* grown on different culture media and under different environments **A)** from cornmeal agar cultures in closed petri dishes; **B)** from cornmeal agar cultures incubated for 48 hr in closed petri dishes and exposed to continuous drying for 3 additional days by removal of cover plate; **C)** from yeast peptone agar cultures in closed petri dishes; and **D)** from tomato stem cankers in the field. Cultures in A, B, and C were incubated at 25 C under 12 hr of light daily (813 lux) and 10-20% ambient RH for 6 days.

agar media, however, was less than half of that on conidia from the field. Conidia produced on CMA that dried to −12.5 to −29.9 bars were larger than those produced on nondried CMA (−2.2 bars). In contrast to conidia formed on CMA or PDA, those formed on pieces of stem tissue did not increase in size as a result of exposure to alternating RH regardless of the manner in which the stem tissue had been prepared.

Effect of water potential.—The results of the experiment with $CaCl_2$ solutions confirmed our previous observations that the size of conidia was increased by drying of the media. For example, conidia from cultures maintained at RH values between 39 to 86% were about 20% longer and about 10% wider than those maintained at RH values between 94.5 and 100%.

The use of a salt mixture (NaCl, KCl,and Na_2SO_4) to reduce the water potential of CMA also increased the size of conidia formed by *A. alternata* (Fig. 5), but the conidia formed in the presence of osmotica were never as large as those formed on CMA that had dried, or on tomato stem tissue. Similar results were obtained when different levels of KCl or sucrose were incorporated into CMA.

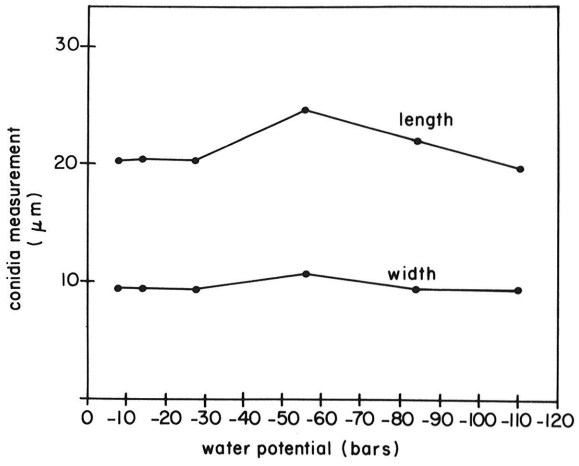

Fig. 5. Effect of water potential of the medium on spore size of *Alternaria alternata*. To provide different water potentials appropriate amounts of a salt mixture (NaCl, KCl, and Na_2SO_4) were added to cornmeal agar in the molal concentrations specified by Robinson and Stokes (15) and Scott (16). Each point in the figure is the average of about 600 measurements made from spores of three pathogenic and three saprophytic isolates. Inoculated plates were incubated at 25 C and 12 hr of light daily (813 lux).

DISCUSSION

The influence of the composition of culture media on the morphology of the conidia of *A. alternata* and other *Alternaria* species has been reported by others (5, 12, 14, 20). Neergaard (14), for example, noticed differences in size of conidia and length of beaks of isolates of *A. tenuis* Auct. (= *A. alternata*). As a result of morphological variability of the conidia of *A. tenuis*, Neergaard (14) described seven types of conidia that he designated beakless oval or ball, short cone, long oval, long cone, and cylinder. Our results suggest that these "types" probably are laboratory-induced artifacts that rarely, if ever, would occur on naturally infected host tissues.

We observed that conidia collected from natural stem cankers were consistently larger, and more uniform in size and morphology than those from synthetic media. Elliott (1) and Hartill (5) have reported similar observations.

The larger conidial size of isolates collected from natural stem cankers could be due to the combined effects of the substrate, fluctuation in ambient humidity, and lower night temperature because the conidia produced in vitro on CMA and on tomato stem pieces were larger when incubated at lower temperature and when exposed daily to various periods of drying.

The beaks of conidia from natural stem cankers were ×3.4 longer than those from CMA cultures. Neergaard (14) also observed that conidia of *A. tenuis* from natural media (seeds, seedlings, or weakened leaves) had longer and more slender true beaks than those from synthetic media.

Increase in size of conidia of *A. tenuis* and *A. longipes* as a result of decrease in temperature also have been reported by Morton (13) and Tisdale and Wadkins (20), and similar effects of temperature on the morphology of conidia of fungi other than *Alternaria* have been reported. For example, in a study of the effect of light and temperature on production of conidia by *Helminthosporium gramineum*, Houston and Oswald (7) found that length of conidiophores grown on plant tissues in the dark was increased and conidium length decreased as the incubation temperature was increased from 8 C to 35 C. *Helminthosporium dictyoides*, grown under 12 hr of light daily, produced small two- to three-celled conidia at 21 and 24 C and typical five- to eight-celled conidia at 15 and 18 C (21). Teviotdale (19) found that length of conidia of *H. gramineum* was correlated directly with the duration of daily exposure to low temperature (6 - 12 C). In our study the size, shape, and number of conidia formed by any of the isolates were not altered significantly by various light regimes that were tested. However, light has been reported to stimulate sporulation of *A. tenuis* (*A. alternata*) (8, 11).

The original description of *A. alternata* is based on measurements of conidia produced on natural substrate under variable environmental conditions (17). In our experience, conidia of *A. alternata* from natural stem cankers and rotted fruits of tomato fit this description quite well, but conidia produced in vitro usually are smaller, scarcely beaked, and more variable in size and shape (2). This variability in morphology of in vitro-produced structures has resulted in considerable confusion in the identification of in vitro cultures of *A. alternata* (12).

In mycological literature, the morphological characters of fungi often are described without adequate definition of the medium used and other growth conditions. Consequently, when identification of a fungus such as *A. alternata* is attempted, often it is difficult to determine whether observed differences are due to innate differences in the fungus or to the effect of environment. The drastic influence of environmental factors on morphology of *A. alternata* as shown in this paper, and by Lucas (12) warrant a thorough reporting of environmental conditions to which the described fungi were subjected during sporulation.

Conidia most similar in size and morphology to those

produced under natural conditions were produced on CMA or PDA at 9 or 12 C or at 25 C for 48 hr prior to drying for 3 additional days. For descriptive studies and for identification of isolates, these conidia were not typical because beaks were shorter than those produced in nature. However, they were sufficiently similar in morphology to allow identification as *A. alternata* with reasonable certainty.

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