Peronospora trifoliorum Sporangium Development and Effects of Humidity and Light on Discharge and Germination

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ABSTRACT

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Peronospora trifoliorum produced sporangia on alfalfa in darkness at ≥ 97% relative humidity (RH). The predominant stages of sporangium development on 11-day-old seedlings observed at 2-hr intervals after induction of sporulation were as follows: at 4 hr sporangiophores were unbranched, at 6 hr sporangiophores had branched, at 8 hr sporangia had formed, and at 10 hr all sporangia were morphologically mature. Sporangium viability was greatest (82%) 12 hr after induction of sporulation (about 2 hr after morphological maturity), decreased slowly to 78% by 16 hr, and then dropped sharply to 52% by 20 hr. Sporangia harvested in darkness 15 hr after induction of sporulation and exposed to 5,400 lux of fluorescent lighting for 0 min, 10 min, 1 hr, or 24 hr followed by darkness for the remainder of a 24-hr period, germinated 43, 72, 69, and 85%, respectively. Light intensities between 540 and 10,800 lux affected germination equally. The viability of sporangia discharged from infected seedlings exposed to drying by laboratory air (about 48% RH) for 0.5 and 3 hr was 31 and 9%, respectively, as compared with 68 and 56%, respectively, for sporangia that remained attached. Nondesiccated sporangia were 81% viable.

Peronospora trifoliorum deBy. causes downy mildew of alfalfa (*Medicago sativa* L.) in the temperate zones of the world. The most practical means to control the disease is to develop and use resistant cultivars. However, the disease is sporadic in Kansas and attempts to increase mildew resistance there by selection during natural epidemics failed. Therefore, the development of procedures to culture *P. trifoliorum* to screen for mildew resistance in the laboratory seemed warranted.

Patel (4) reported that the germination of *P. trifoliorum* sporangia required free water and was optimum at 18 C. Rockett (8), and Rockett and Stuteville (9) found that a dark period of 8-16 hr at a high relative humidity (RH) was required for sporangium production.

We initiated research to improve the production and germinability of *P. trifoliorum* sporangia. During preliminary work, we found that chlorine from tap water in humidity chambers reduced sporangium production and germinability (2). This paper describes sporangium development and release, and reports the RH requirements for sporangium production, the effects of desiccation on germinability, and the effects of light on germination.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa* L. 'Kanza') was seeded 1 cm deep in steam-sterilized masonry sand in three rows (15-18 seeds/row) in 6 × 6 × 5-cm plastic pots. The pots were placed in growth chambers at 20 C, 70-85% RH, and 5,400 lux continuous fluorescent lighting. Four days later, a suspension of 10^5 sporangia/ml in deionized water was sprayed (to the point of run-off) onto the seedlings. Immediately after inoculation, pots of seedlings were placed on the inverted lids of 26 × 35 × 16-cm plastic sweater boxes, and the boxes were inverted over the plants and inside their lids, to form a nearly air-tight seal. Growth-chamber lights were turned off. Fifteen hr later the boxes were removed and the chamber lights were turned on. Plants were watered periodically with deionized water and were watered last (to saturation) 30 hr before sporulation was induced. Eleven days after seeding, sporangium production was induced by replacing the plastic boxes over the plants to increase RH and the chamber lights were turned off.

Inoculum was prepared by excising infected seedlings below the cotyledons, placing them in a jar with deionized water, and shaking to dislodge the sporangia; the resulting suspensions of sporangia and plant debris in water were passed through a tea strainer to remove the plant material.

Sporangium production was estimated by measuring the absorbance at 625 nm (Bausch and Lomb Spectronic-20 spectrophotometer) of a suspension of the sporangia from 30 plants (washed three times in 10 ml of deionized water). Values for the standard curve were determined by actual counts with a hemacytometer.

Unless stated otherwise, sporangia were germinated in darkness at 20 C in drops of water (adjusted to about

35,000 sporangia/ml) on microscope slides on deionized water-soaked filter paper in petri dishes. The germination percentages were recorded 12 hr later and are the means of four replications of 300 observations each.

Relative humidity was measured with a wind-tunnel psychrometer which sampled air from one end of the

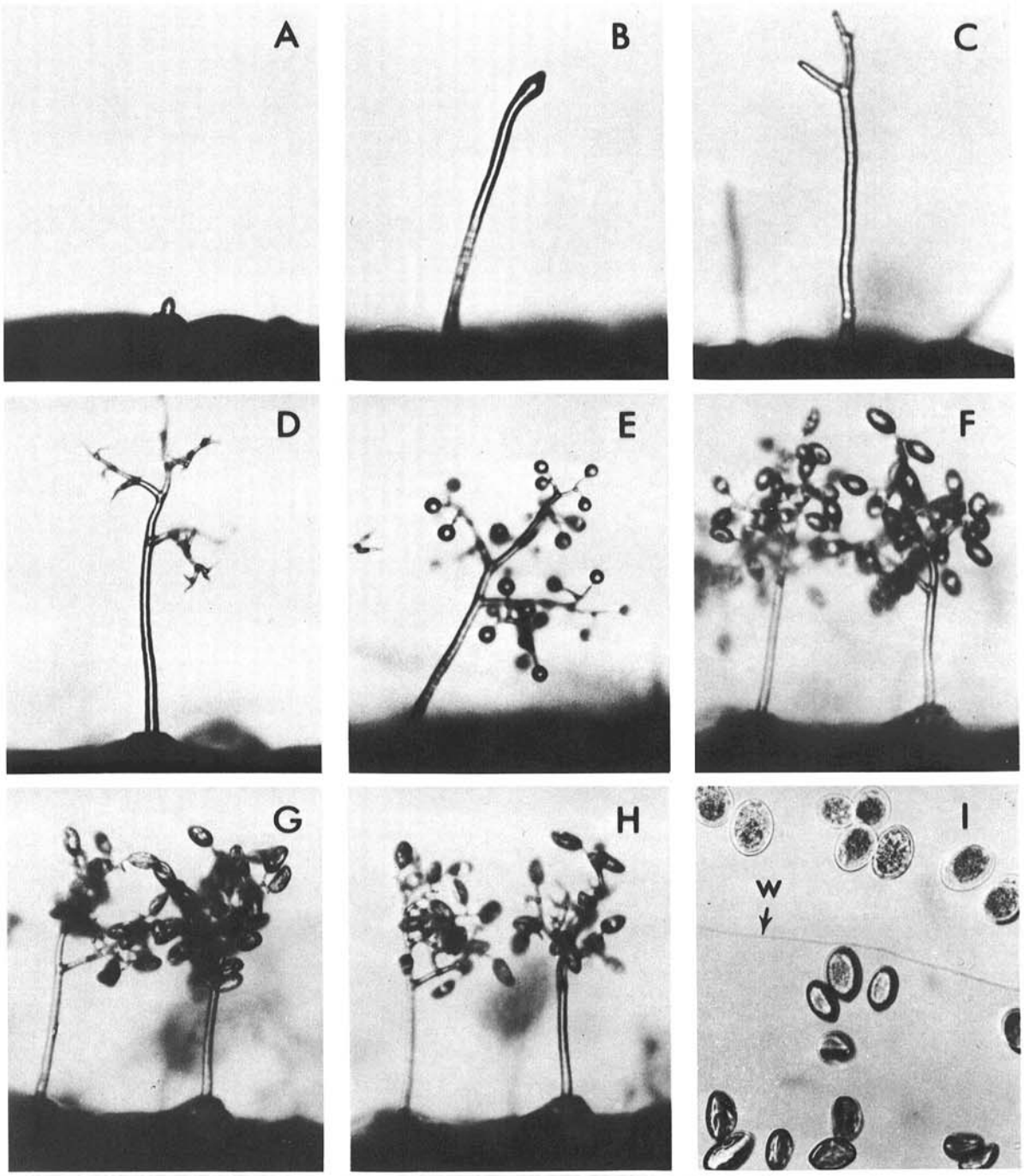

Fig. 1-(A to I). Photomicrographs of stages of development of *Peronospora trifoliorum* sporangiophores and sporangia at 20 C and 100% RH on cotyledons of 11-day-old alfalfa seedlings: **A-B)** 4 hr, **B-E)** 6 hr, **E-F)** 8 hr, and **F)** 10 hr after inducing sporulation. (F-H) Photomicrographs of the same sporangiophores showing **F)** turgid sporangia, **G)** sporangia shriveled by exposure to laboratory air (about 48% RH) for 5-15 sec, and **H)** sporangia still attached after 24 hr of exposure to laboratory air. **I)** Sporangia discharged onto dry glass slide to which a drop of water was added to the upper portion. Note that sporangia on the dry portion near the water line (w), as well as those in the water, have regained turgidity.

humidity chamber and returned it to the opposite end by plastic tubing.

To investigate sporangium development, pots of 11-day-old infected seedlings were placed individually on 1-cm-tall plastic blocks in trays. Deionized water was added to trays to a depth of 0.5 cm and a 500-ml wax-coated paper cup was inverted over each pot to form a dark air-tight seal. Four pots were removed every 2 hr for 24 hr. A few seedlings from each sampling were observed microscopically for sporangium development, and the remainder were used to determine sporangium production and viability as previously described.

To photograph sporangium 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. To observe and photograph sporangium discharge, the cover slip was adjusted to permit entrance of the drier laboratory air.

RESULTS

Effect of relative humidity on sporulation.—*Peronospora trifoliorum* produced sporangia on infected alfalfa seedlings in the plastic humidity chambers when the air drawn through the chambers was at 97% RH or above. However, free water inhibited sporulation. Moisture in the planting medium (sand) from normal watering was sufficient to provide adequate RH for sporulation in the tight chambers.

Sporangium production and germination.—Microscopic observations at 2-hr intervals after induction of sporulation were:

Hour 2–no visible development.

Hour 4–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).

Hour 6–youngest sporangiophores had single branches (Fig. 1-C) and thus were just beyond the same stage of development that the older ones were at 4 hr (Fig. 1-B). This indicated that the stages of development were overlapping by about 2 hr. Most sporangiophores, however, had completed branching (Fig. 1-D) and some were producing sporangia (Fig. 1-E). Sporangia on each sporangiophore formed simultaneously at the apices of all branches, and thus were the same age and size. Sporangiophores at various stages of development often protruded from the same stomate. Immature sporangia were spherical and became egg-shaped when morphologically mature.

Hour 8–all sporangiophores bore sporangia of which about 40% appeared morphologically mature.

Hour 10–all sporangia were morphologically mature (Fig. 1-F).

Our observations were made on plants that previously had not been exposed to light after sporangium production was induced. Thus, to determine the effects of light during sporulation, some cotyledons were returned to darkness after examination and reexamined later. The short exposure to light stopped sporangium production for 2-3 hr; thereafter, it resumed and progressed at the normal rate.

Sporangium production estimated with a spectrophotometer agreed closely with microscopic observations that sporangium production was complete by 10 hr (Fig. 2). However, the germinability was greatest (about 80%) at 12 hr, indicating that the sporangia reached physiological maturity about 2 hr after reaching morphological maturity. Maximum production of viable sporangia occurred 12-16 hr after induction of sporulation and declined thereafter because of a decrease in germinability (Fig. 2).

To determine the duration of sporangium production, infected seedlings were placed at a 9-hr photoperiod, and the sporangiophores and sporangia were removed daily. Sporangia were produced daily on the infected cotyledons until they died, 5-8 days later.

Sporangium discharge.—Sporangia remained turgid and attached to the sporangiophores when kept in a saturated atmosphere (Fig. 1-F). However, when the cover slip was removed from the agar chamber to permit the drier laboratory air to enter, the sporangia shriveled within a few seconds (Fig. 1-G). A counter-clockwise twisting of sporangiophores followed immediately and lasted for 15 sec to 1 min. Some sporangia were discharged during this period but many were still attached to the sterigmata 24 hr later (Fig. 1-H). The entire process was completed in about 2 sec when the observer breathed in the chamber, but the proportion of sporangia discharged apparently was not increased by the more violent twisting.

To observe the sequence of events more closely, the cover slip was adjusted on previously unexposed chambers to permit entry of only a small amount of outside air. After a few minutes the sporangia slowly shriveled, and the sporangiophore branches and branchlets slowly became more upright and the sporangia were grouped into a tight cluster. During this action several sporangia were released. The main sporangiophore stalk did not rotate even after several more minutes exposure to the slowly equilibrating

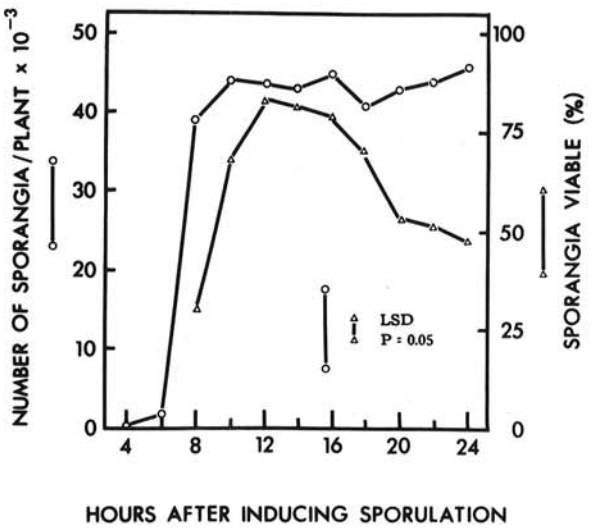

Fig. 2. Production and germinability at 20 C of *Peronospora trifoliorum* sporangia harvested from 11-day-old alfalfa seedlings at intervals after inducing sporulation.

humidity, but it spun counter-clockwise immediately after the cover slip was removed. However, few if any additional sporangia were released during this sporangiophore movement.

When drops of deionized water were pipetted onto leaves with either turgid or shriveled sporangia, the number of sporangia released into the water was at least as great as had been discharged during the drying.

Effect of air-drying on sporangium viability.—Since sporangia were not discharged in a saturated atmosphere, we exposed infected seedlings to drier conditions and determined sporangium viability. Seedlings with freshly-produced sporangia (15 hr after induction of sporulation) were removed from humidity chambers, excised below the cotyledons, and placed on dry microscope slides in open petri dishes in the laboratory at about 540 lux fluorescent lighting, 48% RH, and 25 C. At 0.5 and 3 hr, plants were removed from some slides. Sporangia discharged onto slides and those still attached or entangled in the sporangiophores were germinated separately in drops of water.

Sporangia discharged onto slides during the 0.5 and 3-hr periods germinated 31 and 9%, respectively, compared with 68 and 56%, respectively, for those that remained attached to, or entangled among, the sporangiophores. Nondesiccated controls germinated 81% [LSD ($P=0.05$) = 7]. Thus, germinability in both groups was decreased rapidly by desiccation, but was most pronounced for the discharged sporangia.

Effect of light on sporangium germination.—To determine the influence of light during their germination, sporangia were harvested (15 hr after induction of sporulation) in a darkroom and dispersed in drops of water on microscope slides which were placed on water-soaked filter paper in petri dishes. The dishes were wrapped in aluminum foil, transferred from the darkroom to a growth chamber at 20 C and 5,400 lux fluorescent lighting. The dishes were uncovered and exposed to the light for 0 min, 10 min, 1 hr, or 24 hr and then were covered for the remainder of a 24-hr period.

Mean germination percentages were 43, 72, 69, and 85 [LSD ($P = 0.05$) = 6], respectively. Thus, sporangium germination was enhanced by light, but exposure for 10 min was nearly as beneficial as exposure 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 about 540, 2,700, 5,400, or 10,800 lux.

DISCUSSION

Sporangium morphogenesis of *Peronospora trifoliorum* corresponded closely to that of *P. parasitica* (1) and *Pseudoperonospora cubensis* (3). *Peronospora destructor* (11) and *P. viciae* (6) produced sporangia at 91% RH whereas *P. trifoliorum* required about 97%. A film of water on the host inhibited sporulation of *P. trifoliorum* and *P. tabacina* (7).

The germinability of *P. trifoliorum* sporangia was decreased rapidly by drying. We attributed most of the rapid loss in viability during exposure to laboratory air and the marked difference in viability between discharged and nondischarged sporangia to desiccation. We found no other reports of *Peronospora* sporangia shriveling prior to discharge by the twisting of the sporangiophores during drying. The decrease in moisture tension necessary for mechanical discharge of *P. trifoliorum* sporangia probably reduced germinability. On the other hand, *P. tabacina* sporangia were equally viable at 10 and 95% RH at 24.4 C (10).

Light enhanced sporangium germination in *P. trifoliorum* and *P. manshurica* (5). *Peronospora tabacina* sporangia germinated equally in diffuse light and in darkness but direct sunlight for 1 hr was lethal even though the sporangia were suspended in water in a container on a block of ice (10).

Our work indicates that to produce *P. trifoliorum* sporangia with maximum germinability (at 20 C), it is necessary to harvest the sporangia 12-16 hr after the induction of sporulation, to protect them from desiccation, and to expose them to light. To prevent desiccation while harvesting sporangia and preparing inoculum we place infected shoots in a container with chlorine-free water (2), shake to dislodge the sporangia, and pass the suspended sporangia through a strainer to remove the plant material.

Most or all of the light required for the maximum percentage of sporangium germination is probably provided by the laboratory lighting during the time required to harvest sporangia and prepare inoculum. Therefore, additional light during the infection period may not increase the germination percentage unless the depletion of oxygen in airtight chambers became a limiting factor. Rockett (8) reported that light (5,400 lux) during infection reduced mildew incidence because plants either were too dry or sporangia were washed off by attempts to maintain the free water required for germination. However, this problem should be largely overcome by reducing the light intensity. We found that 540 lux (the lowest intensity we tested) was as effective as 5,400 lux.

LITERATURE CITED

1. DAVIDSON, E. M. 1968. Development of sporangiophores of Peronospora parasitica (Pers. ex Fr.) Fr. Ann. Bot. 32:623-631.
2. FRIED, P. M., and D. L. STUTEVILLE. 1975. Effect of chlorine on Peronospora trifoliorum sporangial production and germination. Phytopathology 65:929-930.
3. KAJIWARA, T., and Y. IWATA. 1957. Observations on the sporulation of cucumber downy mildew fungus. Ann. Phytopathol. Soc. Japn. 22:201-203.
4. PATEL, M. K. 1926. Study of Peronospora trifoliorum de Bary on species of Leguminosae. Phytopathology 16:72 (Abstr.).
5. PEDERSON, V. D. 1964. Light stimulation of conidia germination in Peronospora manshurica. Phytopathology 54:903 (Abstr.).
6. 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.
7. PINCKARD, J. A. 1942. The mechanism of spore dispersal in Peronospora tabacina and certain other downy mildew fungi. Phytopathology 32:505-511.
8. 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. 20 p.
9. ROCKETT, T. R., and D. L. STUTEVILLE. 1969. Effects of photoperiod and temperature on sporulation of Peronospora trifoliorum on seedlings of two alfalfa varieties. Phytopathology 59:1046 (Abstr.).
10. WOLF, F. A., L. F. DIXON, R. Mc LEAN, and F. R. DARKIS. 1934. Downy mildew of tobacco. Phytopathology 24:337-363.
11. YARWOOD, C. E. 1943. Onion downy mildew. Hilgardia 14:595-691.