Conditions Influencing Growth and Sporulation of *Cercospora asparagi* and Cercospora Blight Development in Asparagus

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ABSTRACT

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Sporulation of *Cercospora asparagi* was abundant on V-8 juice agar and carrot decoction agar media when seeded with a spore suspension or when mycelial fragments were placed in molten agar. Sporulation occurred in continuous dark and alternating light/dark treatments. Abundant conidiophores were produced in continuous light, but production of conidia was sparse. The optimal temperature ranges for radial growth and maximum sporulation were 20–28 C and 24–28 C, respectively. Conidial germination occurred between 8 and 36 C with a maximum between 20 and 32 C. Cultures of the fungus could be stored by several methods and remained viable for at least a year at 8 C. Lesions were obtained by inoculating asparagus seedlings with a suspension of 10,000–20,000 spores per milliliter and enclosing the plants in plastic bags to maintain high humidity for at least 96 hr. The optimal temperature for disease development ranged from 25 to 30 C.

Additional key words: *Asparagus officinalis*.

Production of asparagus is an expanding industry in North Carolina, with a proposed establishment of 1,200 ha. Cercospora blight of asparagus (*Asparagus officinalis* L.) caused by *Cercospora asparagi* Sacc. is a serious disease in North Carolina. Blighted ferns become covered with small, oval, grayish tan lesions with purple borders, then turn yellow to brown and eventually die. In 1984, the incidence and severity of *C. asparagi* on the North Carolina asparagus crop were estimated to be 95 and 9%, respectively (14).

The Cercosporae have fastidious requirements for in vitro growth and sporulation (5,17,19), and information about nutritional requirements and environmental factors for sporulation of *C. asparagi* is lacking. Attempts to induce sporulation in other *Cercospora* spp. by varying light and temperature have been reported (2,3,5,7,16). Carrot leaf decoction agar was an effective medium for inducing sporulation of *C. kikuchii* (12), but erratic spore production of the same species occurred on V-8 juice agar (V-8) (19). Other researchers have reported good sporulation using media prepared from host tissues (8,17). Selective subculturing, a method of transferring densely sporulating areas, has induced sporulation in some species of *Cercospora* (5,10,17), but attempts to obtain sporulation with mycelial transfers have generally been unsuccessful (9,12,17). Incident light greatly influences growth and sporulation of many fungi, and exposure often enhances spore production (5,6,13).

Limited knowledge about growth requirements of *C. asparagi* and the effects of environment on release of conidia and conidial germination has hampered progress in managing Cercospora blight. This paper reports optimal cultural conditions for fungal growth, the effects of environment on disease development, and in vitro sporulation and germination.

MATERIALS AND METHODS

Isolates of *C. asparagi* obtained from lesions on asparagus in Sampson County, NC, in 1983 were used. Three isolates were maintained on V-8 culture plates at room temperature or stored on potato-dextrose agar (PDA) slants at 8 C. The culture plates on V-8 were kept under fluorescent light on a 12-hr light/dark cycle. Asparagus seed, 78-syn-2 (provided by Joan of Arc Co., Turkey, NC), was used in all disease development experiments. Seeds were sown in peat mix (W. R. Grace, Travelers's Rest, SC) in 10-cm pots, and plants were thinned to one per pot. All experiments were arranged in a randomized complete block design with three to 20 replicates, and the data were subjected to analysis of variance.

Media and techniques to induce sporulation. Four media and four methods of seeding plates were tested. Conidia removed from Cercospora blight lesions were transferred to cooled but still molten V-8 in petri dishes. The resulting colonies were used to inoculate the test media. Asparagus decoction agar (ASP), carrot leaf decoction agar (CDA), PDA, and V-8 were tested for their effects on spore production. ASP was prepared by placing 225 g of asparagus stems and cladophylls in 700 ml of distilled water and steaming for 1 hr; 600 ml of liquid was strained off and added to 400 ml of distilled water with 12 g of agar. CDA was prepared by steaming 300 g of carrot leaves in 500 ml of distilled water for 1 hr; 500 ml of liquid was strained off and added to 500 ml of distilled water with 12 g of agar (7,12).

The methods of seeding plates were as follows: 1) several transfers of single conidia from sparsely sporulating colonies produced on V-8, 2) transfer of mycelial plugs from the edges of actively growing colonies, 3) plates of growing colonies flooded with 5 ml of sterile water and scraped with a needle and 1 ml of a suspension obtained from the fragmented mycelium and water pipetted into petri dishes and covered with cooled but still molten agar of the test media (2,7,9), and 4) fragments of mycelium scraped from growing colonies and transferred to molten agar plates with a sterile needle (12). All plates were incubated at room temperature under fluorescent lights on a 12-hr diurnal cycle. Each treatment was replicated three times with three or four plates per replicate. Plates were observed daily with a dissecting microscope for sporulation and rated visually on a scale of 0–3, where 0 = no sporulation, 1 = sparse sporulation, 2 = moderate sporulation, and 3 = abundant sporulation.

Effects of temperature on sporulation. A conidial suspension was prepared by pouring sterile water onto 4-day-old V-8 cultures and gently scraping the surface with a rubber spatula. Each plate was rinsed three times. The resulting suspension was filtered

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through one layer of sterile cheesecloth. One milliliter of the conidial suspension (about 25,000/ml) was placed in a 9-cm petri dish, and V-8 agar was poured over the suspension. Plates were individually wrapped with Parafilm when the agar had solidified and placed in incubators adjusted to temperatures from 8 to 36 C at four-degree intervals. Cultures were incubated for 5, 6, or 7 days in total darkness with three plates per treatment. The entire experiment was repeated three times. After each incubation period, 13-mm-diameter disks were cut from each of three plates. Ten disks from each plate were placed in large test tubes with 5 ml of 50% ethanol-distilled water (v/v). The disks and liquid were vortexed for 30 sec. Six counts of the suspension were made for each tube with a hemacytometer. Each treatment was repeated three times to give 18 counts.

Effects of light on sporulation. V-8 was seeded as previously described. Light regimes were as follows: continuous light, 12-hr alternating light/dark cycle, and continuous darkness (three replicates). The continuous and alternating light treatments were placed on shelves under two 30W cool-white fluorescent tubes (Sylvania GTE F30T12/CW/RS) mounted 29 cm above the surfaces of the plates. Plates for the continuous darkness treatment were wrapped in aluminum foil and placed with the other treatments. Ten 13-mm-diameter disks per plate were removed after 4, 6, or 10 days. Six hemacytometer counts were made for each replicate to determine the amount of sporulation.

Effects of temperature on radial growth. Optimal temperature for radial growth of the three *C. asparagi* isolates was assessed in the dark at temperatures ranging from 8 to 36 C at four-degree intervals. Mycelial plugs (6 mm in diameter) taken from the edge of an actively growing colony were placed in the centers of PDA plates containing about 20 ml of agar. Five plates were incubated at each temperature. Fungal growth was measured daily for 8 days by averaging two diameters taken at right angles for each colony.

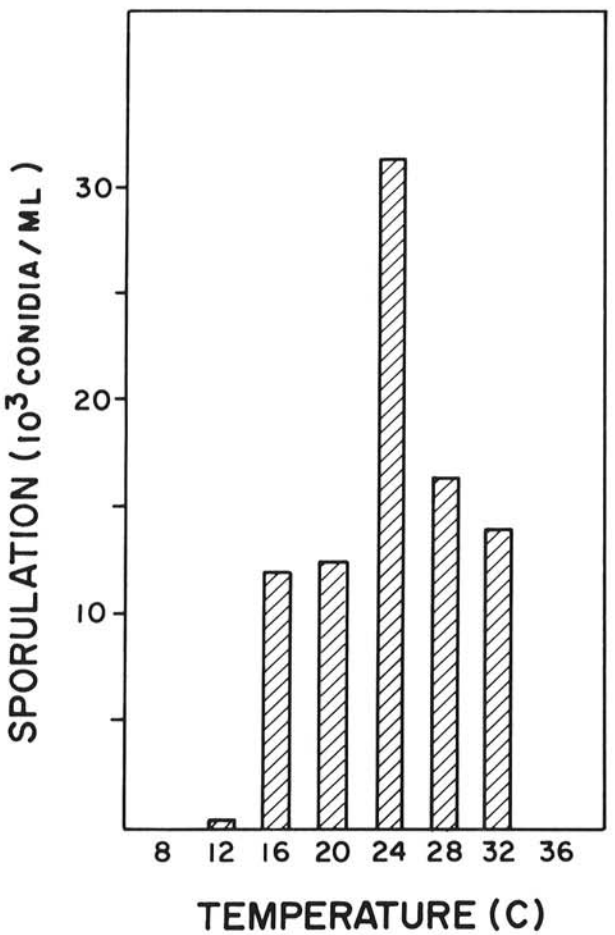

Fig. 1. Effects of temperature on spore production of *Cercospora asparagi* on V-8 juice agar after 5 days.

Effects of temperature and light on spore germination. The effects of temperature on spore germination were measured from 8 to 36 C at four-degree intervals after 4 and 24 hr. The effects of constant fluorescent light and darkness on spore germination were also determined after 4 hr at room temperature (about 24 C). Spores were collected from 3-day-old cultures growing on V-8. Three drops of a spore suspension (2.5×10^4/ml) were placed on glass slides and incubated in petri dishes containing moistened filter paper. Three replicates were used, with separate plates as replicates. A spore was considered germinated if its germ tube was twice as long as the diameter of the spore. A total of 100 spores was counted for each replicate.

Longevity of *C. asparagi* in vitro. Cultures of the fungus were grown on slants of PDA and stored in test tubes of sterile distilled water taken from actively growing colonies on PDA as squares 6× 6 mm or as an aqueous suspension of spores collected from a 3-day-old colony growing on V-8. The tubes were sealed with Parafilm and stored at 8 C.

Inoculum concentration. Inoculum was prepared by flooding plates of 4-day-old cultures growing on V-8 with distilled water and gently scraping with a rubber spatula to dislodge spores. The suspension was collected and filtered through one layer of cheesecloth and concentrated by centrifugation to about 20,000 spores per milliliter. Lower concentrations were prepared by dilution with distilled water. Each of the six spore concentrations, 20,000, 10,000, 5,000, 2,500, 1,200, and 600/ml, was used to inoculate 10 8-wk-old asparagus plants. A pressurized aerosol propellant (Sigma Spray A1147, Sigma Chemical Company, St. Louis, MO.) was used to spray plants to wetness. Plants were covered with plastic bags and placed in a shaded area of a greenhouse. Bags were removed after 6 days, and plants were placed on a greenhouse bench. Infection was rated 14 days after inoculation. A modified Horsfall-Barratt scale where 0 = no disease and 10 = 100% disease was used. The number of lesions on stems and cladophylls was also determined.

Effects of moisture on disease development. To determine the effect of high moisture, 8-wk-old asparagus plants were inoculated with a *C. asparagi* spore suspension of about 20,000/ml. After inoculation, plants were placed in a shaded area of the greenhouse and subjected to high moisture by placing them in plastic bags or to low moisture by leaving them unbagged. Five treatment periods of high humidity were used: 0, 24, 48, 96, and 168 hr, with 10 plants per treatment.

Effects of temperature on disease development. The effects of temperature were studied in controlled-environment chambers and greenhouses in the Southeastern Plant Environmental Laboratories at North Carolina State University, Raleigh. Seedlings of asparagus were grown in a gravel and peat-lite mixture (W. R. Grace) in plastic pots 10× 10 cm in the greenhouse for 6 wk at temperatures of 26 C during the day and 22 C at night. Plants were inoculated with a spore suspension of *C. asparagi* with about

TABLE 1. Degree of sporulation of *Cercospora asparagi* as a result of interaction of medium and inoculation technique

Medium	Method of inoculation			
	Conidial transfer	Flooding suspension	Mycelial plug	Mycelial fragments
Asparagus decoction agar	0[a]	1	0	1
Carrot leaf decoction agar	1	2	0	3
Potato-dextrose agar	0	0	0	0
V-8 juice agar	1	3	0	3

[a] Visual rating of sporulation obtained: 0 = none, 1 = sparse, 2 = moderate, and 3 = abundant.

25,000/ml of water from 3- and 6-day-old cultures grown on V-8. Inoculum was prepared as described before. A drop of Tween 20 was added to 250 ml of the suspension. Germination of conidia in water suspension was determined after 4 hr to verify viability.

Inoculated plants were sprayed to wetness with the spore suspension and controls were sprayed with distilled water. Plants were then placed in clear polyethylene bags to maintain high humidity and set in growth chambers adjusted to 15, 20, 25, 30, and 35 C and a 12-hr photoperiod (cool-white fluorescent lamps). Twenty inoculated and five control plants were placed at each temperature. A thermocouple was used to determine the temperature inside the bags, and the chambers were adjusted to maintain the desired temperatures. Bags were removed after 96 hr. Plants were rated after 14 and 18 days and the scores averaged to produce a disease severity rating.

RESULTS

Media and techniques to induce sporulation. *C. asparagi* was more fastidious in nutritional requirements for sporulation than in its requirements for growth. Mycelial growth of *C. asparagi* occurred on all media and with all inoculation procedures. Abundant, small, grayish colonies developed within 3–4 days of inoculation with either the flooding technique, the conidial transfer, or the mycelial fragment transfer methods; a dense mat of mycelium formed from the mycelial disk transfer. Mycelial disk transfer failed to induce sporulation on any of the media tested (Table 1). Colonies from transfers of single conidia occasionally produced sparse sporulation on CDA and V-8 media but failed to sporulate on subsequent transfers. No sporulation was observed on ASP or PDA from single-conidial transfer. Abundant sporulation occurred on CDA and V-8 with both the flooding technique and with the transfer of mycelial fragments to molten agar. The ability to sporulate could be increased with each successive transfer (up to a limit); however, virulence and ability to sporulate decreased if the culture was transferred 10–20 times. Some initial sporulation, which occurred on ASP with these methods, was overgrown with profuse aerial mycelium after 4 days. No sporulation occurred on PDA with any of the techniques tested.

Effects of temperature on sporulation. The time of peak sporulation was temperature-dependent. The pattern followed the optimal temperature conditions for growth of *C. asparagi* (Fig. 1). Maximum sporulation occurred after 5 days of incubation at 24 C (31,500 spores per milliliter) and after 6 days at 28 C (36,750 spores per milliliter). Many of the spores in the treatments at 20 and 24 C had germinated after 7 days. No growth occured at 8 or 36 C.

Effects of light on sporulation. Cultures sporulated under all light treatments (Fig. 2). Abundant sporulation occurred in the alternating light/dark treatment and the continuous dark treatment, but few spores were produced in the continuous light treatment. Although sporulation under continuous light was very low, abundant conidiophores were produced. Conidia were more abundant on days 4 and 6 for all treatments than on day 10. On day 10, about 50% of the conidia were germinated.

Effects of temperature on radial growth. *C. asparagi* grew in culture over a wide range of temperatures (Fig. 3). All isolates tested had similar growth patterns and grew at temperatures from 12 to 32 C with no growth at 36 C and only negligible growth at 8 C. Optimal growth occured at 24 and 28 C. The fungus did not grow in culture at 36 C, but this temperature was not lethal. When cultures were taken from 36 C 2 wk after the beginning of the test and placed at 24 C, the fungus resumed growth.

A red compound identified as the toxin cercosporin (M. E. Daub, *unpublished*) formed in the medium at temperatures from 16

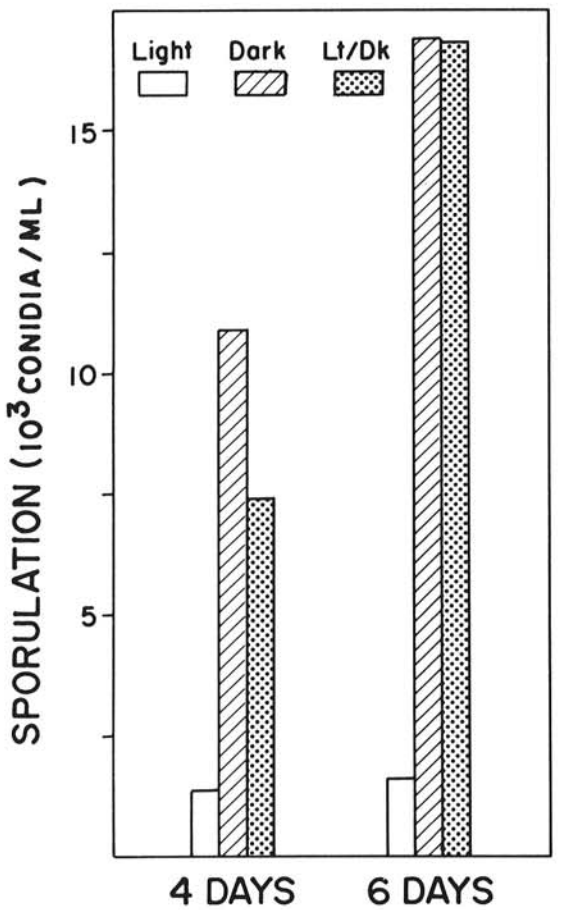

Fig. 2. Effects of light on spore production of *Cercospora asparagi* on V-8 juice agar 4 and 6 days after seeding plates. Light = continuous fluorescent light, dark = continuous darkness, and Lt/Dk = alternating 12 hr of light/12 hr of darkness.

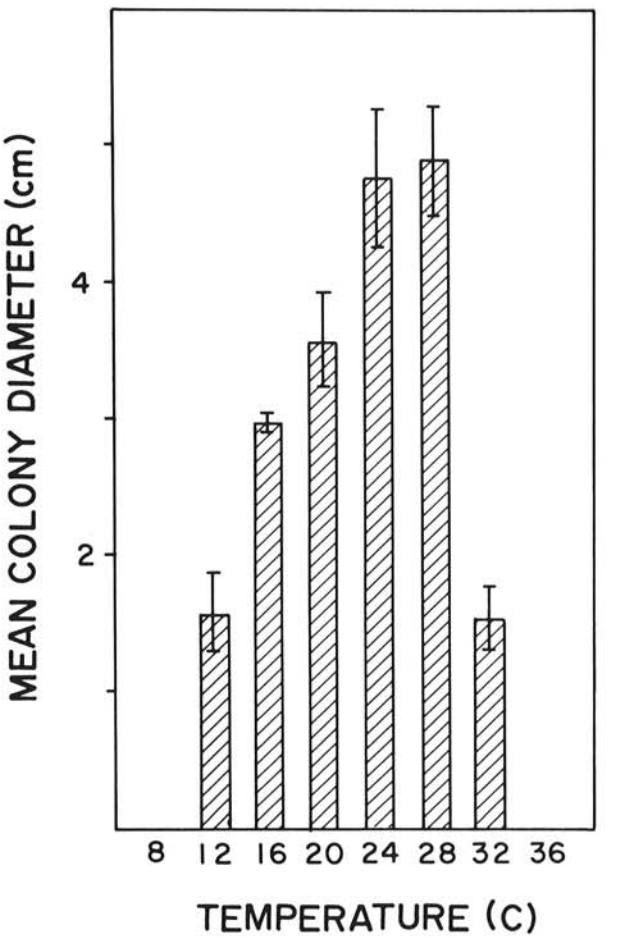

Fig. 3. Effects of temperature on radial growth of *Cercospora asparagi* after 8 days. Bars represent standard error of the mean of five replicates.

to 28 C but not at 12 C or lower or at 32 C or higher. Colony morphology differed somewhat at the different temperatures.

Effects of temperature and light on spore germination. The optimal temperature range for spore germination was between 20 to 32 C (Fig. 4). After 4 hr of incubation, more than 75% of the spores had germinated at temperatures between 20 and 32 C. Low to moderate germination was observed at 8 and 36 C after 24 hr. Light was neither inhibitory nor stimulatory to germination. Germ tubes may arise from any of the cells, but the apical cell usually germinated first, followed next by the basal cell, and then the intercalary cells.

Longevity in vitro. Sporulating cultures of *C. asparagi* were recovered from all treatments up to 1 yr after storage. The recovered isolates had colony morphology similar to that of the original isolates and sporulated readily after transfer to V-8 under proper conditions.

Disease development. Very few infections occurred on plants sprayed with conidial concentrations of 2,500/ml or lower (Table 2). An increase in numbers of lesions on both stems and cladophylls occurred as conidial concentrations were increased. Numerous lesions occurred on the stems with concentrations of 10,000/ml and greater, with an average of 20 stem lesions per plant at 10,000/ml and 30 stem lesions per plant at 20,000/ml. At the high inoculum concentrations, lesions on cladophylls were too numerous to count. Defoliation and chlorosis occurred at spore concentrations higher than 5,000/ml. Disease ratings were low to moderate at spore concentrations of 5,000–10,000/ml and moderate to severe at 20,000/ml.

High moisture periods of 96–168 hr resulted in more numerous lesions than a period of 24 or 48 hr. No symptoms developed on plants receiving 0 hr of high humidity, and very little disease was observed with humidity periods of 48 hr and less (Fig. 5). Disease was low to moderate (severity values of 1–4) for the 96-hr treatment and moderate to severe (disease severity values of 2–9) for the 168-hr treatment. In general, disease severity increased with length of time that plants were kept in a saturated atmosphere after inoculation. *C. asparagi* was reisolated from typical lesions and grew into characteristic colonies on V-8 and PDA. Uninoculated controls remained healthy.

Symptoms first appeared on inoculated plants after 8 days at 20 and 25 C, 11 days at 30 C, and 18 days at 15 C. Cercospora blight developed over a range of temperatures, with the most rapid development and most severe symptoms at 25 C and the slowest development and least severe symptoms at 15 and 35 C (Fig. 6). Uninoculated control plants remained healthy.

TABLE 2. Effects of inoculum concentration on disease rating and severity of Cercospora blight on asparagus 21 days after inoculation (144-hr wet period)

Concentration (spores/ml)	Rating		Average number of lesions/plant	
	Mean	Range	Stems	Leaves
600	0.8[a]	0–1	0.5	1.2
1,200	0.9	0–1	0.7	3.2
2,500	1.4	1–3	3.1	6.5
5,000	2.9	1–4	10.3	260[b]
10,000	3.6	2–6	21.0	>500[b,c]
20,000	4.9	3–7	30.0	>500[b,c]

[a] Average disease severity rating per 10 plants (0 = no disease and 10 = 100% disease).
[b] Defoliation and chlorosis occurred at these concentrations.
[c] Lesions too numerous to count.

DISCUSSION

C. asparagi isolates used in these tests grew over a wide range of conditions but sporulated sparsely or not at all unless specific

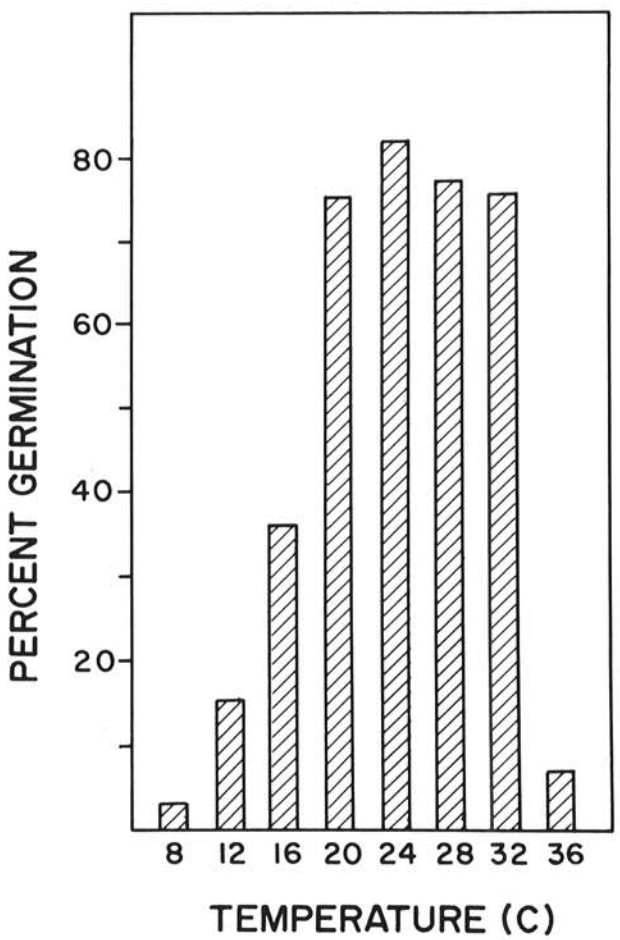

Fig. 4. Effects of temperature on spore germination of *Cercospora asparagi* after 4 hr.

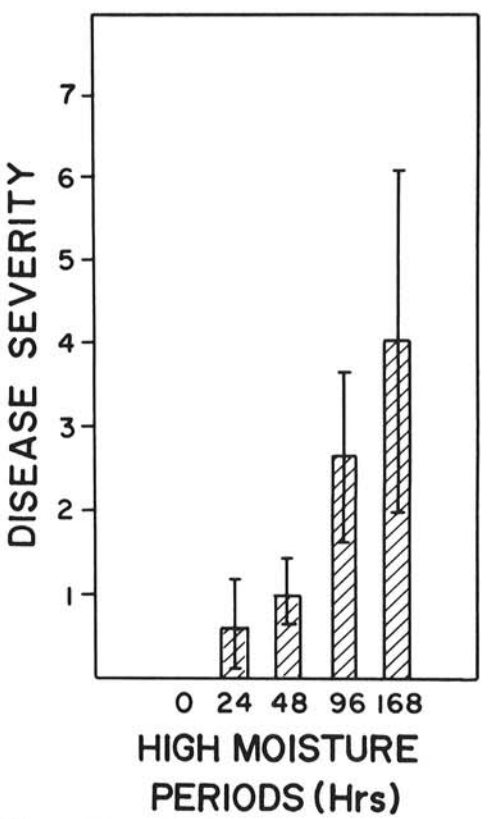

Fig. 5. Effects of high humidity after inoculation (20,000 spores per milliliter) on disease severity of Cercospora blight of asparagus. 0 = No disease and 10 = 100% disease. Bars represent standard error of the mean for 10 replicates.

requirements were met. Culture medium and transfer method influenced growth, colony morphology, and sporulation. The sporulation associated with the flooding technique and mycelial fragment technique could be explained as a direct effect of nutritional depletion caused by the multiplicity of mycelial colonies (9). However, it also may be the result of stress associated with maceration of the mycelium (18).

V-8 as a growth medium has been found to stimulate spore production in numerous *Cercospora* spp. (3,7,9,18). V-8 is recommended as a routine laboratory medium for *C. asparagi* because it is readily available and supports abundant sporulation.

Many factors influence sporulation in fungi, including light, temperature, and pH. Studies of this type have been done for other *Cercospora* spp. (3,5,8,20,22). Cultures of *C. nicotianae* reached maximum spore production sooner as the temperature was increased (21). In this study with *C. asparagi*, maximum sporulation occurred on different days at different temperatures. However, maximum spore production followed a pattern related to that of optimal temperature conditions for growth of the fungus rather than a general trend of reaching maximum sporulation earlier with an increase in temperature.

Germination or disintegration of conidia has been observed for other *Cercospora* spp. (17). Germination of conidia can occur in cultures of *C. asparagi* after 6 days, which decreases the number of viable spores present. It is therefore advantageous to use cultures between 3 and 6 days old as inoculum.

Because sporulation of *C. asparagi* occurred in continuous darkness, no particular wavelength of light is apparently necessary for spore production. Other *Cercospora* spp. differ in their responses to light (1,5–7). Calpouzous and Stallknecht (5) suggested that light is directly involved with spore initiation in *C. beticola*. This does not appear to be true with *C. asparagi*, because sporulation occurred in continuous darkness. However, because abundant conidiophores and few conidia were formed in constant light, a dark period may be necessary for conidial formation. Meredith (15) concluded from studies on *C. musae* that a new crop of conidia could develop overnight under favorable conditions and be available for dispersal the next day. Observations with *C. asparagi* also suggest a distinct diurnal pattern with the release of spores in the early morning hours and a sharp decline in the numbers of spores trapped at night (C. J. Cooperman, *unpublished*).

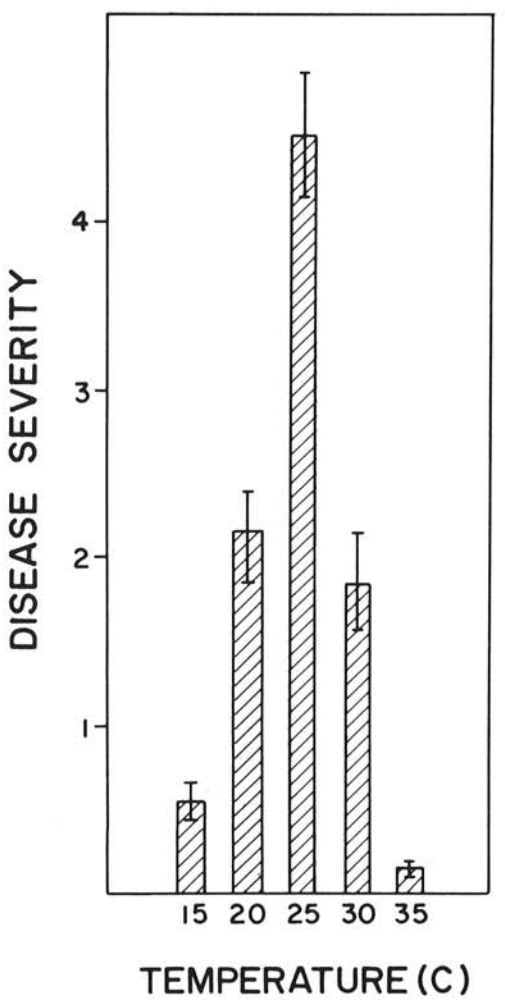

Fig. 6. Effects of temperature after inoculation (25,000 spores per milliliter) on disease severity of Cercospora blight of asparagus. 0 = No disease and 10 = 100% disease. Bars represent standard error of the mean for 20 replicates.

The effect of temperature on mycelial growth followed a pattern similar to that of other *Cercospora* spp. (9). It was suggested that this pattern resembles that of an enzyme reaction, with growth increasing to an optimum, then dropping to zero at very high temperatures (36 C).

Germination studies with other *Cercospora* spp. show that germination of some species is inhibited in the presence of free water (11) and that germination of others is inhibited by light (2). *C. musae* was shown to require a film of water on the leaf surface in order to germinate (15). *C. asparagi* germinated readily in free water at a wide range of temperatures and in the presence or absence of light.

Available moisture is an important factor in many fungal diseases and plays a central role in the development of Cercospora blight of asparagus. *C. asparagi* depends on free water for infection but not for lesion expansion. When asparagus plants were removed from the high-moisture environment, lesions continued to develop. However, the longer the period of high moisture the more extensive the disease. Berger and Hanson (4) found similar results with *Cercospora* spp. of forage legumes. They hypothesized that postinfection exposure to high humidity increased disease severity because the increased water content of tissue or the high relative humidity around the tissue thereby favored the spread of the pathogen in the host. The presence of free water and 100% relative humidity during infection and possibly early stages of Cercospora blight of asparagus development is necessary. Plants atomized with inoculum should be kept in a saturated atmosphere for at least 4 days.

Temperature has a significant influence on the development of Cercospora blight of asparagus. Little or no disease occurred at 35 C, and it can be presumed that 35 C was too high for the infection process to occur. Wallin and Loonan (23) found *C. beticola* caused infection at 35 C with wet periods of 48–72 hr. The temperatures for maximum disease development of Cercospora blight of asparagus coincide with average temperatures in the field under which maximum disease occurs. The temperatures found to be optimal for disease development were also optimal for radial growth and spore development in culture.

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