I. ENVIRONMENTAL EFFECTS ON PERONOSPORA TRIFOLIORUM OOSPORE PRODUCTION IN SEEDLINGS OF TWO ALFALFA CLONES

II. ATTEMPTS TO GERMINATE PERONOSPORA TRIFOLIORUM OOSPORES

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I. ENVIRONMENTAL EFFECTS ON PERONOSPORA TRIFOLIORUM OOSPORE PRODUCTION IN SEEDLINGS OF TWO ALFALFA CLONES

Conidia of Peronospora trifoliorum d By. are short-lived (3) so oospores must be important in long distance dissemination of the fungus. However, oospore germination has not been demonstrated, and little is known about their production. Rockett (10) observed oospores 7 days postinoculation in alfalfa seedlings at 20 C but not at 24 or 28 C. He did not report oospore numbers.

Oospore production by other Peronospora sp. are favored by relative humidities exceeding 90% for P. tabacina (9), P. parasitica (5), and P. viciae (8). High light intensity increased oospore production by P. tabacina (4) but not by P. parasitica (5). Conditions that induced cabbage leaf senescence favored P. parasitica oospore formation (7).

This research was to determine effects of temperature, photoperiod, light intensity, and conidium production on P. trifoliorum oospore production in alfalfa seedlings.

MATERIALS AND METHODS

To determine optimum conditions for oospore production, I used S_1 seedlings of two alfalfa (Medicago sativa L.) clones and a monoconidial isolate (I-7) of Peronospora trifoliorum. Isolate I-7 produced many conidia on all S_1 seedlings of clone 18-21 and moderate numbers on those from clone 275-1.

Seeds were planted 1 cm deep in steam-sterilized, masonry sand in three rows (10 seeds/row) in 9.5 X 9.5 X 8-cm deep plastic pots. Pots were placed in growth chambers at 20 C and about 5,000 lux of continuous cool-white

fluorescent lighting. Four days later, the seedlings were inoculated. Inoculum was prepared by excising infected seedlings below the cotyledons (15 hr after inducing conidium production by placing seedlings in dark at near 100% relative humidity (3)), placing them in a jar with deionized water, and shaking the jar to dislodge the conidia. The resulting suspension was filtered through a tea strainer to remove the plants, adjusted to 10^5 conidia per ml, and then atomized onto the seedlings until runoff. After a 24-hr infection period at 20 C in darkness, plants were placed in growth chambers at 12, 16, 20, or 24 C for photoperiods of 8, 16, or 24 hr of either 6,500 or 10,000 lux of light from cool-white fluorescent tubes. Relative humidity (RH) was not controlled but was low enough (3) to prevent conidium production. Plants were watered with deionized water to avoid adverse effects of chlorine in tap water on P. trifoliorum (2).

To evaluate oospore production, I removed five plants from each pot at 7, 14, and 21 days after inoculation, placed them in test tubes with 5 ml lactophenol (11) with Sudan IV (0.2 g Sudan IV first dissolved in a small amount of 95% ethyl alcohol/liter lactophenol), and autoclaved 15 min at 122 C to stain the oospores.

The plants from each test tube were transferred to a 50-ml centrifuge tube with 5 ml water and homogenized with Brinkmann Polytron (Model 10-20-3500, Brinkmann Instruments; Westbury, NY 11590) for 15 sec at a 3.5 speed setting. The number of fully developed oospores in 0.25 ml of the homogenized material was determined microscopically (100 X) and multiplied by four to estimate the number produced per plant.

All oospore counts were square-root transformed, and treated by analysis of variance; means were separated by Duncan's multiple range test for significance, P=0.05.

RESULTS

Isolate I-7 of Peronospora trifoliorum produced oospores in all of the wide range of treatments used but production was greatest in 275-1 seedlings held at 16 C with 10,000 lux of continuous lighting (Table 1), but production in 18-21 was favored by the 16-hr photoperiod; 16 C favored production regardless of light conditions. Light requirements, especially with 275-1, were less closely defined, although oospore numbers were usually largest with longer photoperiods and/or higher intensities. Thus, total illuminance seemed more important than either photoperiod or light intensity per se. Maximum oospore production (11,081 per 275-1 seedling) was at the highest illuminance used, thus optimum illuminance was not demonstrated for seedlings of that clone. However, oospore concentration was so dense in that significant increases would be doubtful.

More oospores were produced in 275-1 than in 18-21 seedlings under most experimental conditions (Table 1) but downy mildew symptoms were much more severe and oospore production varied more in 18-21. Cotyledons of 275-1 plants were dead three weeks after inoculation and contained most of the oospores (Table 2). The other oospores were in unifoliolate leaves, which likely were forming at inoculation time. There was no evidence of oospore development or systemic invasion above the unifoliolate leaves. However, a high percentage of 18-21 plants in most treatments had typical chlorotic mildew symptoms and stunting from systemic invasion, which was most severe at 12 C and 24-hr photoperiod of 10,000 lux. Stunting was considerably less at 16 C, and decreased further as temperature increased and photoperiod or intensity decreased. Oospores were distributed throughout the systemically invaded 18-21 plants and the greater the stunting, the lower the percentage of oospores formed in the cotyledons (Table 2).

TABLE 1. Effects of temperature, photoperiod, and light intensity on the mean number of Peronospora trifoliorum oospores produced per S_1 seedling of two alfalfa clones in 21 days

Photo period (hr)	Temperature (C)	Clone 275-1 Light intensity (lux) 6,500	Clone 275-1 Light intensity (lux) 10,000	Clone 18-21 Light intensity (lux) 6,500	Clone 18-21 Light intensity (lux) 10,000
8	12	88^{rst} [z]	$2,788^{ge}$	158^{qrst}	$1,005^{lmno}$
8	16	298^{pqr}	$2,090^{ghij}$	254^{qrs}	$1,485^{ijklm}$
8	20	180^{qrst}	$1,461^{ijklm}$	929^{mno}	$1,405^{ijklmn}$
8	24	62^{rst}	857^{no}	76^{st}	670^{op}
16	12	$1,377^{ijklm}$	$3,079^{ef}$	683^{op}	$2,031^{ghij}$
16	16	$2,420^{fgh}$	$6,600^{bc}$	$1,622^{hijkl}$	$2,115^{ghi}$
16	20	$1,001^{lmno}$	$3,851^{de}$	$1,347^{ijklmn}$	361^{pq}
16	24	108^{rst}	$2,017^{ghij}$	38^{t}	232^{qrst}
24	12	$1,787^{hijk}$	$1,811^{hijk}$	262^{qrst}	$1,166^{klmno}$
24	16	$5,885^{c}$	$11,081^{a}$	$1,036^{lmno}$	$2,057^{ghij}$
24	20	$3,901^{de}$	$7,496^{b}$	297^{pqr}	335^{pqr}
24	24	$1,326^{jklmn}$	$4,362^{d}$	151^{qrst}	225^{qrst}

[z]Means followed by the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test.

TABLE 2. The mean percentage of *Peronospora trifoliorum* oospores produced in cotyledons of S_1 seedlings of two alfalfa clones 21 days after inoculation as affected by temperature and photoperiod at 6,500 lux

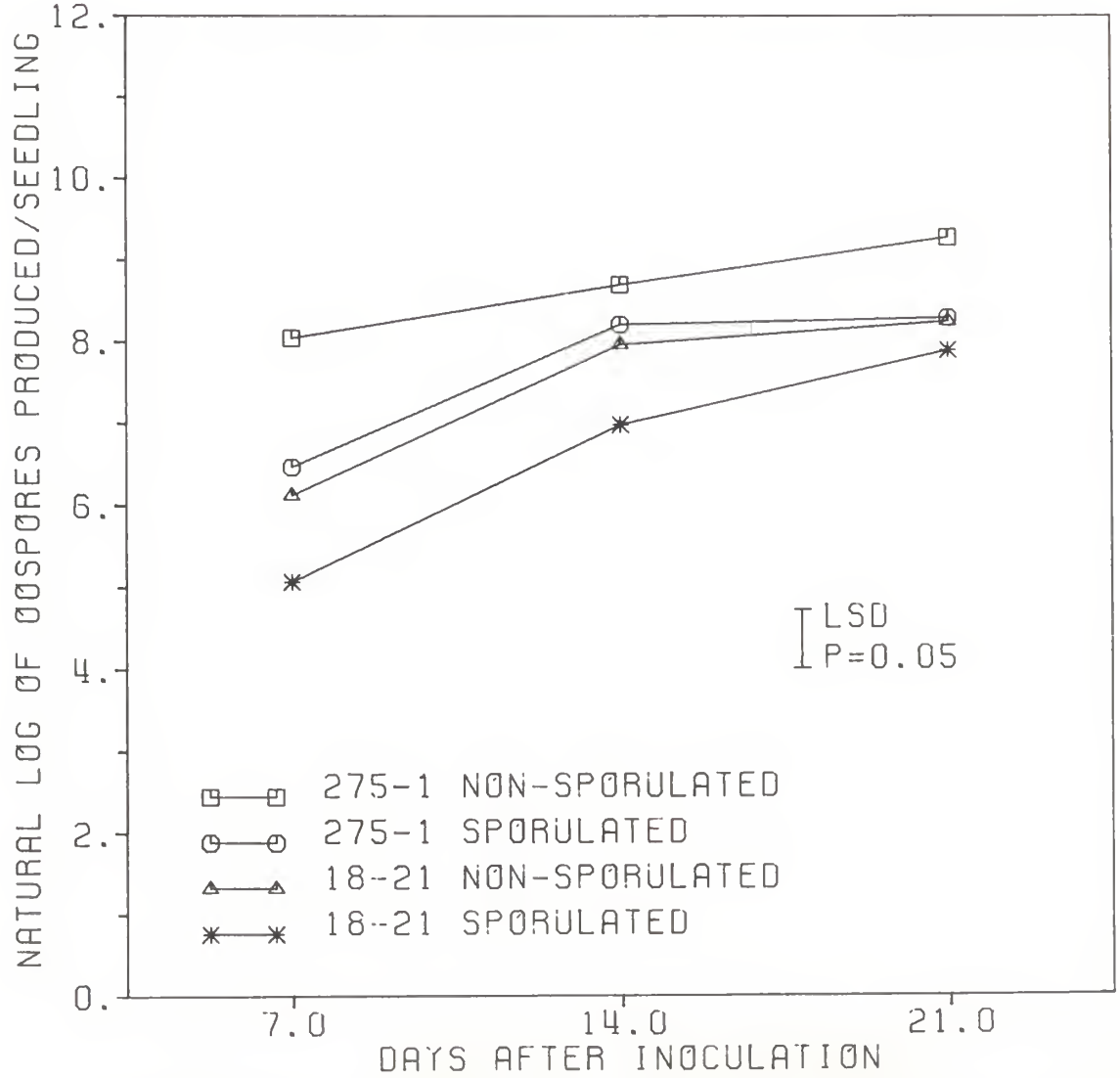
Photo-period (hrs)	Temper-ature (C)	Clone	
		275-1	18-21
8	12	92.5	31.5
8	16	93.9	84.8
8	20	96.9	90.6
8	24	97.3	90.9
16	12	85.8	17.2
16	16	91.1	76.1
16	20	98.2	90.3
16	24	100.0	100.0
24	12	91.4	12.8
24	16	90.6	42.6
24	20	98.6	53.0
24	24	99.6	65.7

Developing oogonia were abundant in the upper trifoliolate leaves of the more severely stunted plants, indicating that oospore production was continuing.

One week after inoculation oospores were found in all treatments except those at 12 C and 6,500 lux. At 20 C and 24-hr photoperiod of 10,000 lux intensity, 2,960 oospores were found per 275-1 seedling and 65 per 18-21 seedling. In addition, the number of oogonia and developing oospores not counted equaled approximately the number of oospores counted. At 6,500 lux there were 648 oospores per 275-1 seedling and 19 per 18-21 seedling. Because those conditions approximated those used to produce conidia for inoculum, the effect of conidium production on oospore production was investigated. Inoculated seedlings, held 24 hr in darkness at 20 C to allow infection, were placed in a growth chamber at 16 C and 12-hr photoperiod of 10,000 lux intensity. To increase relative humidity to induce conidium production, I placed plants in nearly air-tight plastic boxes daily for three weeks during the 12-hr dark period (3). All oospore counts were transformed to natural logs for statistical analyses, and means were separated by the Least Significant Difference test, P=0.05.

One week after inoculation, significantly fewer oospores were produced in 275-1 or 18-21 seedlings during conidium formation than when the fungus produced only oospores (Fig. 1). Significantly fewer oospores were produced by three weeks after inoculation in 275-1 but not 18-21 plants, where the fungus produced conidia. The cotyledons of 275-1 plants in which the fungus was induced to produce conidia senesced earlier than those that produced only oospores. However, cotyledons of 18-21 plants were still light green three weeks after inoculation whether the fungus produced both spore types or only oospores.

Fig. 1. Effects of conidium production (sporulation) on oospore production of Peronospora trifoliorum in S_1 seedlings of two alfalfa clones.


NATURAL LOG OF OOSPORES PRODUCED/SEEDLING
12.
10.
8.
6.
4.
2.
0.
7.0
14.0
21.0
DAYS AFTER INOCULATION
LSD
P=0.05
275-1 NON-SPORULATED
275-1 SPORULATED
18-21 NON-SPORULATED
18-21 SPORULATED

DISCUSSION

The Peronospora trifoliorum isolate used produced oospores over a wide range of environmental conditions but production was clearly favored by 16 C and 10,000 lux of continuous light intensity. The 16-hr photoperiod was optimum for production in 18-21, but 275-1 contained more oospores under continuous light. The fungus was mostly restricted to the 275-1 cotyledons, thus the benefit from increased illuminance may have been nutritional and caused by increased host photosynthesis. Continuous light would aid oospore production under humid conditions by preventing conidium formation.

Rockett (10) observed P. trifoliorum oospores 7 days after inoculation in plants at photoperiods of 8, 16, and 24 h at 5,380 lux at 20 C but not at 24 C. We noted some production at 24 C under the same photoperiods at 6,500 lux so the lower light intensity he used may have delayed production.

Under nearly all of our treatments considerably more oospores were produced in 275-1 than in 18-21 seedlings. However, total production likely would have changed with additional time since oospore production had ceased in most 275-1 plants but was continuing in many 18-21 plants three weeks after inoculation.

I have no explanation why I-7 hyphae quickly ramified throughout the 275-1 seedling cotyledons, yet, with few exceptions did not invade the plants systemically.

Some workers have disagreed on the effects of conidium production on oospore production. McKay (6) observed fewer P. destructor oospores in infected onion tissue, where conidia were also produced, in the field and concluded that conidium production used up food reserves so they were unavailable for oospore production. Our data with P. trifoliorum tend to

support that view. However, Berry and Davis (1) observed occasional P. destructor oospore production in both sporulating and nonsporulating onion tissue in the greenhouse, and concluded production of one spore type was not antagonistic of the other. McMeekin (7) concluded P. parasitica oospores were not formed at the expense of conidial production.

My data may also explain why fewer conidia are produced by the fungus on 275-1 than on 18-21 seedlings at 7 days after inoculation. Under conditions (20 C with about 6,300 lux of continuous lighting) at which we maintain inoculum the fungus produces many conidia on 18-21 seedlings but few oospores. However, while only a moderate number of conidia are produced on 275-1 plants, they produced 6 times more oospores than the 18-21 seedlings did. Thus, the lower conidium production observed on 275-1 seedlings may result from higher oospore production at 7 days after inoculation.

Developing procedures to produce P. trifoliorum oospores that cannot be germinated may seem premature. However, I hope that having the means to readily produce large numbers of oospores in a laboratory will benefit germination studies.

LITERATURE CITED

1. BERRY, S. Z., and G. N. DAVIS. 1957. Formation of oospores by Peronospora destructor and their possible relation to epiphytology. Plant Dis. Rep. 41:3-6.

2. FRIED, P. M., and D. L. STUTEVILLE. 1975. Effect of chlorine on Peronospora trifoliorum sporangial production and germination. Phytopathology 65:929-930.

3. FRIED, P. M., and D. L. STUTEVILLE. 1977. Peronospora trifoliorum sporangium development and effects of humidity and light on discharge and germination. Phytopathology 67:890-894.

4. IZARD, C., and P. SCHILDZ. 1961. Quelques précisions concernant la méthod de culture de Peronospora tabacina Adam, sur des disques découpés dans le limbe des feuilles et premières observatuons "in vivo." Ann. Inst. Exptl. Tabac Bergerac 3(4):659-668.

5. LeBEAU, F. J., and J. A. PINCKARD. 1942. Oospore production in cabbage seedlings by Peronospora parasitica. Phytopathology 32:648 (Abstr.).

6. McKAY, R. 1939. Observations on onion mildew caused by the fungus Peronospora schleideniana W. G. Sm. Roy. Hort. Soc. Jour. 64:272-285.

7. McMEEKIN, D. 1960. The role of the oospores of Peronospora parasitica in downy mildew of crucifers. Phytopathology 50:93-97.

8. PEGG, G. F., and M. J. MENCE. 1970. The biology of Peronospora viciae on pea: laboratory experiments on the effects of temperature, relative humidity and light on the production, germination and infectivity of sporangia. Ann. Appl. Biol. 66:417-428.

9. PERESYPKIN, V. F., and V. A. MARKHAS'OVA. 1966. Ekolohichni osobl-yvosti formuvannya spor hryba Peronospora tabacina Adam. Zakhyst Ruslyn Resp Mizhvidomchyi Temat Nauk ZB. 3:72-77.

10. ROCKETT, T. R. 1970. Some effects of photoperiod, temperature, and humidity on infection, sporulation, and oospore production by Peronospora trifoliorum on seedlings of two alfalfa varieties. M.S. Thesis. Kansas State University, Manhattan. 20p.

11. TUITE, J. 1969. Plant pathological methods fungi and bacteria. Burgess Publishing Co., Minneapolis, MN. 239p.

II. ATTEMPTS TO GERMINATE PERONOSPORA TRIFOLIORUM OOSPORES

Oospore germination has been described for several Peronospora species (6, 8, 13, 15, 20, 26) but not for P. trifoliorum. McKay (15) reported that 1-2% of P. destructor oospores from diseased onion foliage, stored 4-5 years in a box outdoors, germinated in 11 days when placed in water. However, the germination percentage increased to 60-85% when the oospores were placed in a 0.01-0.02% potassium permanganate solution for 48 hours. Germination after 7 years was about 5% in water but 95% in the $KMnO_4$ solution at 20 C. Berry and Davis (6) observed two germinating P. destructor oospores on naturally infected onion foliage collected from the field, sprayed with water, and incubated 4 hours at 10 C and 100% relative humidity. The oospores were surrounded by soil particles indicating they were splashed onto the foliage since the foliage collected was not touching the ground.

Peronospora tabacina oospores in decayed tobacco leaves germinated at 2-24 C after being placed intermittently in cold storage and at room temperature for 7 months (26). However, less than a dozen oospores germinated of several thousand observed. Person and Lucas (17) reported blue mold symptoms on seedling tobacco planted in a soil sterilized and then infested with tobacco leaves (0.33 cm deep) containing P. tabacina oospores. When dried ground pea pods containing P. pisi oospores were buried around and slightly above pea seeds, the resulting seedlings grown at 12-15 C developed mildew symptoms (20). Poppy seedlings in various soils infested with one or two-year-old material containing P. aborescens oospores developed mildew symptoms (13). Fewer symptoms developed if inoculum used was 3 years old, and no symptoms developed if inoculum was less than 1 year old. Attempts to germinate the oospores in vitro failed.

Dunleavy and Snyder (8) reported Peronospora manshurica oospores, in seedcoats of soybeans, germinated after being washed in running tap water for one week and concluded that an inhibitor prevents oospore germination.

The work here was initiated to determine the requirements for Peronospora trifoliorum oospore germination.

MATERIALS AND METHODS

Oospore production method.-Four-day-old alfalfa (Medicago sativa L.) seedlings (planted 1 cm deep in steam sterilized sand) of cultivar Kanza or S_1 seedlings of two alfalfa clones (275-1 and 18-21) were inoculated with conidia of Peronospora trifoliorum. Inoculum sources were monoconidial isolates I-5, and I-7 isolated from Kanza material and El Centro-2 and 3 isolated from mildewed alfalfa sent from El Centro, CA by W. F. Lehman as well as mixed cultures from field collections.

Inoculum was prepared by excising infected seedlings below their cotyledons and shaking them in a jar with deionized distilled water (DDW) to dislodge the conidia. The resulting suspension was passed through a tea strainer to remove plant material and atomized onto the seedlings (10). After a 24-hour infection period at 20 C in darkness, plants were placed in growth chambers at various temperatures, photoperiods, and light intensities.

Infected plants were harvested and either dried at room temperature (RT) and stored in manila envelopes in the laboratory for later use or the oospores were extracted and used immediately in germination experiments.

Blender extraction of oospores from plant material.-Oospores were extracted by grinding infected alfalfa seedlings in 100 ml of DDW with either a Waring Blendor (Model 702 BAW; Waring Products Corp., Winstead,

Conn.) at high speed for 2 min or a Brinkmann Polytron (Model 10-20-3500; Brinkmann Instruments, Inc., Westbury, NY 11590) for 2 min at the No.5 speed setting. The resulting suspensions were poured onto a sieve series composed of a No.270 and No.325, U.S.A. standard Testing Sieve (W. S. Tyler, Inc., Mentor, Ohio 44060) and followed by DDW rinses. Most of the plant debris was retained by the No.270 sieve (53 μm screen opening), and most of the oospores were retained by the No.325 sieve (45 μm screen opening). The oospores were collected from the No.325 sieve and stored in DDW at 5 C in the dark until needed. These are referred to as "blender extracted oospores."

Snail extraction of oospores from plant material.-Live water snails (Pomacia sp.) or pond snails of several species from Carolina Biological Supply, Burlington, NC 27215, were fed infected alfalfa and the oospores were recovered from their feces. Dry, oospore-infested alfalfa seedlings were ground with a mortar and pestle, moistened with DDW, and the resulting slurry was spread on glass microscope slides to dry at RT. The slides were placed in a 1000-ml beaker of DDW where snails, starved for 24-48 hours, ingested the oospore-infested material. The snail feces, contained a high concentration of oospores with their oogonial wall digested away. The feces were collected, crushed with a glass stirring rod, and suspended in DDW. Within a few seconds the heavier particles settled to the bottom and the liquid was decanted and centrifuged for 2 min (supernatant discarded) to collect the oospores. These "snail-extracted oospores," relatively free of plant debris, were resuspended in a small amount of DDW and stored at 5 C in the dark until needed.

Bacterial extraction of oospores from plant material.-Dry, oospore-infested alfalfa seedlings were ground with a mortar and pestle to a fine powder and 0.067 g of the material was added to 100 ml of DDW in a 250-ml

Erlenmeyer flask. The suspension was inoculated with Xanthomonas alfalfae and placed on an orbital shaker in the laboratory. After 14 days the suspension was passed through 4 layers of cheesecloth, rinsed four times in DDW to remove the plant debris, and then treated with vancomycin (200 µg/ml) to inhibit the bacteria. The oospores were collected by centrifuging the filtrates, and then were washed to remove the vancomycin and resuspended in DDW. These "bacterial-extracted oospores" were stored at 5 C in the dark until needed.

RESULTS AND DISCUSSION

Root exudates and soil infestation.-Previous studies, besides those involving members of the genus Peronospora (6, 8, 13, 15, 20, 26), have described the germination of oospores in response to root exudates. Pythium mamillatum oospores germinated in response to turnip seedling exudates (4), and Aphanomyces euteiches oospores germinated in response to root exudates of several plant species (22). Different techniques, based on either the production of root exudates or use of buried oospore-infested material as a source of inoculum, were used to germinate P. trifoliorum oospores.

1. Oospore-infested material (Table 1; F1-2) was buried in non-sterile soil slightly above, below, or at the level at which Kanza seeds were planted. Plants were grown at 16 C under photoperiods of 8, 16, or 24 hours for 6 months. At various times the plants were cut back, then covered to provide the conditions necessary for conidium production (10) while new growth was forming, but no symptoms were observed.

2. Alfalfa seeds from selfed clone 275-1 plants were planted in non-sterile soil infested with an oospore suspension (Table 1; F2-3) and placed

in a growth chamber at 16 C under photoperiods of 8, 16, or 24 hours. Oospores were recovered from portions of the soil every second day for 30 days, placed in DDW at RT, but no germination was observed.

3. Five-ml oospore suspensions (Table 1; E1-8) were placed in boiled, 8-cm long cellulase casings, then buried in a pan of steam-sterilized masonry sand slightly below the level at which Kanza seeds were planted. The casings were removed after 30 days at 20 C with continuous light, and the oospore suspensions were transferred to petri dishes at RT and light. Oospores were examined microscopically daily for 7 days, no germination was observed.

4. Kanza seeds were moistened with DDW and germinated at 20 C under continuous light. The seeds were rinsed daily for 10 days with 100 ml of DDW, and a 5-ml oospore suspension (Table 1; A3-4, D2-3, E1-2, F4-5) was added to the rinse water, then placed on the orbital shaker in the laboratory. The oospores were collected after 24 hours by centrifugation for 2 min, and discarding the supernatant. The pellet was resuspended in 100 ml of DDW which had been used to rinse the germinating alfalfa seeds the second day. This procedure was repeated 9 times using the rinse water of each succeeding day to resuspend the oospores. Oospores were examined with the microscope daily, but no germination was observed.

Foliar inoculation with oospores.-Berry and Davis (6) found germinated P. destructor oospores on onion foliage. Several techniques, based on foliar inoculation with P. trifoliorum oospores, were used in attempts to obtain infection on alfalfa seedlings.

1. An oospore suspension (Table 1; B4-5, B8, E6) was atomized onto four-day-old Kanza seedlings which were covered to provide high relative humidity and placed in the dark at 20 C. Following a 24-hour infection period the plants were uncovered and placed under continuous light. At

TABLE 1. Sources of *Peronospora trifoliorum* oospores used in germination attempts

Oospore source code[a]	Alfalfa cultivar or clone oospores produced in	*P. trifoliorum* isolate(s) used	Age of oospores (days)[b]	Oospore extraction method[c]	Special treatments
A1	Kanza	I-7	17	Blender	None
A2	Kanza	I-7	21	Blender	None
A3	Kanza	I-7	60	Blender	None
A4	Kanza	I-7	61	Blender	None
A5	Kanza	I-7	21	Blender	None
A6	Kanza	I-7	60	Blender	None
A7	Kanza	I-7	42	Blender	None
A8	Kanza	I-7	30	Nonextracted	None
A9	Kanza	I-7	60	Nonextracted	None
B1	Kanza	I-7	42	Blender	None
B2	Kanza	I-7	21	Blender	30 days at 10 C
B3	Kanza	I-7	42	Blender	None
B4	Kanza	I-5 & I-7	21	Blender	None
B5	275-1	I-7	21	Blender	None
B6	275-1	I-7	21	Blender	None
B7	275-1	I-5 & I-7	21	Blender	None
B8	18-21	I-7	21	Blender	None
B9	Kanza	I-7	21	Blender	None

TABLE 1. Continued

Oospore source code[a]	Alfalfa cultivar or clone oospores produced in	*P. trifoliorum* isolate(s) used	Age of oospores (days)[b]	Oospore extraction method[c]	Special treatments
C1	Kanza	I-7	21	Bacterial	None
C2	Kanza	El Centro-2 & 3 I-5 & I-7	48	Blender	None
C3	Kanza	I-7	77	Blender	None
C4	Kanza	I-5 & I-7	77	Blender	None
C5	Kanza	El Centro-2 & 3 I-5 & I-7	48	Blender	Fertilized[d]
C6	Kanza	I-5	77	Blender	None
C7	Kanza	I-5	21	Blender	Dialyzed[e]
C8	Kanza	I-7	21	Blender	Dialyzed[e]
C9	Kanza	I-5	61	Blender	Dialyzed[e]
D1	Kanza	I-7	60	Blender	Dialyzed[e]
D2	Kanza	El Centro-2 & 3 I-5 & I-7	27	Blender	Fertilized[d]
D3	Kanza	El Centro-2 & 3 I-5 & I-7	27	Blender	None
D4	Kanza	Field sample[f]	21	Blender	None
D5	Kanza	Field sample[f]	21	Blender	Fertilized[d]

TABLE 1. Continued

Oospore source code[a]	Alfalfa cultivar or clone oospores produced in	*P. trifoliorum* isolate(s) used	Age of oospores (days)[b]	Oospore extraction method[c]	Special treatments
D6	Kanza	I-7	49	Snail	None
D7	Kanza	I-5	49	Snail	None
D8	Kanza	I-5 & I-7	49	Snail	None
D9	Kanza	I-7	17	Blender	Dialyzed
E1	275-1	I-7	21	Snail	None
E2	18-21	I-7	21	Snail	None
E3	Kanza	I-7	28	Blender	None
E4	275-1	I-7	28	Blender	None
E5	18-21	I-7	28	Blender	None
E6	Kanza	I-7	28	Snail	None
E7	Kanza	I-5 & I-7	28	Bacterial	Feed to snails
E8	Kanza	I-5 & I-7	28	Blender	None
E9	Kanza	I-5 & I-7	49	Blender	Snail enzyme 24 hrs[h]
F1	Kanza	I-5 & I-7	35	Nonextracted	Ground to a fine powder
F2	275-1	I-5 & I-7	35	Nonextracted	Ground to a fine powder

TABLE 1. Continued

Oospore source code[a]	Alfalfa cultivar or clone oospores produced in	*P. trifoliorum* isolate(s) used	Age of oospores (days)[b]	Oospore extraction method[c]	Special treatments
F3	275-1	I-7	21	Nonextracted	Infested cotyledons
F4	275-1	I-7	21	Snail	Stored in liquid nitrogen for 24 hrs
F5	275-1	I-7	21	Snail	Stored at -20 C for 24 hrs
F6	Kanza	I-5 & I-7	35	Nonextracted	None
F7	Kanza	I-7	35	Blender	None
F8	Kanza	I-5	35	Blender	None
F9	Kanza	I-5 & I-7	35	Blender	None

[a]Oospore source code referred to in Tables 2-8.

[b]Days after inoculation oospore infested plants were harvested.

[c]Method used to extract oospores from infected plant material as described in Materials and Methods.

[d]Plants fertilized with Patterson's Soluble Plant Food (20-20-20) from Patterson Chemical Company, Kansas City, MO.

[e]Dialyzed 10 days in running tap water.

[f]Inoculum was a mixture of isolates obtained from sporulating field samples of infected alfalfa.

TABLE 1. Continued

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[g]Dialyzed for 7 days in running tap water.

[h]Beta-glucuronidase (from Helix pomatia) from Sigma Chemical Company, St. Louis, MO.

7 and 14 days after inoculation the plants were again covered and placed in the dark for 16 hours. No mildew symptoms developed.

2. The procedure was repeated as before using seeds of both Kanza and 275-1. However, prior to inoculation the cotyledons were injured by scratching with a needle, but no mildew symptoms developed within 21 days.

3. Four-day-old Kanza seedlings were inoculated with an oospore suspension (Table 1; B7) which had been mixed with a non-sterile soil extract four days prior to use. The remainder of the procedure followed was as described in section 1, but no germination was observed.

Overwintering of oospore-infested material.-Previous studies (15, 26) described oospore germination following exposure, sometimes prolonged, to natural climatic changes or overwintering. McKay (15) reported that the percentage germination of P. destructor oospores increased by treatment with potassium permanganate following an overwintering treatment. P. trifoliorum oospore-infested material was buried outdoors in a garden soil for two years. Oospores were recovered periodically and treated with potassium permanganate. Other tests were also run to simulate the overwintering conditions.

1. Oospore-infested material (Table 1; F6) was buried 1 cm deep with soil in pots and placed outdoors in November 1976. The following April oospores from a portion of the material were recovered using a series of sieves as described in Materials and Methods for oospore extraction from plant material and placed in a 0.01-0.02% solution of $KMnO_4$. Oospores were examined with the microscope daily for 7 days, but no germination was observed. In another portion of the material 275-1 seeds were planted, placed at 16 C under a 24-hour photoperiod for 30 days, but no mildew symptoms developed.

In April 1978, oospores from another portion of the material buried in November 1976 were recovered and placed in a 0.01-0.02% $KMnO_4$ solution. After 24 hours 3 oospores appeared to have thin points along their endospore walls, but no germ tubes were observed.

2. To simulate overwintering conditions oospore-infested alfalfa seedlings (Table 1; F1) were buried 1 cm deep in pots of non-sterile soil between two layers of cheesecloth and alternately frozen and thawed, at 24-hour intervals, for 30 days. Oospores were blender-extracted from seedlings recovered from between the cheesecloth and placed in DDW, sterile tap water, $CaCl_2$ (1 μg/ml), or $KMnO_4$ solutions (0.01-0.02%). Oospores were examined daily for 7 days, but no germination was observed.

Dialysis of oospores.-*Peronospora manshurica* oospores germinated after 1 week of dialysis in running tap water (8). *Peronospora trifoliorum* oospore suspensions and whole, oospore-infested cotyledons were placed in dialysis tubing in running tap water for 7-10 days. Bags were then transferred to a beaker containing 1000 ml of DDW which was changed daily for 52 days. No germination was observed. The dialysis procedure was repeated using only the 1000-ml of DDW, changed daily, for 45 days, but no germination was observed. The sources of oospores used, dialysis treatment, and the length of time the materials were exposed to each treatment are listed in Table 2.

Temperature.-High and low temperatures triggers germination of various spore types of several organisms (25) and were used with *P. trifoliorum* oospores.

1. Oospore suspensions were centrifuged for 2 min and the supernatant discarded. Each pellet was resuspended in 5 ml of 15% DMSO, and 1-ml aliquots of the suspensions were placed in 1-ml semen ampules and sealed with a flame. Some of the ampules were placed in liquid nitrogen (-196 C)

after freezing for 1 hour at -20 C. Ampules were removed from the liquid nitrogen at various intervals and heat-shocked by transferring them directly to water baths at various temperatures. The suspensions were removed from the ampules, washed twice with DDW in the centrifuge to remove the DMSO, and a few drops were placed on glass microscope slides in petri dishes containing moistened filter paper. The oospores were incubated at various temperatures under various photoperiods, and oospores were examined microscopically at 4, 8, 48, 72, and 96 hours. No germination was observed. (Table 3).

2. Oospores were placed in DDW maintained at 40-90 C, at intervals of 10 C, for 1 hour, then incubated at RT and light for 3 days, but no germination was observed (Table 3).

3. Oospore suspensions were frozen at -5 C for 24 hours and then placed at RT for 24 hours. Microscopic examination was made of the material at 48-hour intervals for 30 days. No germination was observed (Table 3).

Light.-Light may influence oospore production and germination in the genus Phytophthora (3, 5, 12, 14, 19). Monochromatic light sources in the blue (450 nm) and far-red (750 nm) regions stimulated oospore germination of P. cinnamoni, P. megasperma var. sojae (race 1 and 2), and P. capsici (19). Blue monochromatic light and various photoperiods at two light intensities were used in attempts to germinate P. trifoliorum oospores, but all failed.

1. The apparatus used to supply the blue monochromatic light (450 nm) has been previously described (18, 19). Oospore suspensions (Table 1; A2, A5, B4, C2-9, D1-9) were placed in two 10-cell microscope slides, covered with glass cover slips, and placed inside of a 16 X 16 X 16-cm wooden box. The top of the box had an 8 X 8-cm window over which a blue primary filter (Carolina Biological Supply, Burlington, NC 27215) was placed. Placed on

TABLE 2. The effects of dialysis on germination of Peronospora trifoliorum oospores

Source of oospores[a]	Dialysis treatment 1[b]	Dialysis treatment 2[c]	Length of treatment (days)	Percent oospore germination
A1	+	-	7	0
A2	+	+	10 (52)[d]	0
A3	+	+	10 (52)[d]	0
A4	+	+	10 (52)[d]	0
A5	+	+	10 (52)[d]	0
A6	+	+	10 (52)[d]	0
A7	+	+	10 (52)[d]	0
A3	-	+	45	0
A4	-	+	45	0
A5	-	+	45	0
A6	-	+	45	0
A8	+	+	10	0
A9	+	+	10	0
B1	+	+	10	0
B2	+	+	10	0
B3	+	+	10	0
B4	+	+	10	0

[a]Code refers to oospore source as listed in Table 1.

[b]Treatment 1; 5 ml of oospore suspension were placed in dialysis tubing and washed under running tap water.

[c]Treatment 2; 5 ml of oospore suspension were placed in dialysis tubing and placed in beaker containing 1000 ml DDW, changed daily.

[d]Dialyzed in running tap water terminated after 10 days, then bags were placed in Treatment 2.

TABLE 3. Effects of cold and/or heat-shock on germination of Peronospora trifoliorum oospores

Sources of oospores[a]	Temperature of cold-shock (C)	Time in cold-shock	Temperature of heat-shock (C)	Time in heat-shock	Incubation temperature (C)	Incubation photoperiod (hours light)	Percent oospore germination[b]
A3,B3,B5[c]	-5[d]	24-96 hours[e]	40-80[f]	5-10 min	16,20,24	16,24	0
A3,B3,B5[c]	-196[d]	24-96 hours[e]	40-80[f]	5-10 min	16,20,24	16,24	0
A3	-196[d]	1 year	40	5 min	16,24,RT[h]	8,RL[i]	0
B6-8	-196[d]	14 days	40	5 min	20,24,RT[h]	24,RL[i]	0
B9	-196[d]	14 days	40	5 min	16	24	0
C1	-196[d]	24 hours	40	5 min	RT[h]	0	0
C1	-196[d]	1 year	40	5 min	20	16	0
C2	-196[d]	24 hours	40	5 min	RT[h]	0	0
C2	-196[d]	18 months	40	5 min	16,20	24	0
A4	-	-	40-90[f]	1 hour	RT[h]	RL[i]	0
A5	-	-	40-90[f]	1 hour	RT[h]	RL[i]	0
A7	-	-	40-90[f]	1 hour	RT[h]	RL[i]	0
A4	-5	24 hours	RT[h]	24 hours	-	-	0
A5	-5	24 hours	RT[h]	24 hours	-	-	0
A7	-5	24 hours	RT[h]	24 hours	-	-	0

[a]Code refers to oospore source as listed in Table 1.

[b]Oospores examined for germination daily, during incubation period, for 14 days.

TABLE 3. Continued

[c] In controls deionized distilled water was substituted for the 15% DMSO.

[d] First hour at -20 C.

[e] Removed from cold-shock at 24-hour intervals.

[f] Range of temperatures used for heat-shock in intervals of 10 C.

[g] Alternately frozen and then thawed daily for 30 days.

[h] Room temperature.

[i] Room light.

top of the primary filter was a 12 X 14 X 12-cm glass tank filled to a depth of 10 cm with an aqueous filter solution consisting of 11.5 g acidified $CuSO_4 \cdot 5H_2O$ in 1.15 liters of distilled water. Light was provided by a 100 W incandescent lamp placed 70 cm from the bottom of the box. The light regime used was 10 days dark/10 days light/10days dark at 16 C followed by an incubation period of 14 days at the same temperature under continuous fluorescent lighting. Oospores were examined daily during the incubation period, but no germination was observed.

2. Oospore suspensions (Table 1; B6, B8) were placed in glass petri dishes under photoperiods of 0, 8, 16, or 24 hours with light intensities of 6,500 or 10,000 lux of fluorescent lighting at RT, 12, 16, 20 or 24 C. Oospores were examined daily for 21 days, but no germination was observed.

Soil extracts.-Dormant Pythium ultimum and P. anphandermatum oospores become increasingly germiable with time of exposure to non-sterile soil extract (2). Peronospora trifoliorum oospores placed in both sterile and non-sterile soil extracts did not germinate (Table 4). To prepare soil extracts 10 g of soil was placed in 1 (opinion) liter of DDW, allowed to stand at RT for 3 days, then passed through a Whatman No.1 filter. A portion of the filtrate was autoclaved at 122 C to provide the sterile soil extract.

Soil extract and root exudates.-Combinations of soil extracts and root exudates were used in attempts to germinate P. trifoliorum oospores.

1. Soil extract agar (500 ml of soil extract and 10 g agar) was autoclaved (122 C) for 25 min and cooled to 45 C. Then a 20-ml suspension of oospores (Table 1; F9), containing vancomycin (200 µg/ml), was added and then poured into glass petri dishes. 275-1 seeds (25 seeds/petri dish) were germinated on the agar at 16 C and a 12-hour photoperiod, RT and light, or at 12 C in the dark. No mildew symptoms developed on the seedlings within 21 days, and no germination was detected by microscopic examination of the oospores in sections of the agar.

2. Both 275-1 seeds and an oospore suspension (Table 1; F9) treated with vancomycin (200 μg/ml) were added to autoclaved (122 C) soil extract agar (500 ml soil extract and 10 g agar), which was poured into glass petri dishes after cooling to 45 C. The seeds were germinated at 12 or 16 C under a 12-hour photoperiod, at RT and light, or at 12 C in the dark. No mildew symtpoms developed on the seedlings within 21 days, and no germination was detected by microscopic examination of the oospores in sections of the agar.

3. Seeds of Kanza and 275-1 were germinated in 100 ml of non-sterile soil extract to which 5 ml of an oospore suspension (Table 1; F7-8) were added. The oospores were examined after 14 days at 12, 16, 20, or 24 C under either dark or continuous light, but no germination was observed.

Calcium.-Stanghellini and Russell (24) reported Pythium aphanidermatum oospores required calcium during the pregermination stage and that calcium at 10-200 μg/ml induced endospore wall absorption. Banihadhemi and Mitchell (3) reported that calcium at 1 μg/ml greatly accelerated the rate of Phytophthora cactorum oospore activation but was inhibitory at concentrations above 25 μg/ml. P. trifoliorum oospores (Table 1; E4-5) were placed in either $CaCl_2$ or $Ca(NO_3)_2$ solutions at concentrations of 1, 20, 100, 200 μg/ml for 24 hours, then a carbon source (1% glucaose or sucrose) was added. Suspensions were maintained at 20 C under continuous light, 16 C in the dark, or at 5 C in the dark. Oospores were examined daily for 7 days, but no germination was observed.

Hydrogen-ion concentration.-Pythium aphanidermatum oospores germinated optimally at pH 6.0 in a selective medium (9) and at or above pH 7.0 in soil (1). Aphanomyces euteiches oospores did not germinate at a pH below 3.2 or above 5.1 (16). Peronospora trifoliorum oospores (Table 1; A3) were placed in a series of test tubes containing 20 ml of tap water adjusted

TABLE 4. Effects of sterile and non-sterile soil extracts on germination of Peronospora trifoliorum oospores

Sources of oospores[a]	Soil extract[b]	Incubation temperature (C)	Incubation photoperiod (hrs)	Length of treatment (weeks)[c]	Percent oospore germination
F4	S,NS	RT[d]	RL[e]	8	0
F3	S,NS	16	0,24	3	0
F3	S,NS (+ 0.01% w/v $KMnO_4$)	16,20	0,24	3	0
A6	S,NS	-10 C for 24 hrs then RT for 24 hrs	0	4	0
B6-7	S,NS	8,12	0,8	3	0
C3-4	S,NS	-10	0	4,8,12[f]	0
B5-6	S,NS	5	0	4	0
B7	S,NS (+ seedling root extract)[g]	RT[d]	0,RL[e]	4	0

[a]Code refers to oospore source as listed in Table 1.

[b]Non-sterile soil extract (NS) was prepared by mixing 10 g soil in 1 liter of tap water and letting it stand for 3 days and filtering it through a Whatman No.1 filter. Sterile soil extract (S) was prepared by autoclaving (122 C) portions of the non-sterile soil extract.

[c]Oospores examined at weekly intervals.

[d]Room temperature.

[e]Room light.

[f]Oospores removed at these intervals, thawed at RT, and examined daily for germination for 7 days.

[g]Seedling root extract was prepared by grinding roots of four-day-old afalfa seedlings in deionized distilled water.

to pH 3 to 10 in 0.5 increments using 0.1 N NaOH or HCl. The pH was buffered with 5 mM potassium phosphate buffer. Solutions were incubated at 20 C in the dark or under continuous light for 30 days. Every second day oospores were examined for germination, but none was observed.

Snail or snail enzymes.-Several snail species are useful in obtaining mycelium-free oospores and for increasing oospore germination of some fungi. Snails (Helsoma sp.) were used to obtain mycelium-free oospores of Aphanomyces euteiches (7), and Planorbarius corneus was used to obtain Phytophthora cactorum oospores and to destroy less resistant structures (23). P. cactorum and P. erythroseptica oospores germinated after passage through the digestive tracts of land snails, Helix aspersa (11). Stanghellini and Russell (24) used water snails (Planorbis sp.) followed by an exogenous carbohydrate source to increase the germination percentage of Pythium aphanidermatum oospores. Salvatore et al. (21) used land snails (Helix aspersa) to consistently increase the percentage germination of Phytophthora megasperma isolates on alfalfa. Similiar results were obtained using the snail enzyme complex, beta-glucuronidase/aryl sulfatase from Helisoma pomatia, and the snail enzyme, beta-glucuronidase. Banihashenii and Mitchell (3) reported increased germination of P. cactorum oospores after treatment with 1% glusulase (glucuronidase from bovine liver and sulfatase).

Peronospora trifoliorum oospores were either passed through the digestive tracts of snails (Pomacia sp.) or pond snails of several species from Carolina Biological Supply, Burlington, NC 27215, or treated with beta-glucuronidase from Helix pomatica (Sigma Chemical Co., St. Louis, MO 63178) in attempts to stimulate their germination. The procedures used to pass oospores through the snails digestive tracts is described in Materials and Methods for snail extraction of oospores from infected plant material.

Snails, particularly Pomacia sp., provided an excellent means of removing the plant material as well as the oogonial wall from oospores, but no oospore germination was observed. Snail enzyme preparations were not as effective as actual snail digestion in removing plant debris or oogonial wall from oospores. The source of oospores used and the snail enzyme treatments are presented in Tables 5-8.

TABLE 5. Effects of snail digestion on germination of Peronospora trifoliorum oospores

Sources of oospores[a]	Calcium treatment[b]	Concentration (µg/ml)[c]	Carbon source (1% w/v)	Temperature (C)	Percent oospore germination[d]
E7	$CaCl_2$	50	Levulose	20	0
E7	$CaCl_2$	50	Levulose	24	0
E7	$CaCl_2$	100	Sucrose	20	0
E7	$CaCl_2$	100	Sucrose	24	0
E7	$CaCl_2$	200	Glactose	20	0
E7	$CaCl_2$	200	Glactose	24	0
E7	$CaCl_2$	50	Levulose	20	0
E7	$CaCl_2$	50	Levulose	24	0
E7	$CaCl_2$	100	Sucrose	20	0
E7	$CaCl_2$	100	Sucrose	24	0
E7	$CaCl_2$	200	Dextrose	20	0
E7	$CaCl_2$	200	Dextrose	24	0
E8	$Ca(NO_3)_2$	50	Dextrose	20	0
E8	$Ca(NO_3)_2$	50	Dextrose	24	0
E8	$Ca(NO_3)_2$	100	Sucrose	20	0
E8	$Ca(NO_3)_2$	100	Sucrose	24	0
E8	$Ca(NO_3)_2$	200	Levulose	20	0
E8	$Ca(NO_3)_2$	200	Levulose	24	0
E8	$Ca(NO_3)_2$	50	Glactose	20	0
E8	$Ca(NO_3)_2$	50	Glactose	24	0
E8	$Ca(NO_3)_2$	100	Sucrose	20	0
E8	$Ca(NO_3)_2$	100	Sucrose	24	0
E8	$Ca(NO_3)_2$	200	Levulose	20	0
E8	$Ca(NO_3)_2$	200	Levulose	24	0

[a]Code refers to oospore source as listed in Table 1.

[b]Length of calcium treatment was 24 hours at 24 C.

[c]Concentration of calcium treatment.

[d]Oospores examined for germination daily for 7 days.

TABLE 6. Effects of the snail enzyme, beta-glucuronidase, on germination of Peronospora trifoliorum oospores

Source of oospores[a]	Snail enzyme conc. (% v/v)	Calcium source[b]	Concentration (μg/ml)	Carbon source (1% w/v)	Incubation temperature (C)	Percent germination[d]
E7	1	$CaCl_2$	50	Sucrose	16,24	0
E7	1	$CaCl_2$	50	Levulose	16,24	0
E7	1	$CaCl_2$	100	Glucose	16,24	0
E7	1	$CaCl_2$	100	Glactose	16,24	0
E7	1	$CaCl_2$	200	Sucrose	16,24	0
E7	1	$CaCl_2$	200	Levulose	16,24	0
E7	1	$Ca(NO_3)_2$	50	Glucose	16,24	0
E7	1	$Ca(NO_3)_2$	50	Glactose	16,24	0
E7	1	$Ca(NO_3)_2$	100	Sucrose	16,24	0
E7	1	$Ca(NO_3)_2$	100	Levulose	16,24	0
E7	1	$Ca(NO_3)_2$	200	Glucose	16,24	0
E7	1	$Ca(NO_3)_2$	200	Glactose	16,24	0
E4-5	1	-	-	-	24	0
E4-5	1	-	-	Sucrose	24	0
E4-5	1	-	-	Levulose	24	0
E4-5	1	-	-	Dextrose	24	0
E4-5	1	-	-	Glactose	24	0
E4-5	1	-	-	Glucose	24	0
E4-5	2	-	-	Sucrose	20	0
E4-5	2	-	-	Levulose	20	0
E4-5	2	-	-	Glactose	20	0
E4-5	2	-	-	Glucose	20	0
E4-5	2	-	-	-	20	0

[a]Code refers to oospore source as listed in Table 1.

[b]Length of calcium treatment was 24 hours at 20 C.

[c]Concentrations of calcium source.

[d]Oospores examined daily for 7 days.

TABLE 7. Effects of various concentrations of the snail enzyme, beta-glucuronidase, on germination of Peronospora trifoliorum oospores

Source of oospores[a]	Vancomycin treatment (25 µg/ml)	Snail enzyme conc. (% v/v)	Incubation temperature (C)	Incubation photoperiod (hrs light)	Percent oospore germination[b]
A2	+	2.0	16,20	0,24	0
A5	+	2.0	16,20	0,24	0
A3	+	2.0	16,20	0,24	0
A4	+	2.0	16,20	0,24	0
A2	-	1.0	16,20	0,24	0
A2	+	1.5	16,20	0,24	0
A5	+	1.5	16,20	0,24	0
A3	+	1.5	16,20	0,24	0
A4	+	1.5	16,20	0,24	0
A5	-	1.0	16,20	0,24	0
A2	+	1.0	16,20	0,24	0
A5	+	1.0	16,20	0,24	0
A3	+	1.0	16,20	0,24	0
A4	+	1.0	16,20	0,24	0
A3	-	1.0	16,20	0,24	0
A2	+	0.5	16,20	0,24	0
A5	+	0.5	16,20	0,24	0
A3	+	0.5	16,20	0,24	0
A4	+	0.5	16,20	0,24	0
A4	-	0.5	16,20	0,24	0

[a]Code refers to oospore source as listed in Table 1.

[b]Oospores examined for germination daily for 8 days.

TABLE 8. Effects of various treatments on germination of *Peronospora trifoliorum* oospores following snail digestion or snail enzyme, beta-glucuronidase, treatments[a]

Source of oospores[b]	Treatment	Incubation photoperiod (hrs light)	Incubation temperature (C)	Percent oospore germination[c]
D8,E9	Root extract[d]	0	RT[g]	0
		12	16	0
		24	RT[g]	0
D8,E9	Soil extract	0	RT[g]	0
		12	20	0
		24	RT[g]	0
D8,E9	Soil and root extracts[f]	0	RT[g]	0
		12	24	0
		24	RT[g]	0
D8,E9	Deionized water	0	RT[g]	0
		12	24	0
		24	RT[g]	0
D8,E9	Glucose (1% w/v)	0	RT[g]	0
		12	24	0
		24	RT[g]	0

[a]Oospores were either passed through snails (*Pomacia* sp.) and the oospores recovered from their feces or were treated with the snail enzyme, beta-glucuronidase (1% v/v) for 24 hours at 20 C, followed by placement in liquid nitrogen (-196 C) for 24 hours then heat-shocked at 40 C for 5 min.

[b]Code refers to oospore source as listed in Table 1.

[c]Oospores examined for germination daily for 21 days.

TABLE 8. Continued

[d]Extract prepared by grinding 5 g of seedling alfalfa roots in 100 ml deionized water in a Waring Blendor, then passing it through four layers of cheesecloth.

[e]Extract prepared by soaking 10 g of soil in 1000 ml deionized water for 3 days, then filtering through a Whatman No.1 filter.

[f]Combination of root and soil extracts prepared as previously described.

[g]Room temperature.

LITERATURE CITED

1. ADAMS, P. B. 1971. Pythium aphanidermatum oospore germination as affected by time, temperature, and pH. Phytopathology 61:1149-1150.

2. AYERS, W. A., and R. D. LUMSDEN. 1975. Factors affecting production and germination of oospores of three Pythium species. Phytopathology 65:1094-1100.

3. BANIHASHEMI, Z., and J. E. MITCHELL. 1976. Factors affecting oospore germination in Phytophthora cactorum the incitant of apple collar rot. Phytopathology 66:443-448.

4. BARTON, R. 1957. Germination of oospores of Pythium mamillatum in response to exudates from living seedlings. Nature 180:613-614.

5. BERG, L. A., and M. E. GALLEGLY. 1966. Effect of light on oospore germination in species of Phytophthora. Phytopathology 56:583 (Abstr.).

6. BERRY, S. Z., and G. N. DAVIS. 1957. Formation of oospores by Peronospora destructor and their possible relation to epiphytology. Plant Dis. Rep. 41(1):3-6.

7. BHALLA, H. S., and J. E. MITCHELL. 1970. A method of obtaining viable, mycelium-free oospores of Aphonomyces euteiches using live water snails. Phytopathology 60:1010-1012.

8. DUNLEAVY, J., and G. SNYDER. 1962. Inhibition of germiantion of oospores of Peronospora manshurica. Iowa Acad. of Sci. 69:118-121.

9. FLOWERS, R. A., and R. H. LITTRELL. 1972. Oospore germination of Pythium aphanidermatum as affected by casein, gallic acid, and pH levels in a selective medium. Phytopathology 62:757 (Abstr.).

10. FRIED, P. M., and D. L. STUTEVILLE. 1977 Peronospora trifoliorum sporangium development and effects of humidity and light on discharge and germination. Phytopathology 77:890-894.

11. GREGG, M. 1957. Germination of oospores of Phytophthora erythroseptica. Nature 57:454.

12. KLISIEWICZ, J. M. 1970. Factors affecting production and germination of oospores of Phytophthora drechsleri. Phytopathology 60:1738-1742.

13. KOTHARI, K. L., and N. PRASAD. 1970. Downy mildew of opium poppy in Rajasthan. Indian Phytopath. 23:764-788.

14. LEAL, J. A., and B. GOMEZ_MIRANDA. 1965. The effect of light and darkness on the germination of the oospores of certain species of Phytophthora on some synthetic media. Trans. Brit. Mycol. Soc. 48(3): 491-494.

15. McKAY, R. 1939. Observations on onion mildew caused by the fungus Peronospora schleideniana W. G. Sm. J. Roy. Hort. Soc. 64:272-285.

16. OLOFSSON, J. 1968. Influence of hydrogen-ion concentration on germination of naturally produced oospores of Aphanomyces euteiches. Plant Dis. Rep. 52(4):264-267.

17. PERSON, L. H., and G. B. LUCAS. 1953. Oospore germination in Peronospora tabacina. Phytopathology 43:701-702.

18. POFF, K. L., and K. H. NORRIS. 1967. Four low cost monochromatic sources of known equal intensities. Plant Physiol. 42:1155-1157.

19. RIBERIO, O. K., G. A. ZENTMYER, and D. C. ERWIN. 1975. Comparative effects of monochromatic radiation on the germination of oospores of three Phytophthora spp. Phytopathology 65:904-907.

20. RYAN, E. W. 1971. Two methods of infecting peas systemically with Peronospora pisi, and their application in screening cultivars for resistance. Ir. J. Agric. Res. 10:315-322.

21. SALVATORE, M. A., F. A. GRAY, and R. B. HINE. 1973. Enzymatically induced germination of oospores of Phytophthora megasperma. Phytopathology 63:1083-1084

22. SCHAREN, A. L. 1960. Germination of oospores of Aphanomyces euteiches embedded in plant debris. Phytopathology 50:274-277.

23. SHAW, D. S. 1967. A method of obtaining single-oospore cultures of Phytophthora cactorum using live water snails. Phytopathology 57:454.

24. STANGHELLINI, M. E., and J. D. RUSSELL. 1973. Germination in vitro of Pythium aphanidermatum oospores. Phytopathology 63:133-137.

25. SUSSMAN, A. O., and H. O. HALVORSON. 1966. Spores: Their dormancy and germination. Harper and Row, New York. 354 p.

26. WOLF, F. A., R. A. McLEAN, and L. F. DIXON. 1936. Further studies on downy mildew of tobacco. Phytopathology 26:760-777.

I. ENVIRONMENTAL EFFECTS ON PERONOSPORA TRIFOLIORUM OOSPORE PRODUCTION IN SEEDLINGS OF TWO ALFALFA CLONES

II. ATTEMPTS TO GERMINATE PERONOSPORA TRIFOLIORUM OOSPORES

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ABSTRACT

Peronospora trifoliorum d By. causes downy mildew of alfalfa (Medicago sativa L.) in the temperate zones of the world. P. trifoliorum conidia are short-lived suggesting that oospores are important in long distance dissemination and survival. I determined optimum conditions for P. trifoliorum oospore production but was unable to germinate them.

I inoculated S_1 seedlings of two alfalfa clones (275-1 and 18-21) with a monoconidial isolate (I-7) and determined oospore production at weekly intervals at 12, 16, 20, and 24 C under photoperiods of 8, 16, and 24 hours of cool white fluorescent lighting at 6,500 and 10,000 lux intensity. I-7 attacks both clones but produces considerably more conidia on the 18-21 seedlings.

Oospore production at three weeks postinoculation was greatest in 275-1 seedlings (11,081 per seedling) at 16 C and 10,000 lux of continuous lighting. The 16 C temperature was optimum at nearly all light conditions. Optimum light requirements were less clearly defined. However, oospore production usually increased with photoperiod or light intensity. Thus, illuminance may be more important than either photoperiod or intensity per se.

More oospores were produced in 275-1 than in 18-21 seedlings in most treatments and over 6 times more under optimum conditions. At three weeks postinoculation, 275-1 plants contained nearly all their oospores in their dead cotyledons. Most 275-1 seedlings showed no other symptoms and had outgrown the fungus. However, 18-21 plants in most treatments had generalized systemic chlorotic mildew symptoms with stunting which was most severe at the lowest temperature and high light conditions (12 C and 24-hour photoperiod of 10,000 lux). Oospores were distributed throughtout the systemically invaded tissue of 18-21 plants. In the most stunted plants nearly 90% of

the oospore formed above the cotyledons, and developing oogonia were apparent in the uppermost trifoliolate leaves.

One week after inoculation significantly fewer oospores were produced in 275-1 and 18-21 seedlings on which the fungus was induced to form conidia. At three weeks conidium formation significantly reduced oospore production in 275-1 but not in 18-21 plants.

Attempts to germinate P. trifoliorum oospores failed. Unsuccessful treatments included many used to germinate the oospores of other Peronospora spp. and other Oomycetes. Treatments included heat-shock; cold-shock; soil extracts; seedling exudates; light of different quality, intensity, and photoperiod; dialysis; pH; digestion by snails; snail enzymes; overwintering and storage in soil outside for 2 years; calcium treatments; and oxidizing agents.