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Effect of temperature, hydrogen ion concentration and osmotic potential on zoospore production by three *Pythium* species isolated from pond water

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Pythium fluminum **produced zoospores most abundantly at 15°C, whereas the optima for *Pythium* 'group F' and *P. marsipium* were 20 and 25°C, respectively. Increasing the incubation temperature above the optimum resulted in the decrease of the duration of zoospore production. In *Pythium* 'group F' the ability to produce zoospores was not lost even after incubation at 40°C for 24 h. On the other hand, *P. marsipium* and *P. fluminum* lost the ability under these conditions. Zoospore production was inhibited at pH 4.5 and 10.5 in all the species tested. *Pythium fluminum* and *P. marsipium* were found to have two pH optima for zoospore production (7.5 and 9.5 for the former and 5.5 and 8.5 for the latter). The optimum pH for zoospore production by *Pythium* 'group F' was 6.5–7.5. Moderate osmotic potentials (−0.27∼−0.47 MPa) appeared to favor zoospore production by the pythia tested. The effect of temperature, pH and osmotic potential on zoospore production was discussed in relation to pollution of pond water.**

Key Words——pH and osmotic potential; pond water; *Pythium* spp.; temperature; zoospore production.

During an ecological study on *Pythium* species in aquatic habitats (Abdelzaher et al., 1993), more than 20 species and 500 isolates have been obtained by baiting from water of three ponds in Osaka, Japan. About 95% of the species isolated were found to produce zoospores. Grass leaf blades are commonly used for the production of zoospores by *Pythium* species (Emerson, 1958; Waterhouse, 1967; Webster and Dennis, 1967; Saleem and Dick, 1990). *Pythium* fluminum Park var. *fluminum* (*P. fluminum*) was first isolated in North Ireland (Park, 1975, 1977). Abdelzaher et al. (1994a) isolated this fungus from Tatsumi pond water in Sakai, Osaka, for the first time outside Ireland. Park (1975) used carboxymethyl cellulose (CMC) agar disks, which had been invaded by *P. fluminum* and placed in sterilized deionized water to induce zoospore formation. *Paspalum* leaf blades colonized by *Pythium* spp. in sterilized distilled water were found to be good material for obtaining adequate zoospores for the present work.

The aim of this work is to examine the effect of temperature, hydrogen ion concentration and osmotic potential on the zoospore production of three aquatic *Pythium* spp., which might elucidate the effect of pollution upon fungal spread in an aquatic ecosystem.

Materials and Methods

Pythium fluminum, P. marsipium Drechsler and *Pythium* 'group F' were isolated from pond water by baiting technique using a variety of natural and artificial cellulolytic and pectinolytic baits (Abdelzaher et al., 1994a, b; Park, 1977; Pittis and Colhoun, 1984).

Pythium fluminum is a unique cellulolytic species, but data is lacking about its behavior in aquatic habitats (Park, 1977; Abdelzaher et al., 1994a). *Pythium* 'group F' was chosen since it has been isolated every month under various environmental conditions (Abdelzaher et al., unpublished data) and generally occurs in open water (Plaats-Niterink, 1981). *Pythium marsipium* was chosen because it is a typically aquatic species with proliferating zoosporangia (Abdelzaher et al., 1994b).

Grass blades of *Paspalum thunbergii* Kunth were cut into 3 × 14 mm pieces and then autoclaved at 121°C for 20 min. *Pythium marsipium* and *Pythium* 'group F' were inoculated on Petri dishes containing 3% water agar, while *P. fluminum* was inculated on CMC-agar. After the fungal colonies reached about 4 cm diam, the autoclaved *Paspalum* leaf blades were layed over each inoculum in contact with the actively growing margin and incubated at 25°C. After 1 day (in the case of *P. marsipium* and *Pythium* 'group F') or 4 days (in the case of *P. fluminum*) of incubation the colonized grass blades were transferred to Petri dishes (7 cm diam) containing 10 ml of sterilized deionized water to determine the effect of temperature on zoospore production.

To study the effect of hydrogen ion concentration on zoospore production, the colonized *Paspalum* leaf blades were transferred to Petri dishes (7 cm diam) containing 10 ml of 0.025 M MES [2-(N-morpholino) ethanesulfonic acid] buffer which had been adjusted to pH values of 4.5

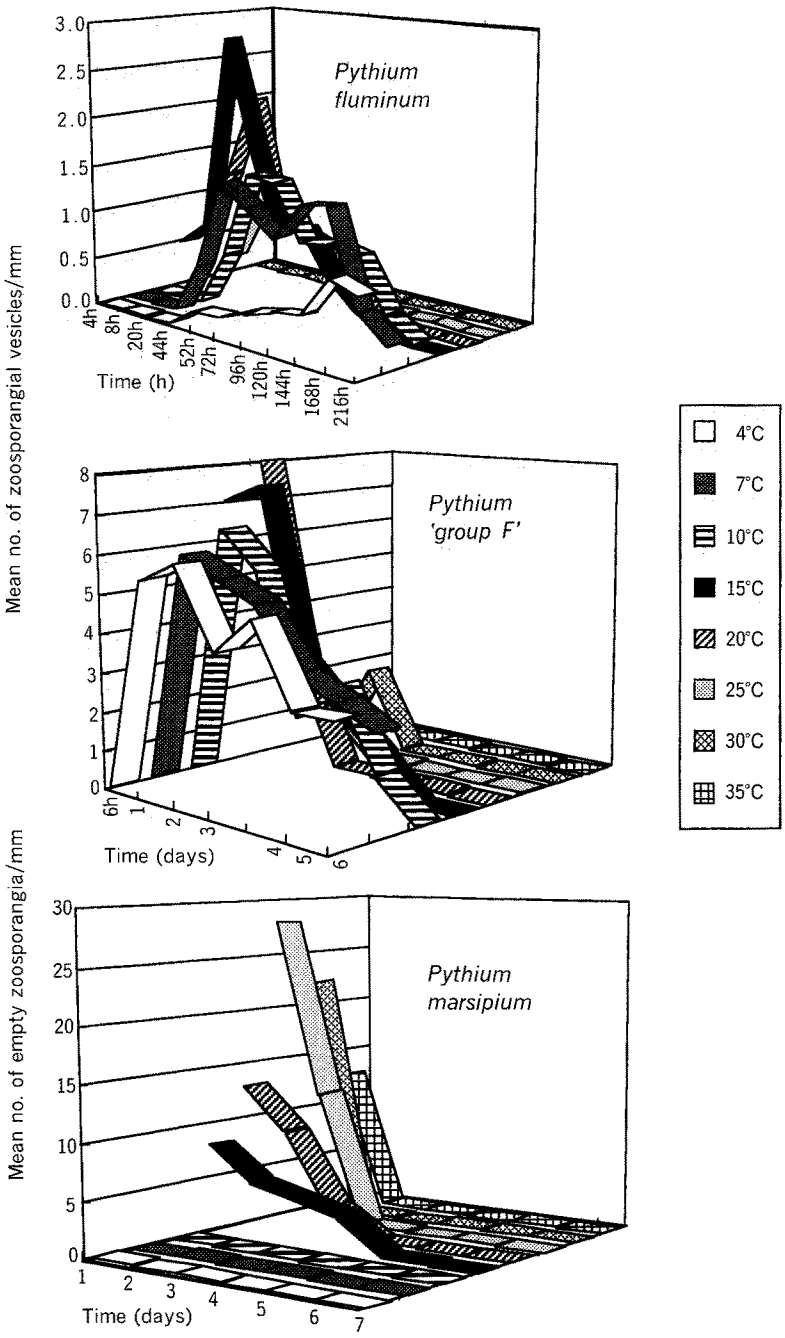

Fig. 1. Effect of temperature on zoospore production of three *Pythium* spp.

to 7.5 with 1 N HCl or 1 N NaOH. CHES [2(cyclohexylamino) ethanesulfonic acid] buffer was used to make pH values of 8.5, 9.5 and 10.5 with the aid of 1 N HCl or 1 N NaOH. Since these buffers had an inhibitory effect on zoospore production of *P. fluminum*, solutions of different pH values were prepared using HCl and NaOH. The solutions and buffers were sterilized by filtration.

To study the effect of osmotic potential on zoospore production, the colonized *Paspalum* leaf blades were transferred to Petri dishes (7 cm diam) containing 10 ml of sterilized mannitol solution dissolved in distilled water, which is inactive to *Pythium*, of different osmotic potentials. The osmotic potential was determined with a Dew Point Microvoltmeter (HR-33T; Wescor, Logan, USA). Deionized water and distilled water were also used as test solutions. Deionized water which contains traces of salts that cannot be detected by the instrument (zero MPa osmotic potential) had been used for zoospore production of *P. marsipium* (Abdelzaher et al., 1994b). Distilled water was also used since it does not contains

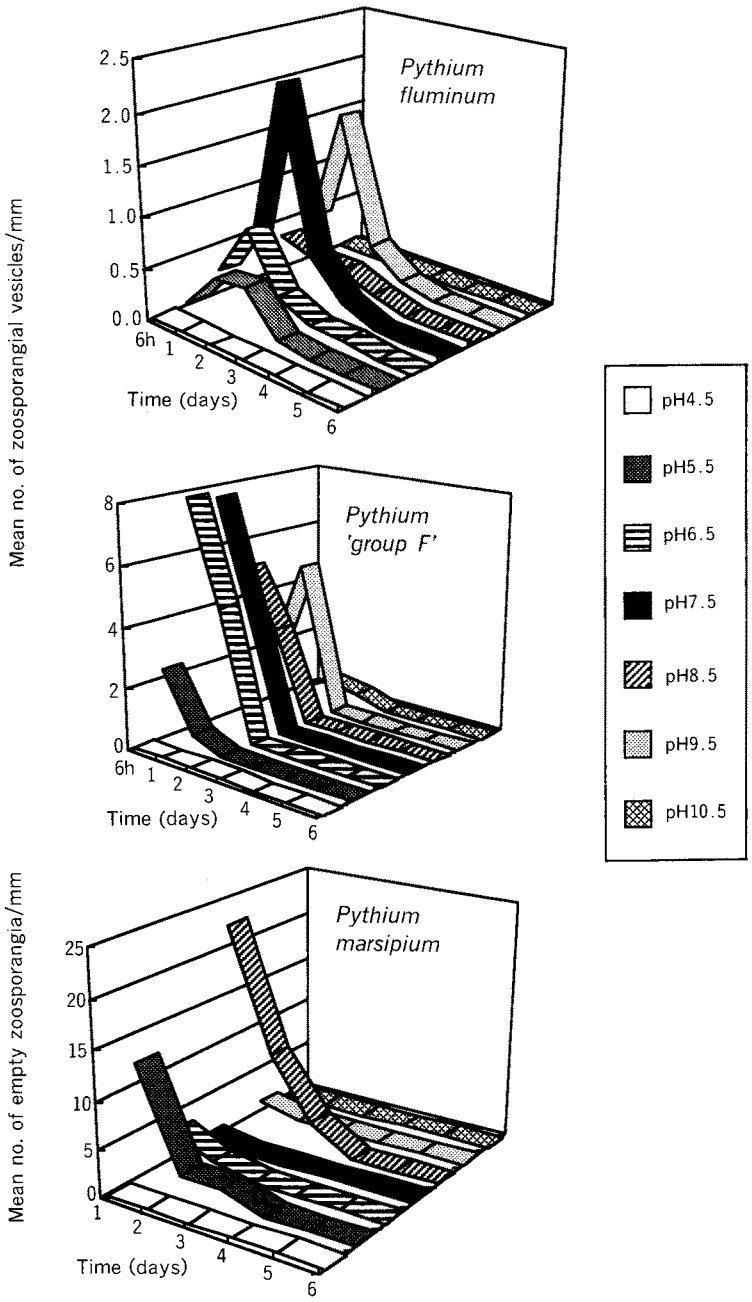

Fig. 2. Effect of hydrogen ion concentration on zoospore production of three *Pythium* spp.

any salts (zero MPa osmotic potential). To evaluate the usefulness of Robertson and Howard's solution, which they recommend [cited by Singleton et al., 1992] for inducing zoosporangia and zoospores of *Pythium* species, this solution was also used for comparison. Robertson and Howard's solution contains salts in an amount that can not be detected by the instrument (zero osmotic potential).

Cultures were observed microscopically along the *Paspalum* leaf margin through the lid of the Petri dish at different intervals. *Pythium fluminum* and *Pythium* 'group F' have filamentous, non-inflated sporangia which produce vesicles at the top of evacuation tubes. Each active vesicle contains zoospores ready to discharge after a specific time. An active vesicle can be determined by its zoospore differentiation just before discharge. The method of Saleem and Dick (1990) was followed in which the number of active zoosporangial vesicles along the leaf margin was recorded. A mean value was calculated for each treatment and expressed as number of vesicles per mm. In *P. marsipium*, which has internally proliferated zoosporangia, zoospore production was expressed as the number of empty zoosporangia with discharge tubes per mm. The whole experiment was

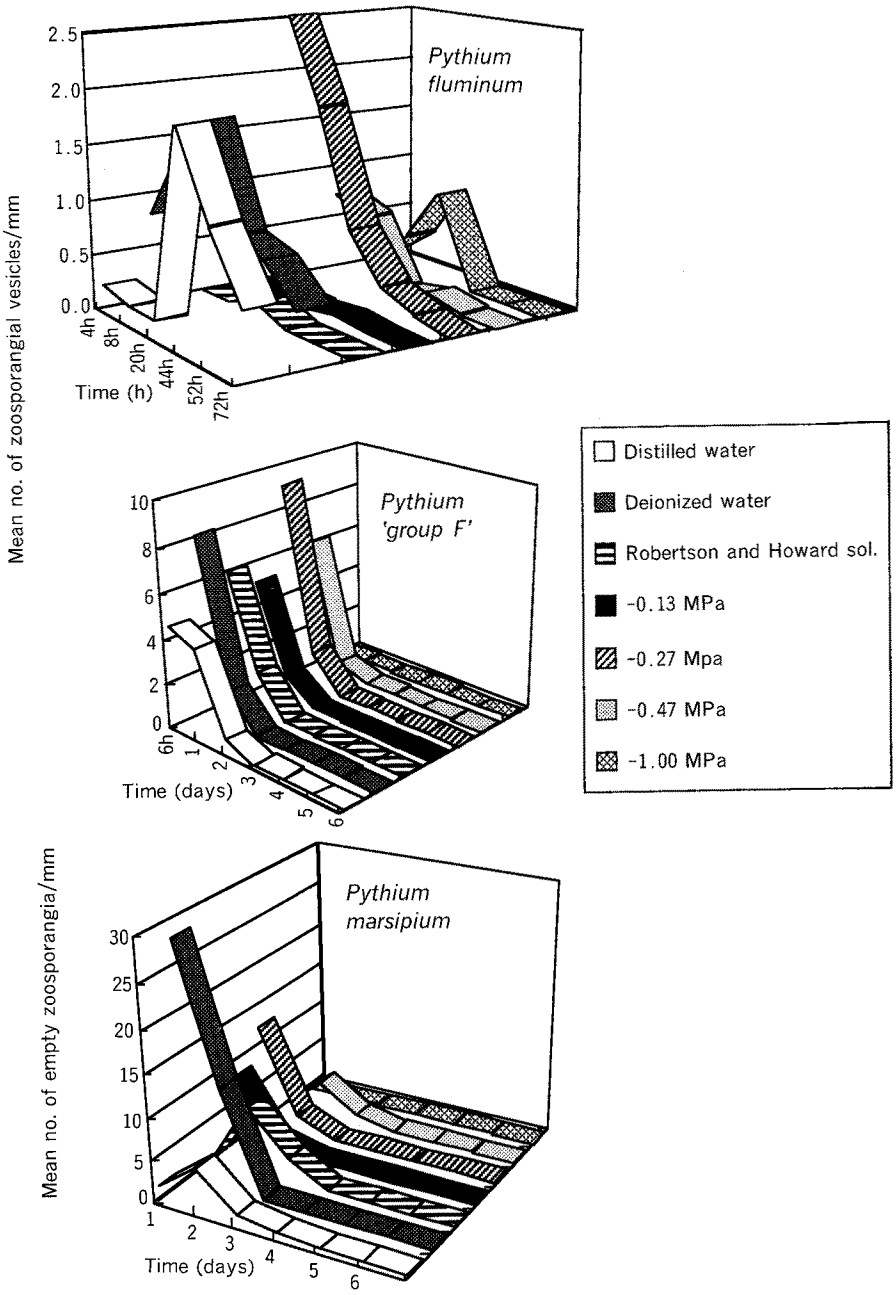

Fig. 3. Effect of osmotic potential on zoospore production of three *Pythium* spp.

repeated twice with three replications, and partial data from a third replication were obtained for confirmation.

Results

Figure 1 shows that the zoospore production was generally highest initially and gradually declined during incubation. *Pythium fluminum* started to produce zoospores after 4 h of incubation at 15, 20 or 25°C. At below 15°C the fungus produced zoospores after 44–52 h. Production continued for many hours, particularly at lower temperatures. The fungus continued to produce zoospores up to the end of the experiment at 4, 7 or 10°C, and above 10°C the production declined drastically. *Pythium* 'group F' began to produce zoospores after about 6 h between 15 and 30°C, but it failed to produce at 35°C. At lower temperatures of 4, 7 or 10°C, *Pythium* 'group F' produced zoospores after 24 h, and low temperature supported a longer time of production than high temperature. *Pythium marsipium* did not produce zoospores at 4, 7 or 10°C, but did produce from 15 to 35°C. The optimum temperatures for zoospore production were 15, 20 and 25°C for *P. fluminum, Pythium* 'group F' and *P. marsipium*, respectively.

In *Pythium* 'group F', the ability to produce zoo-

spores was not lost even after incubation for 24 h at 40°C. On the other hand, *P. marsipium* and *P. fluminum* lost the ability under these conditions.

Figure 2 shows that production of zoospores was inhibited at pH 4.5 and 10.5 for all the three species tested. The pythia tested could produce zoospores within the range of pH 5.5 to 9.5. In solutions prepared, using HCl or NaOH, *P. fluminum* produced zoospores in the range of pH 5.5 to 9.5 with two basic optima for production of 7.5 and 9.5. *Pythium* 'group F' has a wide range of pH values from 5.5 to 10.5 with the optimum at 6.5 to 7.5 for zoospore production. *Pythium* 'group F' produced a small amount of zoospores at pH 10.5 after 24 h. *Pythium marsipium*, however, produced a small amount of zoospores at pH 9.5 and 7.5. Since pH 5.5 and 8.5 were found to be optimum for zoospore production in *P. marsipium*, the fungus apparently had two optima, one acidic and the other basic.

Figure 3 presents the effect of osmotic potential on zoospore production by the species tested. Robertson and Howard's solution [cited by Singleton et al., 1992], which contains salts in an amount that cannot be detected by the instrument, supported very poor zoospore production by *P. fluminum*, fairly high production by *Pythium* 'group F' and moderate production for *P. marsipium*. Deionized water (zero osmotic potential) was found to have a good effect on zoospore production by the species used. In deionized water amended with a non-ionic mannitol to adjust osmotic potential between −0.13 MPa and −3.40 MPa, *P. marsipium* and *Pythium* 'group F' were able to produce zoospores between −0.13 to −0.47 MPa but not above this range. On the other hand, *P. fluminum* produced zoospores above −0.47 MPa. The highest zoospore production was found at −0.27 MPa for all species tested.

Discussion

One of the important roles of zoospores is in dissemination of plant pathogens. The influence of water currents on the spread of zoospores is clearly illustrated by Tomlinson (1958a, b). Our experiment shows clear evidence that certain aquatic habitats play an important role in the establishment of *Pythium* spp. in the aquatic ecosystem. Because of the vesicle membrane, the behavior of vesicles is correlated to the contacting atmosphere. Heavily polluted water, with a shifted hydrogen ion concentration and high osmotic potential, represents an obstacle against zoospore production. Temperature had a great influence on zoospore production of the pythia tested. Minimum, optimum and maximum temperatures were different from one species to the other. *Pythium* 'group F' was found throughout one year of field study at water temperatures ranging from 4–30°C (Abdelzaher et al., unpublished data). *Pythium marsipium* was isolated in October, 1992 (Abdelzaher et al., 1994b), when the water temperature was 21°C but it could not be isolated during the winter (Abdelzaher et al., unpublished data). *Pythium fluminum* is a low temperature species isolated in winter (Feb., 1993) while the temperature of the pond water wad 6°C (Abdelzaher et al., 1994a). Suzuki (1960, 1961) pointed out that the production of zoospores of aquatic fungi was much influenced by water temperature, and that the amount of zoospores of *Pythium* in lake water fluctuated seasonally.

Values of pH during our field survey ranged from 6.3–9.5, sometimes 10.5, and this range allows zoospore production (Abdelzaher et al., unpublished data). The acidic (pH below 4.5) pond is not suitable for zoospore production by *Pythium* species. Zoospores of *Pythium* species were very rare in acidotrophic lakes (Suzuki, 1961). Total soluble salts (conductivity) ranged from 0.24–0.61 Ms/ml, within which range zoospores can be produced (Abdelzaher et al., unpublished data). Our results support the data that *Pythium* spp. differ in their occurrence in different seasons. Although the inhibitory effect of MES and CHES buffers on zoospore production of *P. fluminum* has been clearly detected, no alternative buffer could be given at present.

Deionized water was a good medium for zoospore production by the species tested, especially with colonized grass blades. Diluted salt solution (Robertson and Howard sol.) for zoospore discharge whose effectiveness had been suggested by Robertson and Howard [cited by Singleton et al. 1992], did not give good results in our case, suggesting that it might be used for mycelial mats than colonized grass blades. The fungi can meet their nutritional requirements for zoospore production from the grass blades themselves.

Moderate osmotic potential appeared to favor zoospore production. This phenomenon correlated with the influence of osmotic strength on the formation of the vesicles, because at higher concentration levels the fungi could produce vesicles but at the same time these vesicles failed to produce zoospores. Harvey (1952), in his study of the role of fungi in stream sanitation and processes of natural purification, concluded that no *Pythium* spp. have been detected in heavily polluted water and have seldom been found in partially polluted places. For the above reasons, it is speculated that highly polluted water with high osmotic potential acts as a barrier against zoospore production by *Pythium* spp.

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