Infectivity of *Pythium* spp. Zoospores in Snow Rot of Wheat

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We acknowledge the support of the Washington State Wheat Commission. Scientific Paper 5492, Project 0142, College of Agriculture Research Center, Washington State University, Pullman 99164.

Accepted for publication 18 January 1980.

ABSTRACT

LIPPS, P. E., and G. W. BRUEHL. 1980. Infectivity of *Pythium* spp. zoospores in snow rot of wheat. Phytopathology 70:723-726.

Pythium iwayamai and *P. okanoganense* were isolated from naturally infected wheat plants only after water from snow melt had drained through the field. Infections by zoospores of *P. iwayamai* resulted in the death of wheat seedlings if leaves were inoculated and plants were maintained under flooded conditions at 0.5 C in the dark for a total of 90 days. The length of time plants were maintained at 0.5 C under water after inoculation influenced disease development more than did either the length of time plants remained under these conditions prior to inoculation or the amount of inoculum used (500, 1,000, 5,000, or 10,000 zoospores per milliliter). Zoospores of both *P. iwayamai* and *P. okanoganense* encysted on guard cells of stomata; germ tubes growing from the cysts penetrated the stomatal apertures. More zoospores encysted around stomata of old leaves than of young leaves. In the field, zoospores of *P. iwayamai* and *P. okanoganense* are released into the snow melt water and zoospores accumulate on plant surfaces and penetrate via stomata.

Diseases caused by *Pythium* spp. on aboveground plant parts are uncommon but not rare (1,6,14,20). Fruits and foliage contact infested soil and infections are caused by direct germination of oospores, sporangia, or chlamydospores at the soil surface (16,17). Zoospores of soilborne *Phytophthora* spp. infect aboveground plant parts submerged in contaminated irrigation or flood water (7,11,12,19).

Zoospores of specialized foliage pathogens accumulate on guard cells of stomata and germ tubes growing from them penetrate stomatal openings (2,13,23). Kim et al (6) demonstrated that zoospores of *P. aphanidermatum* accumulate on stomata when suspensions of zoospores were sprayed on the surface of bean leaves. Hirane (4) reported that zoospores of *P. iwayamai* Ito encysted on stomata of wheat leaves in vitro, and that germ tubes penetrated stomatal openings. The consistent association of diseased plants with water from snow melt and the ability of *P. iwayamai* and *P. okanoganense* Lipps to produce zoospores in near-freezing water (9) indicate that zoospores may be the major infective propagules in the field.

The purpose of this study was to determine if zoospores of the snow rot-causing *Pythium* spp. are infective propagules in nature, and to establish when infection occurs, whether zoospores could incite the snow rot disease under laboratory conditions, and the mode of penetration by zoospores.

MATERIALS AND METHODS

Isolation from naturally infected plants. Two field plots were located in low-lying fields where water from snow melt could drain or collect. One plot near LaFleur, Okanogan County, Washington was planted 25 August 1977, the other near Pullman, Whitman County, Washington, was planted 21 September 1977. Both plots were identical in design and wheat cultivars. From 19 November 1977 to 6 April 1978, plants were collected at random, placed in plastic bags, put into a chest with ice and transported to the laboratory. To assay for the presence of *Pythium*, plants were washed briefly to remove soil, then rinsed in cold running tap water for 24 hr. Leaves were cut into pieces 5 cm long, blotted dry with paper towels, and placed in petri dishes containing either the P_{10} VP selective medium (21) or 2% water agar. The P_{10} VP plates were incubated at 15 C for 3 days and the water agar plates were incubated at 1 C for 5 days (10). For identification, hyphal tip isolations were transferred to Difco corn meal agar (DCMA) and incubated at 10 C.

Inoculations with zoospores. Zoospore suspensions were prepared by transferring 2- to 3-mo-old DCMA cultures of *P. iwayamai* (isolate IMI 209669) to Difco lima bean agar (LBA) and incubating at 10 C until the colony diameter reached 5 cm. Four to five agar disks (1 cm in diameter) were cut from the periphery of the colonies, placed into each glass petri dish with 20 ml of deionized water (DW), and incubated at 5 C. The DW in each dish was decanted 24 hr later, and replaced with fresh DW precooled to 5 C. Forty-eight hours later, dishes with motile zoospores were put at 0.5 C and after 2-3 hr zoospore suspensions were decanted into 250-ml beakers and the concentration was adjusted by diluting with DW precooled to 0.5 C.

Winter wheat (*Triticum aestivum* L. 'Nugaines') seeds were planted in sand in 237-ml styrofoam cups (one seed per cup) on 28 February 1978 and placed on the greenhouse bench. After emergence, the plants were grown outdoors to harden. On 13 April plants in cups were brought into the grenhouse, flooded with tap water to a depth of 1 cm above the sand, and a wet pad of absorbent cotton was placed over each plant. Plants were then maintained at 0.5 C in the dark. After 0, 20, 40, 60, and 80 days of predisposition, 100 plants were washed from the sand with cold tap water. Four plants at a time were completely submerged in 200-ml zoospore suspensions containing 0, 500, 1,000, 5,000, and 10,000 spores per milliliter at 0.5 C in the dark. A total of 20 plants were submerged in suspensions of each inoculum level for 24 hr. Afterwards, plants were transplanted into styrofoam cups in sand (one plant per cup), flooded with tap water to a depth of 1 cm above the soil surface, covered with cotton, and placed in the 0.5 C chamber. After 90 days (preinoculation + postinoculation times) all cups were taken from the low-temperature chamber and placed on the greenhouse bench. Cups were drained, the cotton covers were removed, and plants were watered with 10% Hoagland's Solution No. 2 (5) to facilitate recovery. The number of surviving plants was recorded after a 2-wk recovery period.

Host penetration. Wheat plants were submerged in a zoospore suspension (10,000 spores per milliliter) at 0.5 C for 4 or 24 hr, or were collected from a field plot when water from snow melt covered the plot to a depth of 15 cm (15 March 1978). Plants were dipped in 0.1% Trypan blue in lactophenol for 30 sec, then dipped in DW to remove excess stain. Strips of epidermal cells were lifted from leaf blades and mounted on glass slides in lactophenol for microscopic examination.

0031-949X/80/08072304/$03.00/0

RESULTS

Isolation from infected plants. In Okanogan County several light snows occurred in December 1977. Fluctuating temperatures did not maintain a persistent snow cover, and fields became waterlogged due to accumulated water from rain and snow melt. Subsequently, ice formed over the plot, and plants were beneath ice in the furrows left from the packer wheels of the deep furrow drill. Some of the upper leaves of plants were imbedded in the ice, but the lower leaves were in contact with the frozen soil or in the air space below the ice. The field plot remained frozen through February and about 10 cm of snow collected over the ice layer during this time. The snow and ice began to melt in early March and the resulting water covered the plants in the seed furrows beneath the ice crust. By the time the water drained away the plants in the previously flooded portion of the field were dead.

Pythium ultimum was the only species isolated from plants collected before snow fall (19 November) or by the time the plot was covered with ice (3 January) and its frequency of isolation did not increase during snow melt (3 March to 6 April) (Table 1). *P. okanoganense* was not recovered from plants until the beginning of snow melt (3 March) and it was isolated from nearly all plants during snow melt. On 15 March, 18 plants were collected from sites near the plot, but where water from snow melt had not drained. Four of these plants yielded *P. ultimum* and no other *Pythium* spp. were isolated. *P. iwayamai* was not isolated from any plants from the plot in Okanogan County.

In Whitman County a persistent snow cover was not maintained. Only *P. ultimum* was isolated from plants collected before the first snow cover. However, after a short period of snow cover on 20 January, *P. iwayamai* was isolated as well as *P. ultimum*. After a second snow in mid-February, which had melted by the first week in March, *P. iwayamai, P. okanoganense,* and *P. ultimum* were isolated, and none of the plants died from snow rot.

Inoculations with zoospores. At 0.5 C, zoospores remained motile, but at 5 C zoospores could not be decanted without many of them encysting, therefore, all tests and preparations were performed at 0.5 C.

Plants were flooded and kept in the dark at 0.5 C before and after inoculation for a total of 90 days. Zoospores of *P. iwayamai* encysted on leaves of all plants regardless of the predisposition period prior to inoculation. Infections by zoospores resulted in the death of more plants if the leaves were inoculated after zero and 20 days of predisposition (90 and 70 days of incubation, respectively) (Fig. 1). Seventy-six percent fewer plants were killed if inoculated after 40 days of predisposition (50 days of incubation) than after 20 days of predisposition and fewer plants died if inoculated after 60 or 80 days of predisposition (30 or 10 days of incubation, respectively). There was no difference in the survival of plants inoculated with different concentrations of zoospores (500, 1,000, 5,000, or 10,000 zoospores per milliliter).

Mode of penetration. Four hours after intact wheat plants were

TABLE 1. Isolation of *Pythium iwayamai, P. okanoganense,* and *P. ultimum* from leaves of winter wheat collected at various times during 1977–1978 from field plots in Okanogan and Whitman Counties, Washington

Location and date isolated	Field conditions	Plants (no.)	*Pythium* spp.		
			P. iwayamai	*P. Okanoganense*	*P. ultimum*
Okanogan Co.					
19 Nov 77	No snow cover	25	0[a]	0	6
3 Jan 78	Ice covered	35	0	0	3
3 Mar 78	Beginning snow melt	10	0	8	3
15 Mar 78[b]	Under 15 cm water	17	0	15	2
6 Apr 78[c]	Water drained away	64	0	62	5
Whitman Co.					
5 Dec 77	No snow cover	61	0	0	3
20 Jan 78	After short snow cover	40	1	0	6
3 Mar 78	After short snow cover	44	26	7	1

[a]Number of plants from which each *Pythium* spp. was isolated.
[b]On this date 18 plants were collected from a site near the plot where snow-melt water had not drained, four plants yielded *P. ultimum* and no other *Pythium* spp. were isolated.
[c]2% water agar (10) was used for isolation on this date, P_{10} VP selective medium (21) was used on all other dates.

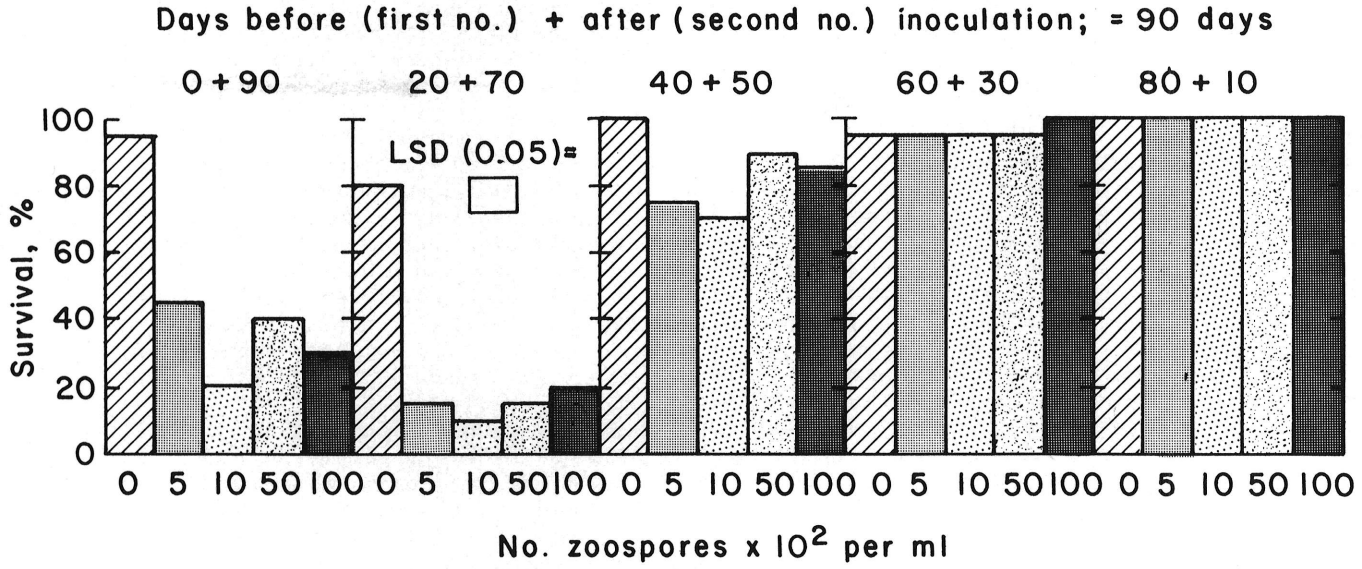

Fig. 1. The effect of the time under water at 0.5 C in the dark before and after inoculation and of zoospore inoculum level (*Pythium iwayamai*) on survival of wheat seedlings. Twenty plants per treatment, one plant per replicate.

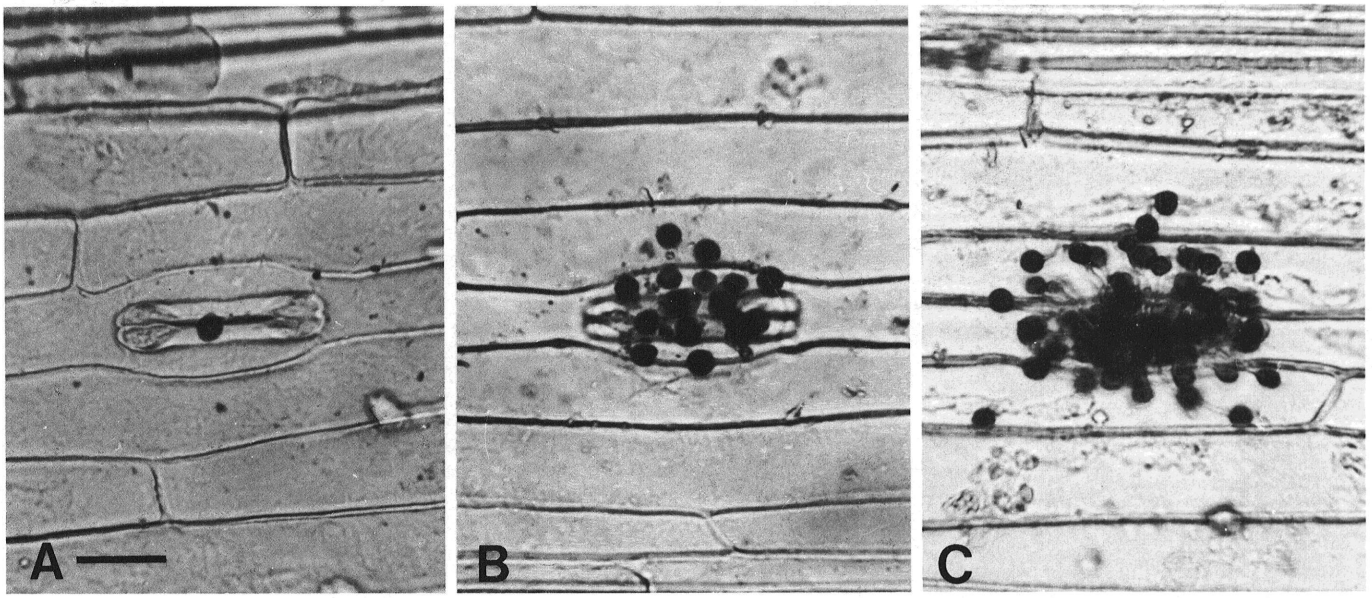

Fig. 2. Zoospores of *Pythium iwayamai* encysted on stomata of inoculated wheat leaves at 0.5 C. **A, B,** leaves remained in zoospore suspensions (1×10^4 spores per milliliter) for 4 hr. **C,** zoospore cysts with germ tubes entering stomatal apertures after 24 hr in zoospore suspensions. Bar = 30 μm.

submerged in zoospore suspensions at 0.5 C, from one to nearly 100 zoospores of *P. iwayamai* encysted on and around guard cells of individual stomata (Fig.2A, B). Zoospores also encysted on splits between epidermal cells, disrupted or damaged cells, and other surface wounds. Zoospores encysted more frequently on stomata near the leaf tip and the number of zoospore cysts decreased towards the base of the leaf. More zoospores encysted around stomata of older leaves than around those of younger leaves, and young leaves frequently had no zoospores encysted on their stomata. Zoospore cysts germinated and produced single germ tubes which grew into the stomatal aperture (Fig. 2C). Stomatal penetration was complete within 24 hr after inoculation. Zoospores also encysted on cut ends of roots and root tips, and around damaged epidermal cells where secondary roots emerged.

Encysted zoospores of *P. okanoganense* (identified after isolation) were found on guard cells of stomata with germ tubes penetrating the stomatal aperture of plants collected from the Okanogan County plot under 15 cm of water from melted snow (15 March 1978) (Fig. 3). No zoospore cysts were found on roots of plants collected from the field plot.

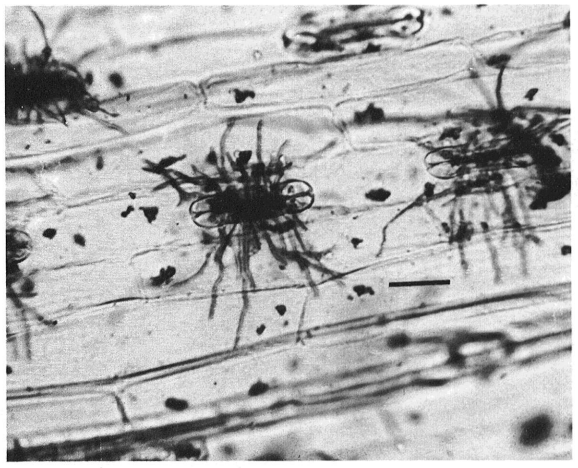

Fig. 3. Stomatal penetration by germ tubes from zoospore cysts of *Pythium okanoganense.* These plants were from a field plot flooded by water from snow melt and infected by natural zoospore inoculum. Epidermal strip viewed from the underside to show numerous germ tubes entering the stomatal aperture. Bar = 30 μm.

DISCUSSION

Direct evidence that zoospores of *Pythium* spp. cause infections of aboveground plant parts in nature does not exist (6,15,16). Zoospores of some *Phytophthora* spp. occur in flood and irrigation water and serve as infective propagules in nature (7,11,12,19). The field distribution of diseased plants indicates that zoospores are the main infective propagules of snow-rot *Pythium* spp. (4,10). *P. iwayamai* and *P. okanoganense* were isolated only after water from snow melt had drained through the field plots. Plants collected at the beginning of snow melt from the Okanogan County plot (4 March 1978) did not have the severe soft rot symptoms usually associated with diseased plants. This indicates that infection had occurred recently and that the fungi had insufficient time to extensively invade the leaf tissues. Plants collected at later dates exhibited severe soft rot symptoms and were dead by the time the water from the snow melt drained away. In the Whitman County plot no plants were killed at the end of snow melt, presumably snow melt occurred too quickly and temperatures rose too rapidly, preventing further invasion by the *Pythium* spp.

P. ultimum was isolated from leaves of 8–24% of the plants in the Okanogan County plot and from 2–15% of the plants in the Whitman County plot. This fungus invaded plants before snow fell and the frequency of isolation did not increase during snow melt (Table 1). These results confirm previous evidence (10) that *P. ultimum* invades tissues but is unable to incite the snow rot disease.

Infections by zoospores of *P. iwayamai* resulted in the death of wheat seedlings if the incubation period after inoculation was 70 days or longer under flooded conditions at 0.5 C in the dark. Shorter incubation times resulted in more plants surviving 90 days at low temperatures. Under natural conditions, plants remained under snow or ice for 60 to 90 days and an additional 30 to 40 days under water from melted snow. Therefore, the rotting of leaves and crowns required 70 days after initial infection under artificial conditions compared with an estimated 30 to 40 days under natural conditions. It is likely that either the larger field plants were more susceptible than the smaller plants used in inoculation trials or plants predisposed under natural conditions were more susceptible than those maintained under artificial conditions. The nature of the predisposition is unknown, and it is probably not closely simulated in the low-temperature chamber.

There was no difference in the number of surviving plants inoculated with different concentrations of zoospores (500-10,000 spores/ml) (Fig. 1). In nature, zoospores are probably released into

the water during snow melt from numerous infected plants over a period of several weeks, resulting in high concentrations of inoculum in the water stream.

Although zoospores of *P. iwayamai* encysted on stomata of wheat leaves, they also encysted on root tips, cut ends of roots, and other damaged cells, indicating that zoospore chemotaxis was nonspecific (3,15). More zoospores accumulated on stomata of older leaves, indicating that more exudate escapes from them than from stomata on younger leaves (3). Tukey (22) reported that the amount of substances leached from leaf tissues depended on the physiological age of the leaf. Young leaves are relatively immune to loss of minerals and carbohydrates, and mature leaves loose greater amounts of these substances (22). Tomiyama (18) demonstrated that more openings, either wounds or abnormal stomatal openings, occured in older leaves than in younger leaves. His results suggested that the essential difference between hyphal penetration of old leaves and young leaves by *Typhula incarnata* was due to the predominance of natural openings on older leaf surfaces. Both the increased number of natural openings and the greater amount of exudates from older leaves probably increases the chance of zoospores encysting on and penetrating older leaves. Under flooded conditions during snow melt, predisposed tissues probably exude large amounts of nutrients into the water, and zoospores follow the chemical gradient to its source and accumulate on stomata or wounds having the greatest nutrient leakage.

Although inoculations with zoospores of *P. okanoganense* did not result in the death of seedlings in previous experiments (8), the response of zoospores of *P. okanoganense* to stomatal openings was identical to those of *P. iwayamai.*

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