SOME EFFECTS OF PHOTOPERIOD, TEMPERATURE, AND HUMIDITY ON INFECTION, SPORULATION, AND OOSPORE PRODUCTION BY PERONOSPORA TRIFOLIORUM ON SEEDLINGS OF TWO ALFALFA VARIETIES

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INTRODUCTION

Downy mildew of alfalfa (Medicago sativa L.), caused by Peronospora trifoliorum deBary, occurs throughout the world's temperate regions wherever alfalfa is grown. The disease is endemic in cool, humid climates such as that of the northern United States, but is sporadic in Kansas (5).

Chlorosis is the first symptom of the disease and is followed by necrosis and leaf collapse. Other symptoms include reduced internode length, smaller stems, and malformed leaves. Seedlings may be killed if conditions are favorable to the disease (10).

Resistant varieties appear to be the only practical means of control (10). Resistance varies among varieties and tends to be greater in improved varieties developed in the northern states while those developed in Kansas and Nebraska are less resistant (6, 7). The endemic nature of the disease in the northern area allows the more susceptable breeding material to be recognized and thus discarded.

The sporangia of P. trifoliorum are deciduous and germinate by germ tube. Optimum germination occurs in free water at 18 C (14, 15). Sporulation is favored by cool, moist conditions and may continue for several days (10, 14). Sporangiophore production occurs through the stomata on both leaf surfaces and on the stem and petiole (21), but is most prevelant on the lower leaf surface (10).

Little is known about the development of P. trifoliorum but studies on related species of Peronospora have shown that light (17), humidity (14, 26), and temperature (2, 18) affect the germination of sporangia and germ tube growth. Direct penetration appears more common in the group (11); however, P. destructor penetrates through the stomata (27). Sporulation of Peronospora species is generally favored by low temperature (3, 19, 23, 24, 25, 27) and

high humidity (1, 4, 12, 19, 23, 24, 27). P. destructor requires a diurnal cycle of light for normal sporulation (25).

The purposes of this study were: (i) to determine the effects of sporangial concentration in inoculum on the number of seedlings that become infected, (ii) to determine the effects of light and humidity on infection, (iii) to determine the optimal photoperiod and temperature for sporulation, and (iv) to determine the effects of photoperiod and temperature on oospore production. Seedlings of two alfalfa varieties with noted differences in their resistance to mildew were used in all experiments to compare their reactions under a range of environmental conditions.

MATERIALS AND METHODS

Two varieties of alfalfa, 'Buffalo' and 'Narragansett', were selected for all experiments. Buffalo was selected for its high degree of susceptability and Narragansett for its relative resistance to P. trifoliorum (6, 7). Seed from lots NCC 63 and BCC 63 obtained from W. R. Kehr, University of Nebraska, was used.

Plants were grown in steam-sterilized masonry sand in $7\frac{1}{2}$ X 11 X 1 inch aluminum pans with holes in the bottom for drainage, Five replicates of 2 rows each were included per pan, with the varieties randomized within the pair of rows of each replicate. Seeds were planted $\frac{1}{2}$ inch deep, 70-80 seeds per row. The first 50 plants in each row were used for the experiments. After planting, the sand was watered until saturated and the pans were placed in a growth chamber maintained at 16 C, 500 ft-c of incandescent and flourescent lighting, and an 8-hr photoperiod. The plants were watered daily until the 7th day. Seedlings were inoculated 7 days after seeding and were in the cotyledon stage of growth.

For inoculum, infected shoots from 2-week-old seedlings (1 week after inoculation) were placed in distilled water in a bottle and shaken until the sporangia were in suspension. The plant material was removed by a tea strainer. The sporangial concentration was estimated by hemocytometer, and the suspension was adjusted to 50,000 sporangia/ml except for experiments involving incoulum load. The inoculum was sprayed onto plants with a DeVilbiss atomizer until most plants had a drop of inoculum resting between the cotyledons. This required ca. 15 ml of inoculum/pan.

The pans were then placed on $\frac{1}{2}$-inch-thick wood slats in metal trays containing about $\frac{1}{4}$ inch of water. To provide high relative humidity, a chamber

consisting of a Mylar plastic hood supported by a metal frame was placed over the pans and situated in the tray to form a water seal. Relative humidity was determined using a wind-tunnel type psychrometer which sampled air from one end of the moist chamber and returned it to the moist chamber at the opposite end. The plants were incubated in darkness for 12 hr at 20 C after inoculation and before being placed under experimental conditions except in the experiment involving infection period. Free water formed readily on the leaves of plants in moist chambers during periods of darkness.

To test the effect of various inoculum loads, plants were inoculated with suspensions of 4,000 to 200,000 sporangia/ml and incubated 7 days at 20 C, 8-hr photoperiod and 500 ft-c lighting.

To test viability of the sporangia used for each experiment, a portion of the inoculum was sprayed onto petri plates of 2% water agar and incubated at 18 C for 12 hr, with the per cent germination estimated from the average of 5 counts of 100 sporangia on each plate. Plates were exposed to white flourescent and incandescent light and dark to test the effect of light on germination.

To test the effect of light and moisture on infection, plants were inoculated and placed either under continuous light or dark conditions in moist chambers at 20 C for 8, 12, 16, or 24 hr. Plants kept in the light were kept wet by spraying them with water every 4 hr to compensate for water vapor which condensed on plants kept in the dark. After these infection periods, leaves from some plants kept 24 hr in the light or dark were harvested, stained with ploxine, and examined for sporangial germination and penetration. All other plants were placed in a growth chamber at 20 C, 8-hr photoperiod, and 70-85% relative humidity for 5 days. The plants were then put back into the moist chamber for 12 hr to induce sporulation, and infection was determined using chlorosis and sporulation as criteria. The data was statistically analyzed

using analysis of variance.

For experiments involving light-temperature interactions, wooden boxes covered with aluminum foil were placed over pans in the moist chambers and were changed manually to regulate photoperiod. Temperatures used were 16, 20, 24, and 28 C and photoperiods were 8, 16, and 24 hr. Water was atomized onto plants grown under continuous light to compensate for water vapor which condensed on the leaves in dark. Criteria used for determining infection were chlorosis and sporulation.

The effects of photoperiods of 8, 16, and 24 hr at 20, 24, and 28 C on oospore production was determined. Cotyledons from inoculated plants were submerged in lactophenol (20) with 0.2 g Sudan IV (dissolved in 95% ethyl alcohol)/liter added, autoclaved for 15 min at 15 psi pressure, and examined microscopically for oospores.

RESULTS

Number of infected seedlings increased with inoculum concentrations up to 25,000 viable sporangia/ml. Further increasing the concentration did not increase the number of resulting diseased seedlings. Fewer Narragansett than Buffalo seedlings became diseased at each inoculum level used (Fig. 1). Sporangial germination on watar agar ranged from 50-60% throughout the experiments; most germinated 4-12 hr after inoculation. The presence or absence of light did not affect sporangial germination on watar agar.

On cotyledons, germination of sporangia averaged 41 and 45% in light and dark, respectively, while sporangia from the same lot germinated 52% on water agar. Following germination on the cotyledons, appressoria formed, and the fungus penetrated directly through the epidermis. However, germ tubes from many sporangia did not form appressoria and did not penetrate the host. Many germ tubes grew over stomata, but no stomatal penetration was observed.

Maximum infection occurred in plants kept dark and wet for at least 12 hr following inoculation (Table 1). Here, 32 and 67% of Narragansett and Buffalo seedlings became infected in all treatments.

P. trifoliorum sporulated within 5 days after inoculation on some plants and by one week on all plants with mildew symptoms grown at 20 C and 8-hr photoperiod (Table 2). In a subsequent experiment at an 8-hr photoperiod and 16 and 20 C, sporulation occurred on all Narragansett and Buffalo plants with mildew symptoms. As the temperature or photoperiod increased, sporulation decreased until it was nil on plants under continuous light or at 28 C (Table 2).

To determine if the apparent inhibition of sporulation during a 7-day incubation period at a 24-hr photoperiod or 28 C was reversible, some plants

Figure 1. Effect of inoculum load of P. trifoliorum sporangia on Narragansett and Buffalo alfalfa seedling infection.

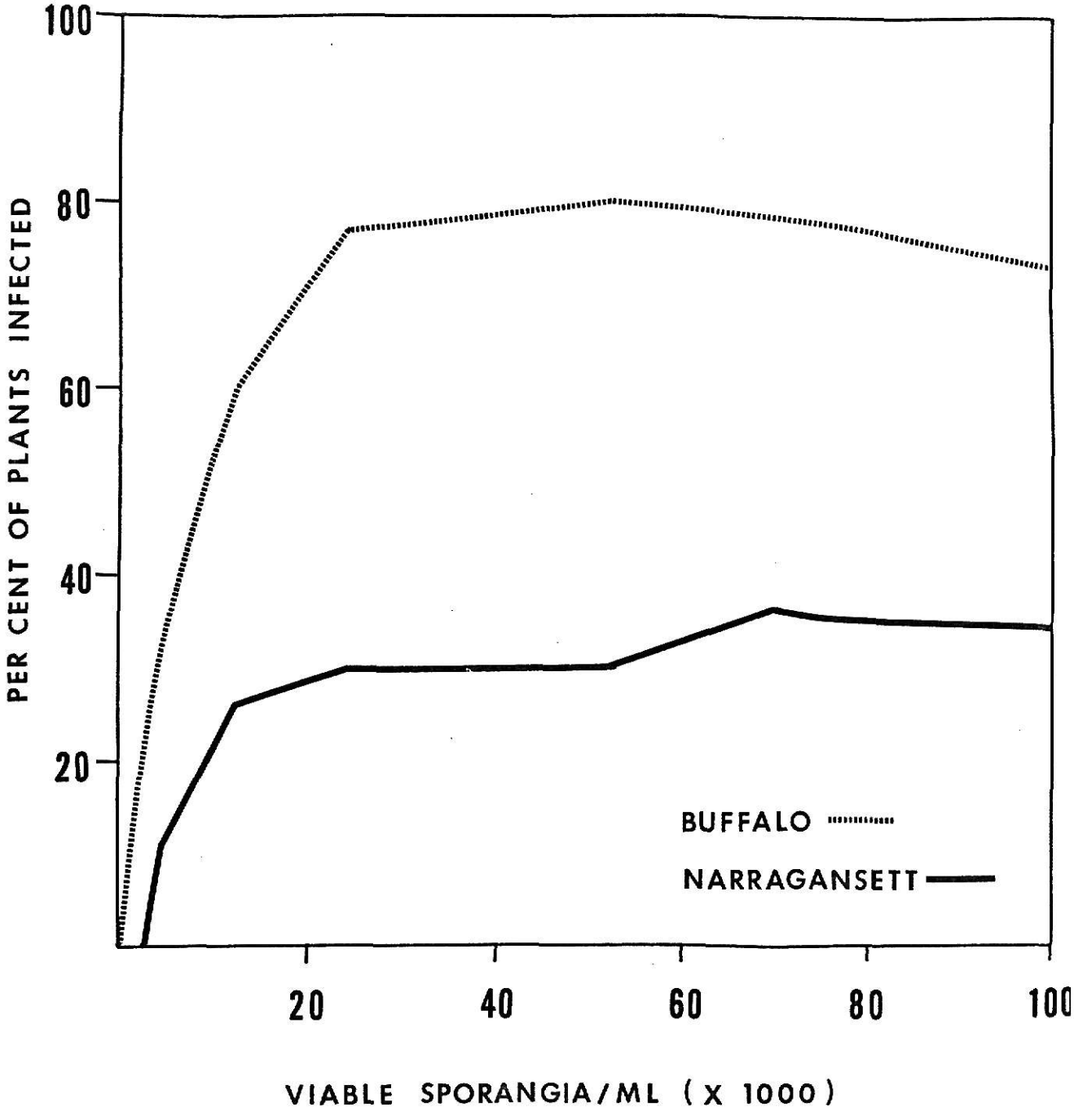
100
80
60
40
20
PER CENT OF PLANTS INFECTED
BUFFALO
NARRAGANSETT
20
40
60
80
100
VIABLE SPORANGIA/ML (X 1000)

Table 1. Effects at 20 C of light and the period of time plants are kept wet following inoculation with Peronospora trifoliorum on the resulting infection of Narragansett and Buffalo alfalfa seedlings.

Period (hr) of free water	% of seedlings infected				Overall free water period means[a]
	Narragansett		Buffalo		
	Light	Dark	Light	Dark	
8	25	28	44	67	41a
12	26	32	64	67	47b
16	26	30	64	68	47b
24	20	34	69	70	45b
Overall variety means[a]	27a		69b		
Overall light or dark means[a]	Light-41a		Dark-49b		

[a] Mean figures followed by different letters are significantly different at the .05 level.

Table 2. Effect of photoperiod and temperature on sporulation of *Peronospora trifoliorum* on infected Narragansett (N) and Buffalo (B) seedlings.

	% of infected seedlings with sporulation						
	Photoperiod (hr)						
	8		16		24		Overall
Temp C	N	B	N	B	N	B	temp mean[a]
20	100	100	77	90	0	I	62a
24	86	92	69	86	0	0	56b
28	0	3	0	0	0	0	Ic
Overall photoperiod means[a]	64a		54b		0c		
Overall variety means[a]	Narragansett-37a		Buffalo-41b				

[a] Mean figures followed by different letters are significantly different at the .05 level.

were placed in conditions more favorable for sporulation while others were continued another week under the original conditions. Sporulation did not occur on infected plants during 14 days under continuous light (Table 3), but did occur on 85 and 97% of infected Narragansett and Buffalo seedlings, respectively, kept 7 days under continuous light followed by 7 days at an 8-hr photoperiod. Water atomized on plants kept under continuous light did not promote sporulation.

Sporulation did not occur on infected seedlings during 14 days at 28 C but occurred on 24 and 63% of infected Narragansett and Buffalo seedlings, respectively, kept 7 days at 28 C followed by 7 days at 20 C (Table 4).

Oospore production in infected seedlings was affected by temperature but not by photoperiod (Table 5). By 7 days after inoculation, oospores were produced at 20 C and photoperiods of 8, 16, and 24 hr but were not produced at 24 or 28 C at any of these photoperiods. Oospores were not found in infected Narragansett seedlings grown at 20 C and an 8-hr photoperiod.

Table 3. Effect of photoperiod on sporulation of Peronospora trifoliorum at 20 C on infected seedlings of two alfalfa varieties.

Photoperiod (hr)		% of infected seedlings[a] with sporulation			
		Narragansett		Buffalo	
1st week	2nd week	1 week	2 weeks	1 week	2 weeks
24	24	0	0	0	0
24	8	0	85	0	97

[a] Seedlings with the chlorotic symptoms of downy mildew.

Table 4. Effect of temperature on sporulation of Peronospora trifoliorum on infected seedlings of two alfalfa varieties at an 8-hr photoperiod.

Temp (C)		% of infected seedlings[a] with sporulation			
		Narragansett		Buffalo	
1st week	2nd week	1 week	2 weeks	1 week	2 weeks
28	28	0	0	0	0
28	20	0	24	0	63

[a] Seedlings with the chlorotic symptoms of downy mildew.

Table 5. Effect of temperature and photoperiod on production of oospores by Peronospora trifoliorum in infected cotyledons of Narragansett (N) and Buffalo (B) alfalfa seedlings.

Temp (C)	Photoperiod (hr) 8 N	8 B	16 N	16 B	24 N	24 B
20	-	+[a]	+	+	+	+
24	-	-	-	-	-	-
28	-	-	-	-	-	-

[a] (+) indicates the presence of oospores 7 days after inoculation.

DISCUSSION

An inoculum load of 25,000 viable sporangia/ml provided nearly as many infected plants as did 100,000/ml. Resistance appeared to be maintained at viable sporangia concentrations of 100,000/ml.

I have no explanation for the low per cent of sporangial germination. There was no evidence of inhibition due to sporangial concentration. Uppal (22) reported failure of P. trifoliorum to germinate in the absence of oxygen, but lack of oxygen did not appear to be a factor in this case since germination did not increase on water agar where water was readily available.

No evidence of a light response was noted on the germination of P. trifoliorum sporangia. This is in contrast to P. manshurica sporangia which Pederson (17) reported were stimulated to germinate by light.

The direct penetration by P. trifoliorum is similar to that described for P. tabacina by Henderson (8). It is contrasted with P. destructor which penetrates through the stomata (27).

The period of time that leaves were kept wet after inoculation appears to be a critical factor for infection. Eight hr was not sufficient for maximum infection by P. trifoliorum, but 12 hr was. On the other hand, Hill (9) reported 4 hr was sufficient for infection by P. tabacina.

The reduction of sporulation under continuous light has also been noted on both P. destructor (25) and P. tabacina (3). Cruickshank (3) reported that continuous light at intensities of only 4 ft-c reduces sporulation of P. P. tabacina 50%. The reduction of sporulation of P. trifoliorum was reversible in most plants when returned to a favorable photoperiod. Perhaps both a light inhibitory mechanism and a diurnal cycle of sporangiophore production are involved similiar to that found in P. destructor by Yarwood (25).

The unfavorable effect of high temperature on sporulation of P. trifoliorum is similiar to that reported for other species of Peronospora. Yarwood reported 13 C optimum and 22-25 C maximum for sporulation of P. destructor (27). Jadot (13) reported 30 C for 5 days irreversibly inhibited sporulation of P. tabacina. The inhibition of sporulation by P. trifoliorum at 28 C was reversible in some infected plants when they were returned to 20 C. However, P. trifoliorum failed to sporulate on 76 and 36%, respectively, of the Narragansett and Buffalo seedlings with mildew symptoms when they were returned to 20 from 28 C. Because of this extreme variation in varietal response, more than temperature per se was apparently involved. More likely the fungus was less able to compete with the more resistant plants at 28 C and may have died although this was not determined. Pederson and Barnes (16) have postulated that resistance to downy mildew in alfalfa is conditioned by one tetrasomically inherited, incompletely dominant gene (Dm). Therefore, resistance is due to a dosage effect, with the nulliplex genotype (dmdmdmdm) being most susceptible and the quadriplex genotype (DmDmDmDm) being homozygous for resistance.

On this basis, infected plants on which the fungus did not sporulate when returned from 28 to 20 C would have predominantly duplex or simplex genotypes. Plants with triplex or quadriplex genotypes would be less likely to become infected while those on which sporulation occurred would have nulliplex genotype.

The higher level of resistance to P. trifoliorum reported for Narragansett compared to Buffalo under field conditions (6, 7) seemed to hold with seedlings under the range of environmental conditions tested. This suggests that seedlings can be screened for resistance to P. trifoliorum anytime, provided a means of controlling temperature and an initial inoculating source is available.

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ABSTRACT

Downy mildew of alfalfa (Medicago sativa L.), incited by Peronospora trifoliorum deBary, occurs during cool, moist conditions and causes chlorosis, necrosis, and stunting, and may kill young seedlings.

The purposes of this investigation were to determine the effects of light, temperature, and moisture on infection, sporulation, and oospore production by this obligate parasite, in order to facilitate subsequent research on disease resistance.

One-week-old seedlings of the relatively resistant alfalfa variety 'Narragansett' and the susceptible variety 'Buffalo' were sprayed with a suspension containing 25,000 viable sporangia/ml water. Higher concentrations did not increase disease incidence. Sporangial germination was not affected by light and averaged 55% on water agar in closed petri dishes and 43% on cotyledons. On cotyledons, the fungus formed appressoria and penetrated directly within 12 hr at 20 C. Maximum infection occurred in seedlings kept dark and wet for 21 hr following inoculation; 32 and 67% of Narragansett and Buffalo seedlings, respectively, became infected.

After the initial 12-hr infection period and subsequent 8-hr photoperiods at 20, 24, and 28 C, for one week, sporulation occurred on 100, 86, and 0%, respectively, of Narragansett seedlings and on 100, 92, and 3%, respectively, of infected Buffalo seedlings. Sporulation was most profuse at the lower temperatures. At 20 C and photoperiods of 8, 16, and 24 hr, sporulation occurred on 100, 77, and 0% respectively, of infected Narrangansett seedlings and on 100, 90, and 1%, respectively, of infected Buffalo seedlings.

The inhibition of sporulation by a 24-hr photoperiod or at 28 C was partially reversible. At an 8-hr photoperiod, sporulation occurred on 24 and

63% of infected Narrangansett and Buffalo seedlings, respectively, when returned from 7 days at 28 C to 20 C for 7 more days. At 20 C, sporulation occurred on 85 and 97% of infected Narrangansett and Buffalo seedlings, respectively, when returned from 7 days under continuous light to an 8-hr photoperiod for 7 days.

Oospores were found 7 days after inoculation in cotyledons of plants grown at 20 C and photoperiods of 8, 16, and 24 hr, but were not found in those grown at 24 or 28 C at these photoperiods.