INFLUENCE OF LIGHT, HUMIDITY AND CHLORINE ON ASEXUAL REPRODUCTION OF PERONOSPORA TRIFOLIORUM

by

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I. INFLUENCE OF LIGHT AND HUMIDITY ON ASEXUAL REPRODUCTION OF PERONOSPORA TRIFOLIORUM

Sporangial production by Peronospora trifoliorum d By., the causal organism of downy mildew of alfalfa (Medicago sativa L.), is favored by a dark period between 8 and 16 h at 16-20 C in a high relative humidity (RH) (7). Sporangia require a film of water for germination; they will not germinate on a dry slide in 100% RH (3). This paper reports on sporangial development and some effects of light and humidity on sporangial release and germination as a means of improving sporangial production by this obligate parasite, in the laboratory, and for a more thorough understanding of the disease.

MATERIALS AND METHODS

'Kanza' alfalfa was seeded 1 cm deep in steam-sterilized masonry sand in 3 rows (15-18 seeds/row) in 6 x 6 x 5 cm plastic pots. The pots were placed in growth chambers at 20 C, 70-85% RH, and 5382 lx (500 ft-c) continuous fluorescent lighting. Four days later a water suspension containing ca. 100,000 sporangia/ml was sprayed until run-off onto the seedlings. Immediately after inoculation, seedlings were placed inside the lids of 26 x 35 x 16 cm plastic sweater boxes and the boxes were inverted over the plants and inside the lids, to form a nearly air-tight seal. Growth chamber lights were turned off. Fifteen h later the boxes were removed from over the plants and the chamber lights turned on. Pots of plants

were watered as needed with deionized water and were last watered (to saturation) 30 h before inducing sporulation. Eleven days after seeding, sporangial production was induced by replacing the plastic boxes over the plants and turning off chamber lights.

Inoculum was prepared by cutting off mildewed seedlings below the cotyledons, placing them in a jar with deionized water, shaking to dislodge the sporangia and passing the sporangial suspension through a tea strainer to remove the plant debris.

Sporangial production was determined by measuring the turbidity (Spectronic 20) of 30 ml of sporangial suspension secured by washing 30 plants three times in 10 ml of deionized water.

Sporangial germination at 20 C in darkness, was determined 12 h after placing drops of sporangial suspension (adjusted to ca. 35,000 sporangia/ml) on microscope slides on deionized water-soaked filter paper in petri dishes, unless stated otherwise.

Relative humidity was measured with a wind-tunnel psychrometer which sampled air from one end of the humidity chamber and returned it to the opposite end by plastic tubing.

To investigate sporangial development, 48 pots of 11-day-old infected seedlings were individually placed on 1-cm tall plastic blocks in trays. Deionized water was added to trays to depth of 0.5 cm and a 500 ml wax-coated paper cup was

inverted over each pot forming a dark air-tight seal. Four pots were removed every 2 h thereafter for 24 h. A few seedlings from each pot were studied microscopically to observe sporangial development, and the balance was used to determine sporangial production and viability as previously described.

To photograph sporangial development, infected cotyledons were removed from plants and placed immediately inside a 3-mm tall water agar ring (1-cm diam) on a microscope slide and covered with a cover slip. When photographs of sporangial discharge were taken, the cover slip was removed to permit the drier laboratory air to enter the chamber.

RESULTS

Effect of RH on sporulation.-P. trifoliorum produced sporangia on infected seedlings in the plastic humidity chambers when the air drawn through the chambers was at 97% RH or above. However, sporulation was greatly reduced in free water on leaves. Moisture in the planting medium (sand) was sufficient to provide adequate RH for sporulation in the tight chambers.

Sporangial production and germination.-General microscopic observations at given intervals after inducing sporulation were:

2 h) No visible development.

4 h) Sporangiophores ranged in size from those emerging through stomata (Fig. 1-A) to those starting to produce the first side branch just behind the growing point (Fig. 1-B).

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Fig. 1. (A-I) Photomicrographs of developing Peronospora trifoliorum sporangiophores and sporangia at 100% RH on cotyledons of 11-day-old alfalfa seedlings at 4 h A-B), 6 h C-D), 8 h E), and 10 h F) after inducing sporulation. F-H) Photomicrographs of the same sporangiophores showing turgid sporangia at F), sporangial shriveling and no sporangial release after exposure to laboratory air (ca. 48% RH) for 5-15 sec at G) and sporangia still attached after 24 h exposure to laboratory air at H). I) Discharged sporangia in dry (lower) and in water (upper portion of a glass slide). Note that shriveled sporangia near the water line (w) regain inflated form without contact with water.

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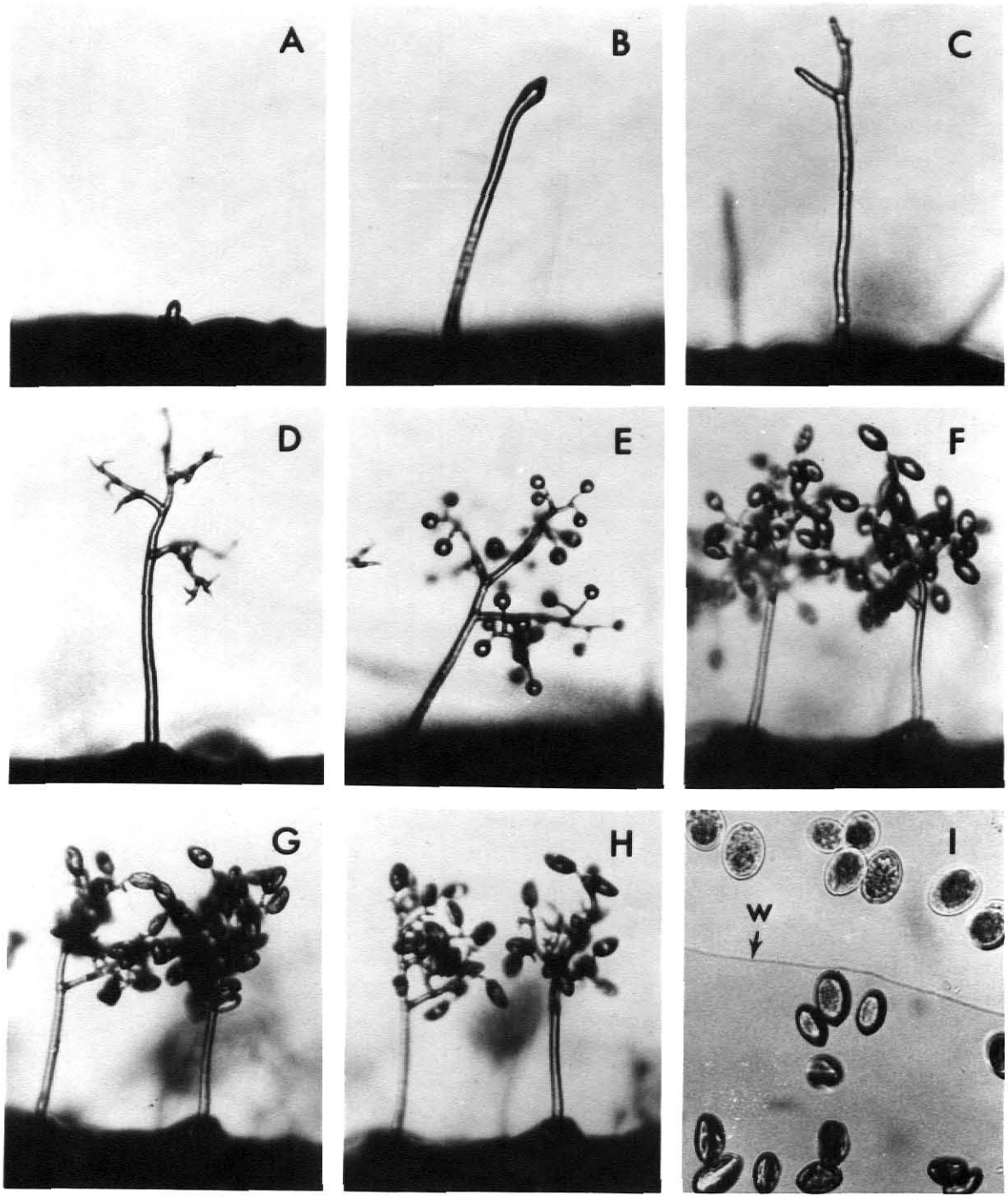
A
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W

6 h) Youngest sporangiophores observed had single branches (Fig. 1-C) or were just beyond the same stage of development as the older ones were at 4 h (Fig. 1-B). This indicated the stages of development were overlapping by about 2 h. Most sporangiophores, however, had completed branching (Fig. 1-D) and some were producing sporangia. Sporangia on a sporangiophore formed simultaneously at the apices of all branches, and thus were the same age and size (Fig. 1-E). Sporangiophores at various stages of development were often protruding from the same stomate. Immature sporangia were spherical and became egg-shaped when morphologically mature. A few sporangia appeared to be morphologically mature (Fig. 1-F).

8 h) All sporangiophores were bearing sporangia of which ca. 40% appeared to be morphologically mature.

10 h) All sporangia were morphologically mature (Fig. 1-F).

The observations above were made on plants that previously had not been exposed to light after sporangial induction. However, to determine the affects of light during sporulation some of the cotyledons were placed back into the dark after examination and reexamined later. In those cases, the short exposure to light stopped sporangial production for about 2-3 h, after which time it progressed at the normal rate.

Sporangial production determined at 2-h intervals after inducing sporulation agreed with microscopic observations that

sporangial production was complete by 10 h (Fig. 2). However, the germinability was greatest (ca. 80%) at 12 h, or 2 h later, indicating that the sporangia reach physiological maturity about 2 h after becoming morphologically mature. Maximum production of viable sporangia thus occurred 12-16 h after inducing sporulation and declined thereafter due to loss in germinability (Fig. 2).

To determine longevity of sporangial production, infected seedlings were placed at a 9-h photoperiod, and the sporangiophores and sporangia were harvested daily by vacuum. P. trifoliorum sporulated daily on the infected cotyledons until they died, 5-8 days later.

Sporangial discharge.-Following sporulation, sporangia remained turgid and attached to the sporangiophore as long as kept in a saturated atmosphere (Fig. 1-F). However, when exposed to the drier laboratory air, the same sporangia shriveled within a few seconds (Fig. 1-G). Counter-clockwise twisting of sporangiophores started immediately thereafter and lasted for 15 sec to 1 min, during which time sporangia were discharged violently. However, many of the sporangia were not discharged and were still attached to the sterigmata 24 h later (Fig. 1-H).

Effect of air-drying on sporangial viability.-Since sporangia are not discharged in a saturated atmosphere in nature, we exposed mildewed seedlings to drier conditions and determined their viability. Seedlings bearing freshly-produced sporangia (15 h after inducing sporulation) were removed from humidity chambers, excised below the cotyledons, and placed on dry

Fig. 2. Production and germinability at 20 C of Peronospora trifoliorum sporangia harvested from 11-day-old alfalfa seedlings at given time after inducing sporulation.

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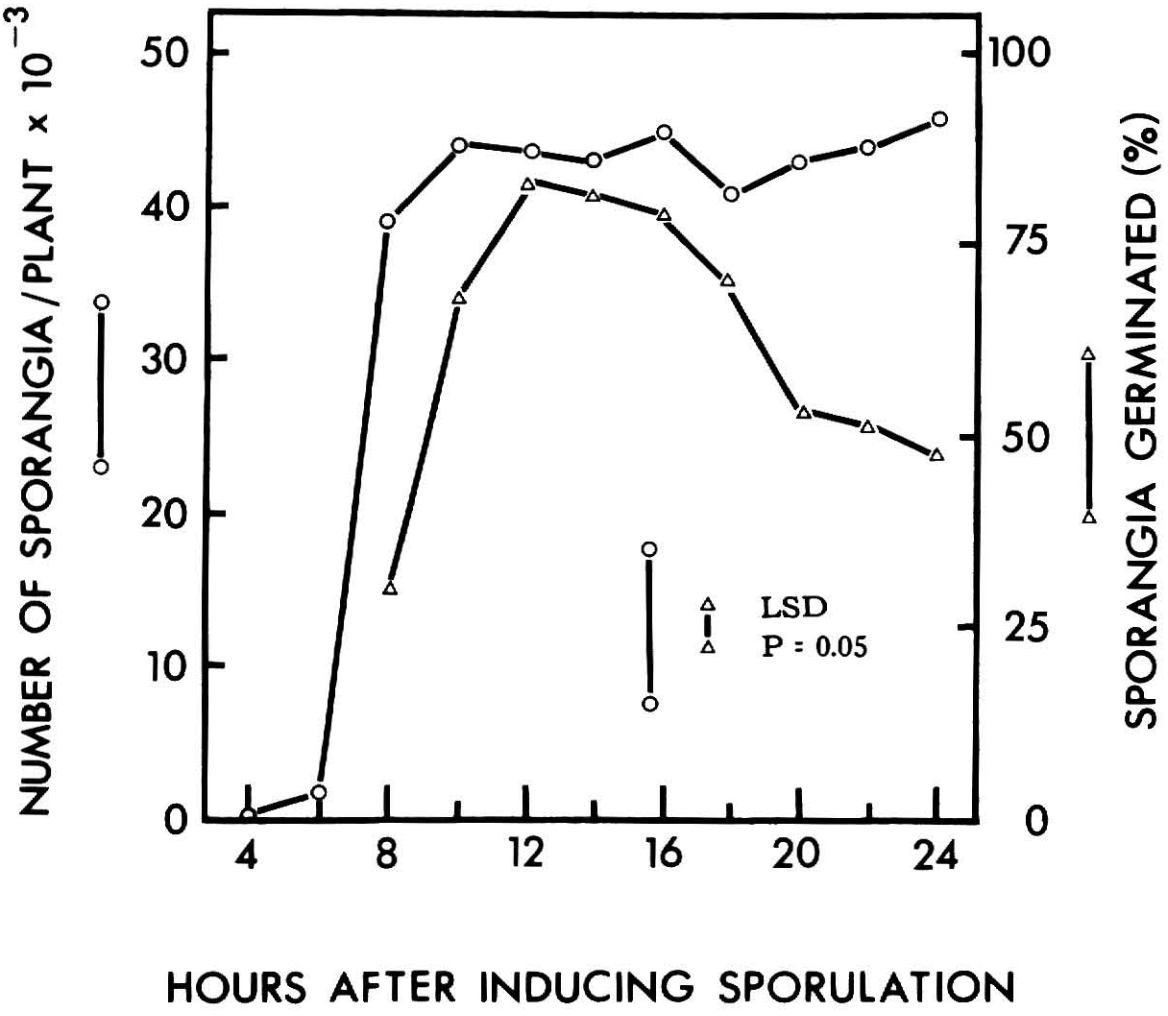
NUMBER OF SPORANGIA/PLANT x 10^{-3}
SPORANGIA GERMINATED (%)
50
40
30
20
10
0
100
75
50
25
0
4
8
12
16
20
24
LSD
P = 0.05
HOURS AFTER INDUCING SPORULATION

microscope slides in open petri dishes on a laboratory table. After 30 min and 3 h, plants were removed from the slides and the sporangia discharged onto the slides and those either still attached or entangled in the sporangiophores were germinated separately in drops of water.

Sporangial germinability decreased rapidly under the laboratory conditions and was most pronounced for the discharged sporangia (Table 1).

Effect of light on sporangial germination.-To determine light influence during sporangial germination, a sporangial suspension was prepared and dispersed as drops on microscope slides in petri dishes in a darkroom. The petri dishes were wrapped in aluminum foil and transferred from the darkroom to a growth chamber at 20 C and exposed to given light periods at 5382 lx fluorescent lighting followed by continuous darkness for the balance of 24 h at which time germination percentages were determined.

Light enhanced sporangial germination (Table 2). Germination was highest in sporangia exposed to light during their entire germination period. In subsequent experiments we found that germination percentages were not significantly different among sporangia germinated under continuous fluorescent lighting intensities of ca. 538, 2691, 5382, or 10,764 lx. Germination ranged between 87 and 95% in those tests.

TABLE 1. Per cent of *Peronospora trifoliorum* sporangia from mildewed alfalfa seedlings viable after given exposure periods in laboratory at ca. 25 C, 48% RH, and 538 lx of fluorescent and daylight

Exposure period	Per cent sporangia viable [y]	
	Discharged	Non discharged [z]
None (control)	---	81.0 *a*
30 min	31.1 d	67.8 b
3 h	9.0 e	55.7 c

[y]Values followed by unlike letters are significantly different, P = 0.05.

[z]Sporangia attached to, or entangled in, sporangiophores.

TABLE 2. Per cent of *Peronospora trifoliorum* sporangia germinated after 24 h in drops of water on microscope slides in petri dishes exposed to 5382 lx of fluorescent lighting for the specified period followed by continuous darkness

Time exposed to light	Per cent sporangia germinated
0 min	43.2
10 min	71.6
60 min	68.7
24 h	85.3
LSD (P = 0.05 ANOV)	5.9

DISCUSSION

In summarizing our data, procedures for optimal sporangial production and germination at 20 C include maintaining a RH of 97% or greater during sporulation, harvesting sporangia 12-16 h after inducing sporulation, keeping them in a saturated atmosphere, and incubating them for germination in light. These conditions are easily met by harvesting plants as soon as possible after removing from the humidity chamber, placing them with water in a container, and shaking it to dislodge the sporangia, passing the sporangial suspension through a strainer to remove plant debris, and germinating the sporangia in water (3) in the light. Rockett reported that 5382 lx did not improve the germination of P. trifoliorum on water agar (7). However, he made no mention of preparing the inoculum in the dark; therefore much of the photosensitive inhibition of germination may have been overcome by exposure to light during the period of inoculum preparation. Light also stimulated the germination of P. manshurica (4). Rockett found that 5382 lx of lighting during the infection period reduced mildew incidence because the plants either dried off, or sporangia were washed off in an attempt to deep the plants moist (7). However, this problem should be overcome by reducing the light intensity, as we found 538 lx (the lowest intensity tested) during the germination period was as effective as 10 times that amount. The required intensity may even be much lower than the ones tested.

Sporangial morphogenesis and discharge by P. trifoliorum corresponds fairly well to descriptions for other Peronospora species (1, 2, 6). However, other workers never mentioned that during the process of sporangial discharge due to a water vapor pressure deficit, sporangia shriveled before the sporangiophores began their counter-clockwise twisting action. Also, P. destructor (8) and P. viciae (5) produce sporangia at as low as about 91% RH, while P. trifoliorum requires about 97%.

In view of the rapid loss of viability after discharge, it appears that P. trifoliorum sporangia are not as well suited for long range dispersal as for local spread by splashing rain.

In trying to relate the present laboratory data on P. trifoliorum to the sequence of events leading to infection in the field, we can safely assume that sporangia are produced at or near the dew point during the night and at 20 C are physiologically mature and most viable the next morning. Further interpretations are speculations without more specific information on other temperature and humidity influences on sporangial viability and, or, actual field data.

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II. EFFECT OF CHLORINE ON PERONOSPORA TRIFOLIORUM SPORANGIAL PRODUCTION AND GERMINATION

Peronospora trifoliorum d By. produces sporangia on infected alfalfa (Medicago sativa L.) only during darkness and at a high relative humidity (RH), but does not sporulate on wet leaves (2). To meet the RH requirements, we placed flats of infected seedlings on blocks 3-cm tall in trays and added tap water to trays to ca. 1 cm deep. Then we inverted an airtight plastic container over the flats and inside the tray to form a water-sealed humidity chamber. We later found that when tap water was omitted from the trays or replaced by deionized water, sporangial production increased and varied much less, which indicated that one or more volatile substances in tap water affected sporulation. That, in turn, led to the research reported here.

MATERIALS AND METHODS

'Kanza' alfalfa was seeded 1 cm deep in steam-sterilized masonry sand in 5.5 x 5.5 x 5 cm plastic pots (ca. 40 seeds/pot, in 3 rows), and placed in growth chambers maintained at 20 C with 5382 lx (500 ft-c) continuous fluorescent lighting. Four days later a water suspension containing ca. 100,000 P. trifoliorum sporangia /ml was sprayed onto the seedlings to run-off. Pots with inoculated plants were placed immediately into the lids of plastic boxes and the boxes (35 x 25 x 16 cm) were inverted over the pots and fitted into their lids to give a nearly airtight seal. Then, the boxes

were placed into dark growth chambers. Fifteen h later the boxes were removed and the chamber lights turned on. Five days later (11th day after seeding), sporangial production was induced by again covering the plants with the boxes and turning off lights.

For sporulation studies with water, 500 ml was poured into each lid and pots were blocked up so water never touched the pots of plants.

Inoculum was prepared by cutting off mildewed seedlings (16 h after inducing sporulation) below cotyledons, placing them in a jar with deionized water, shaking to dislodge the sporangia, and passing the sporangial suspension through a tea strainer to remove plant debris.

Plants were watered as needed, the last time 30 h before inducing the pathogen to sporulate. The avg number of sporangia produced per inoculated plant was determined by measuring the A_{625} of a sporangial suspension from 30 plants (washed 3 times in 10 ml deionized water) with a Bausch and Lomb Spectronic 20. Values for the standard curve were determined by hemocytometer counts.

For germination tests, 3 drops of sporangial suspension, standardized at 0.05% light absorbance (approx. 35,000 sporangia /ml), was placed on a microscope slide previously cleaned in ethanol. The slides were placed on bent glass rods in petri dishes with a filter paper and deionized water in the bottom. The petri dishes were then incubated in dark at 20 C for 12 h. The percentage of germination was estimated by counting 100 sporangia in each drop,

altogether 300 sporangia/replicate or 1200/procedure.

Chlorine content of water was measured by the orthotolidine method with a Hach Test Kit, Model DR-EL, manufactured by Hach Chemical Company, Ames, Iowa 50010.

RESULTS AND DISCUSSION

Peronospora trifoliorum produced three to four times more sporangia of higher viability in humidity chambers with either deionized or no water than with fresh tap water (Table 1). Mean percentages of alfalfa seedlings with visible sporulation were 87, 85, and 11 when humidity chambers contained deionized, no water, or tap water, respectively.

Neither sporangial production nor viability was increased, however, by using deionized instead of tap water for plant irrigation previous to inducing sporulation.

As plants never touched the water in the humidity chambers, one or more volatile substances in tap water apparently reduced sporulation. Also, tap water exposed to air in shallow trays for 24 h before being used in humidity chambers failed to reduce either sporangial production or germinability.

We first investigated chlorine because it is widely used to purify water. About 1 μg/ml of chlorine gas is added to the Manhattan, Kansas, municipal water supply. Tap water in our laboratory avg 0.5 μg/ml and ranged from 0.46 to 0.78 μg/ml during periodic sampling for 3 months. Fresh tap water treated with sodium thiosulfate (to bind the chlorine) and placed in

TABLE 1. Effects on sporangial production and germinability of water sources for irrigation, and for humidity during sporulation of Peronospora trifoliorum in humidity chambers

Water source		Mean no. sporangia produced per inoculated plant (x 10^{-3})	Mean viable sporangia (%)
Plant irrigation pre sporulation	Humidity chambers during sporulation		
Deionized	Deionized	46.5	76.9
	None	45.5	78.4
	Tap[a]	15.6	65.2
Tap[a]	Deionized	41.8	79.4
	None	49.2	76.8
	Tap[a]	10.7	60.0
LSD (P = 0.05 ANOV)		6.3	6.7

[a] Tap water chlorine content = 0.5 µg/ml.

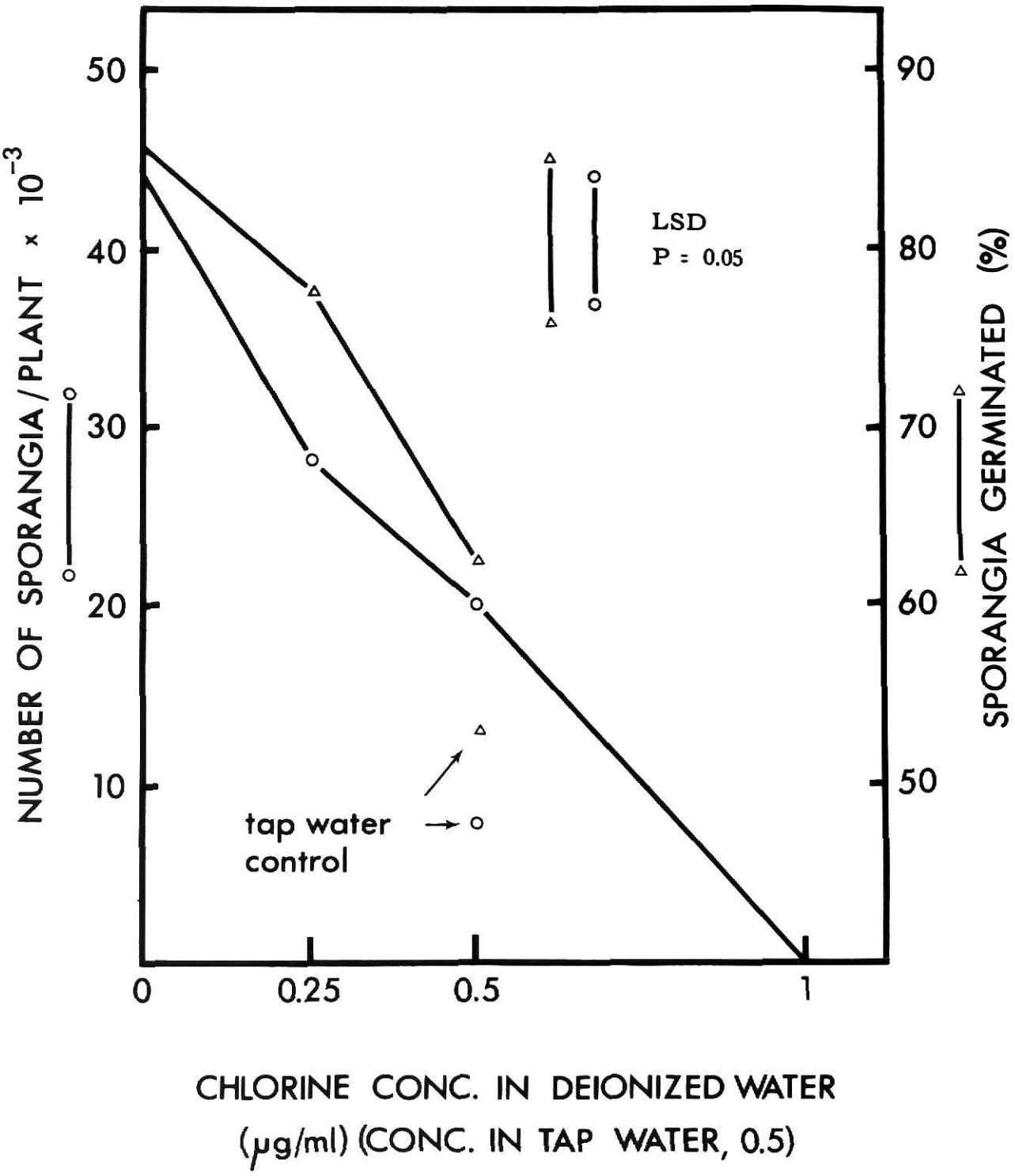
humidity chambers did not reduce sporangial production or germination.

To determine influence of various concentrations of chlorine on sporulation, we added chlorine gas to deionized water and compared results with those from deionized and tap water in humidity chambers during a 15-h sporulation period. Sporangial production decreased sharply as chlorine concn increased and was nil at 1 µg/ml (Fig. 1). Sporangial germination was 86, 78, 62, and 52% at chlorine concn of 0, 0.25, 0.5 µg/ml and tap water, respectively. Chlorine concn in water in the humidity chamber trays after the sporulation period was ca. 10% of the amounts added. Sporulation was significantly less in humidity chambers with tap water (containing 5 µg/ml chlorine) than in chambers with the same amount of chlorine added to deionized water. The difference was consistent in several experiments, indicating that the tap water contains one or more other volatile materials that are bound by sodium thiosulfate, perhaps chloramines, and reduce P. trifoliorum sporulation.

The humidity chamber air volume (excluding the area used by water and pots of plants) was ca. 12.5 liters or 25 times that of the 500 ml of water used. We did not determine the chlorine concn in the air inside the humidity chamber. However, if all chlorine from the 1 µg/ml concn in the water, which prevented sporulation, was dispersed in the chamber space, the avg concn in the air could not have exceeded 0.04 µg/ml.

Among higher plants, alfalfa is one of the most sensitive

Fig. 1. Effects on sporangial production and germination of Peronospora trifoliorum on alfalfa seedlings of indicated chlorine concn added to deionized water to increase relative humidity in humidity chambers.


50
40
30
20
10
90
80
70
60
50
0
0.25
0.5
1
NUMBER OF SPORANGIA / PLANT x 10^{-3}
SPORANGIA GERMINATED (%)
LSD
P = 0.05
tap water
control
CHLORINE CONC. IN DEIONIZED WATER
(μg/ml) (CONC. IN TAP WATER, 0.5)

to chlorine, being visibly injured by 2-h exposure at an atmospheric concn of 0.1 μg/ml (1). We never observed chlorine damage on the alfalfa seedlings, even at the 5 μg/ml concn in the humidity chamber water, which further indicates that sporulation of P. trifoliorum is affected by extremely low chlorine concn in the atmosphere.

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ABSTRACT

Peronospora trifoliorum produced sporangia on alfalfa seedlings only at 97% relative humidity (RH) and above. Microscopic observations on 11-day-old seedlings, at 2-h intervals after inducing sporulation, revealed that predominate stages at given times were: 4 h) unbranched sporangiophores, 6 h) branching sporangiophores, 8 h) sporangial formation, and 10 h) only morphologically mature sporangia. Germination tests indicated that the sporangia reached physiological maturity about 2 h after morphological maturity. Thus the max number of viable sporangia (82% germination) were harvested 12 h after inducing sporulation. Germination decreased slowly to 78% at 16 h and then dropped sharply to 52% at 20 h.

Sporangia harvested in dark 15 h after inducing sporulation and germinated in water while exposed to 5382 lx (500 ft-c) of fluorescent lighting for 0 min, 10 min, 1 h, and 24 h followed by darkness for the balance of 24 h, germinated 43, 72, 69, and 85%, respectively. Light intensities between 538 and 10,764 lx affected germination equally. Discharged sporangia collected 30 min and 3 h after exposing mildewed seedlings to the laboratory air (48% RH) germinated 31 and 9%, respectively, compared to 67 and 55%, respectively for undischarged ones. Non-exposed controls germinated 81%.

Tap water containing ca. 0.5 μg/ml chlorine placed below, but not touching, infected plants in an airtight chamber,

released chlorine into the chamber and reduced sporangial production by 83%. Sporangial germination was reduced 40%. Increasing chlorine concn in deionized water decreased both sporulation and sporangial viability. The tap water had no toxic effect with sodium thiosulfate added or after exposure in a large, shallow tray for 24 h.