

Effects of Inoculum Density, Plant Age and Temperature on Disease Severity Caused by Pythiaceous Fungi on Several Plants

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Alfalfa, maize, sorghum and sugarbeet plants were inoculated with zoospores of *Phytophthora* and *Pythium* species in order to assess the effects of inoculum density, plant age and temperature on disease severity. Seedlings were grown axenically in test tubes and inoculated with zoospore suspensions. Disease severity was assessed by measuring the root growth and discoloration of treated and control seedlings. The incremental root length of all plants decreased and root discoloration increased as inoculum concentration of the pathogen increased. Changes were more intensive among low levels of zoospore concentrations and no significant differences in disease severity were found for inoculum densities higher than 10^4 zoospores ml^{-1} . Disease severity was negatively related to plant age. Disease development on sugarbeet seedlings infected with *Pythium* and *Phytophthora* species was affected by temperature, but the pattern of response was determined by the pathogen's temperature preferences. The incremental root length decreased as temperature increased up to 25°C. The effect of *Pythium dissimile* and *Phytophthora cactorum* on root length was significantly lower at 35°C than at 25°C, whereas *Pythium aphanidermatum* and *Phytophthora nicotianae* caused significant damage to roots even at 35°C.

KEY WORDS: Alfalfa; maize; sorghum; sugarbeet; root growth; root discoloration; *Phytophthora*; *Pythium*.

INTRODUCTION

Members of the genera *Pythium* and *Phytophthora* are very commonly isolated from diseased or healthy plant roots, from both agricultural and virgin soils, from terrestrial or aquatic environments. Although the biology and pathology of certain *Pythium* and *Phytophthora* species have been studied extensively and a great amount of information has been accumulated over the last 50 years, there are still questions to be answered. The nature and quantification of the infective propagules in field conditions, the variation of spatial and temporal patterns of propagules in relation to pathogenicity and saprobic survival of essentially pathogenic species, the limits of saprophytism or parasitism in natural conditions including their role as minor pathogens, are only some of these questions. The main reason for the fragmentary information is the biodiversity of the fungal species in these genera.

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An understanding of the influence of inoculum density (ID) on plant infection is important before attempting to elucidate quantitatively the overall interactions of the host, pathogen and the environment because of the complexity of factors influencing infection and then disease development (19). A range of inoculum densities should be selected that will characterize better the relationship between inoculum density and root infection (7,17,18). For some *Phytophthora* and *Pythium* species, infection and disease may result from very low zoospore numbers (21). The use of high ID may break down host barriers that otherwise would remain intact (2,22).

Also, plant age affects the pathogen – host relationship and physiological plant age has a greater influence on the expression of host susceptibility than chronological age (5). Older plants may show symptoms upon inoculation but later recover and grow to maturity (21).

Diseases caused by pythiaceous pathogens occur under conditions unfavorable both to other pathogens and to the host (12). Temperature is a critical parameter for the development of infective propagules and for a successful infection and although most species grow well at a wide range of temperatures, the optimum conditions are found in a restricted spectrum (24). Temperature of the substratum affects the speed and severity of root symptom development. With few exceptions root-rot symptoms increase with rising temperatures to ~ 30°C, while the symptoms may be suppressed at very low and high temperatures (5). Damping-off and other *Pythium* diseases occur over a wide range of temperatures, which determine which species of *Pythium* may be active.

The aim of this study was to analyze the effects of zoospore concentration, plant age and temperature on pathogenicity of several plants infected with *Phytophthora* and *Pythium* zoospores.

MATERIALS AND METHODS

Media Cleared V8 broth was prepared as follows: a solution of 200 ml V8 juice (Campbell Soup Co.) and 2 g CaCO₃, was centrifuged at 5000 g min⁻¹ for 20 min and the supernatant filtered through Whatman No. 1 paper, diluted tenfold with double glass-distilled water, pH = 6–6.5 (adjusted with NaOH), and autoclaved at 15 psi for 20 min, at 121°C. After cooling, 10 ml of a 95% ethanol solution containing 0.02 g β-sitosterol was added. As water quality is critical, double glass-distilled water free of toxic ions was used.

TABLE 1. Isolates of *Pythium* and *Phytophthora* species used in this study (Isolates were obtained from the Aquatic Phycomycetes Culture Collection (APCC), held in the UK by the second author, the International Mycological Institute (IMI), UK, and the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands)

Species	Author	APCC	IMI	CBS
<i>Pythium aphanidermatum</i>	(Edson) Fitzp.	na	na	634.7
<i>Pythium deliense</i>	Meurs	D162 ADAS	na	na
<i>Pythium dissimile</i>	Vaartaja	4204f	345186	na
<i>Phytophthora cactorum</i>	(Lebert & Cohn) Schröt.	na	325063	na
<i>Phytophthora nicotianae</i>	van Breda de Haan	na	NR17	na

The mineral salts solution contained 0.48 g MgSO₄, 0.5 g KNO₃, 1.64 g Ca(NO₃)₂ per liter of distilled water (4). The solution was autoclaved and then the filter-sterilized

TABLE 2. Plant species and varieties used in this study

Scientific name	Common name	Variety	Supplier
<i>Beta vulgaris</i> L.	Sugarbeet	Saxon	Hilleshog (Norfolk, UK)
<i>Sorghum bicolor</i> (L.) Moench	Sorghum	Dabar	Sudan, Africa
<i>Zea mays</i> L.	Maize	Energy	PBI (Cambridge, UK)
<i>Medicago sativa</i> L.	Alfalfa	Vela	DLF-Trifolium UK and Ireland, Ltd. (Gloucestershire, UK)

chelated iron solution was added at 1 ml l^{-1} of mineral salts solution (1 l of chelated iron solution consisted of 13.05 g of EDTA (ethylenediamine-tetraacetic acid), 7.5 g KOH, 4.9 g $FeSO_4 \cdot 7H_2O$, distilled water to 1 l).

Pathogens and zoospore production Isolates were obtained from the Aquatic Phycomycetes Culture Collection (APCC) held by the second author in the UK, the International Mycological Institute (IMI) and the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands (Table 1).

All stock cultures were maintained on hemp seeds immersed in 50 ml sterile distilled water, in 100 ml sealed flasks at 5°C, in darkness (6). Corn meal agar or potato dextrose agar was also used for storage of cultures for up to 10 days. To limit variability, isolates were obtained from a single hyphal tip of a germinated cyst that had been spread thinly on the surface of hard water agar. Bacteria were eliminated by placing a small piece of mycelium on the inside of a Raper's glass ring that had been placed in the middle of a petri dish filled with water agar. *Phytophthora* and *Pythium* spp. can grow below the glass ring, but bacteria and actinomycetes usually do not.

Prior to use in experiments, cultures were removed from the stock solution and plated on 2% water agar for 2–6 days at 20°C, in the dark. With the aid of a scalpel, small orthogonal pieces (1 × 3 cm) were cut from the colony area around the periphery, transferred to cleared V8 broth and incubated at 25°C for 1 day or more, in darkness. The colonized agar pieces were placed in sterile petri dishes and washed three times (at 30-min intervals) with mineral salts solution and then incubated at 25°C, under light. Finally, the sporangia bearing mycelia were washed with three changes of double glass-distilled water and incubated at 18°C for 1 h or chilled for 15 min at 8°C and returned to room temperature; zoospores were released in ~ 1 h.

Zoospores were counted using either the hemocytometer or the microsyringe method. When using the hemocytometer method, a small amount of zoospore suspension was pipetted to both chambers of the hemocytometer and then zoospores were induced to encyst by slight heating on a hot plate for 3–5 s. This time was enough to induce encystment and immobilize the zoospores without destroying them (3). Counts were made in the center and four corner squares, the mean was calculated, and the concentration of zoospores ml^{-1} was estimated. With the microsyringe method, three drops of the same amount of zoospore suspension (10 μ l) were placed on one side of a microscope slide. Then the number of zoospores was counted for each one and an average was calculated and transformed to zoospores ml^{-1} . Zoospore suspensions were diluted to appropriate concentrations using sterile double glass-distilled water of the same temperature.

Plant material The plant species and cultivars used in this study are shown in Table 2. Seeds were stored at 4°C, sealed in moisture-proof containers (glass or plastic jars, polyethylene bags). Standard germination tests were carried out to assess the viability

of seeds. Seed vigor was also evaluated for potential of rapid, uniform emergence and development of normal seedlings. Seeds were surface-sterilized before they were used, to establish *in vitro* axenic cultures. In brief, seeds were thoroughly rinsed under running tap water for 10–20 min and then placed in a 70% ethyl alcohol solution for 1–5 min. Afterwards, they were submerged in the disinfectant solution (sodium hypochlorite 1–3%, plus 2–3 drops of wetting agent Tween 20) for 10 min, in a sealed sterile bottle and gently agitated. Under sterile conditions, the solution was decanted and seeds were washed with sterile distilled water several times.

For germination, aseptic seeds were placed in petri dishes containing a 5-mm layer of water agar and sterile Whatman No. 1 filter papers laid over the agar surface. Petri dishes were covered with their lids and placed in incubators to allow germination (darkness, 20°C). Periodic inspection of plant material was carried out in order to detect any contaminants. Seed health was assessed using visual evaluation (discoloration, sclerotia, insects, etc.) and incubation on petri dishes filled with water agar for growth of contaminants. Any cultures showing colonies of bacteria or fungi were discarded.

Each germinated seed (root length was less than 5 mm, because longer radicles made handling more difficult) was transferred to a 1.5 × 15 cm test tube containing 5 ml of half-strength Hoagland's nutrient solution and Whatman No. 1 moist filter paper strips, which had been fashioned into platforms. Filter paper edges were long enough to extend near the tube bottom in order to maintain constant moisture supply. Tubes were plugged with plastic caps and sterilized. After autoclaving, tubes were supported in a fashioned base of black plastic plug trays (Plantpak, UK). The pH of the nutrient solution was 6.5. Plants were incubated using a 16-h photoperiod. Light was provided by cool warm fluorescent lamps (40W, RS Components, UK) situated above the plants. Photosynthetic photon flux density at leaf height was $180 \pm 30 \mu\text{mol m}^{-2} \text{s}^{-1}$. The growth chamber was ventilated and the temperature was maintained at $25 \pm 2^\circ\text{C}$ and the relative humidity at $70 \pm 10\%$.

Disease assessment The root length (RL) and a disease severity index (DSI) based on root discoloration were used as criteria for disease assessment. The length of the primary root instead of the total root length was measured. Plants were spread over a white plastic surface, then straightened, and their length was measured to the nearest millimeter using a ruler. Root length of inoculated plants was expressed as percentage of the control plants and the data were arcsine-transformed for further analysis. In order to have a common DSI, a scale was devised based on root discoloration, including water soaking. Plants were rated for symptom development according to the following scale: 0 = no root discoloration, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 75–100% of the root system discolored.

Effect of inoculum density Two fungal species were selected: *Pythium deliense*, a species showing high accumulation rates, and *Pythium dissimile* – with relatively low encystment rates. Alfalfa var. 'Vela' and sorghum var. 'Dabar' seedlings were used in this study. Zoospore suspensions were added to the test tubes, resulting in a final density of 10^2 , 10^3 , 5×10^3 , 10^4 , 5×10^4 and 10^5 zoospores ml^{-1} . Plants were left in the incubators for 10 days after inoculation. The DSI of inoculated plants was compared with the corresponding controls.

Effect of plant age The varieties alfalfa 'Vela', sugarbeet 'Saxon', maize 'Energy' and sorghum 'Dabar' were chosen for their representation of plant families as well as their relatively similar and fast growth rates. Great care was taken to use plants with similar RLs

and growth stage. Zoospore suspensions of *Pythium aphanidermatum* were added after 3, 6, 9 or 14 days, to the test tubes containing the seedlings of the corresponding age. The final zoospore concentration in test tubes was 5000 zoospores ml⁻¹. Plants were left in the incubators for 10 days after inoculation. The DSI of inoculated plants was compared with the corresponding controls.

Effect of temperature Fungal species were selected for their temperature optima. *P. aphanidermatum* and *Phytophthora nicotianae* are typical plant parasites of warm regions, whereas *P. dissimile* and *Phytophthora cactorum* have been recorded mainly in temperate areas (10,23). Sugarbeet (var. Saxon) seedlings were germinated and grown until the root system was 3 cm long (2-leaf stage). All plants were grown in incubators at 25 °C, under a 12-h light-dark cycle. Twelve hours before inoculation, during the dark cycle, plants were transferred to the corresponding temperature regimes, in separate incubators, and left to equilibrate. The temperatures tested were 5, 15, 25 and 35 °C. Zoospore suspensions were added to the plants and were left for 10 days before measurements were taken. Data were expressed as percentage of the RL of control plants grown at the corresponding temperature.

Experimental design and analysis Experiments were designed as completely randomized and blind, with three replications of 30 seedlings for each treatment. Frequent re-randomization of the locations of test tubes batches inside the growth chamber was applied to guard against a uniform or non-random environmental variable. All data were checked for normality of errors by plotting residuals *versus* predicted values, as well as using the Kolmogorov-Smirnoff test for goodness of fit. Variances of data were tested for homogeneity using the Bartlett's and Levene tests. Percent data did not always satisfy the assumptions of analysis of variance and subsequently they were subjected to suitable transformations for statistical analysis, but actual percentages are given in the tables and figures. When the assumptions of ANOVA were not satisfied even after transformations, non-parametric methods were employed. The Kruskal-Wallis test was used to test for differences among groups in single classification analysis and the Nemeyi test to compare the groups (32). All data were subjected to analysis of variance using General Linear Models procedures and, when appropriate, means were compared with Tukey's multiple comparisons test (26). All tests for significance were conducted at $P = 0.05$.

RESULTS

Effect of inoculum density The incremental root length of all plants decreased as inoculum concentration of the pathogen increased (Fig. 1). This decline was steeper among low levels of zoospore concentrations. No significant differences in incremental RL were found for inoculum densities higher than 10⁴ zoospores ml⁻¹ in all plant-pathogen combinations. Sorghum seedlings infected with *P. dissimile* showed significantly higher incremental root growth, for all inoculum densities, compared with three other plant-pathogen combinations. Root discoloration, expressed as DSI, of alfalfa and sorghum plants inoculated with *P. dissimile*, did not differ significantly among them; however, root discoloration increased with inoculum concentration for plants inoculated with *P. deliense*, although at concentrations higher than 10³ zoospores ml⁻¹, differences in DSI were not significant.

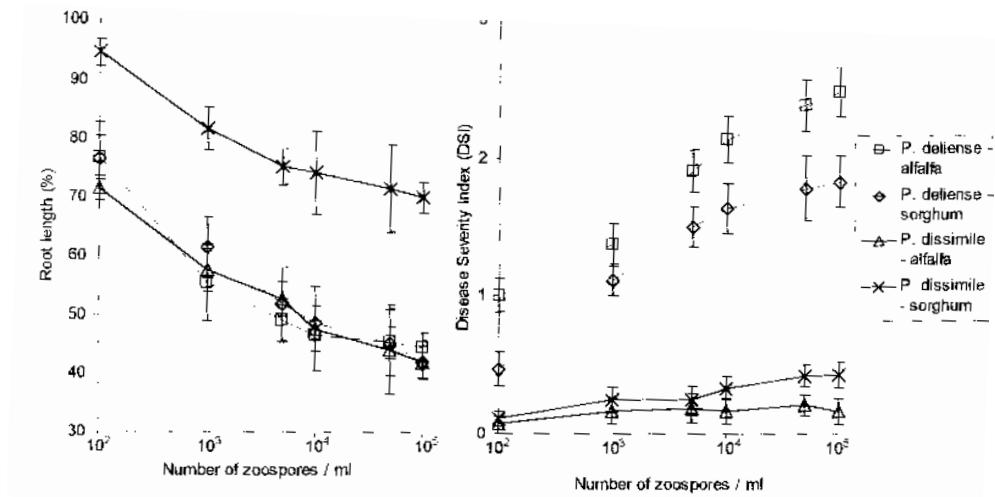


Fig. 1. Effect of different zoospore concentrations of *Pythium deliense* and *Pythium dissimile* on roots of alfalfa and sorghum plants, measured as root length expressed as percentage of control (left) and root discoloration expressed as disease severity index (right). Each point represents the mean \pm 1 SD of 30 observations.

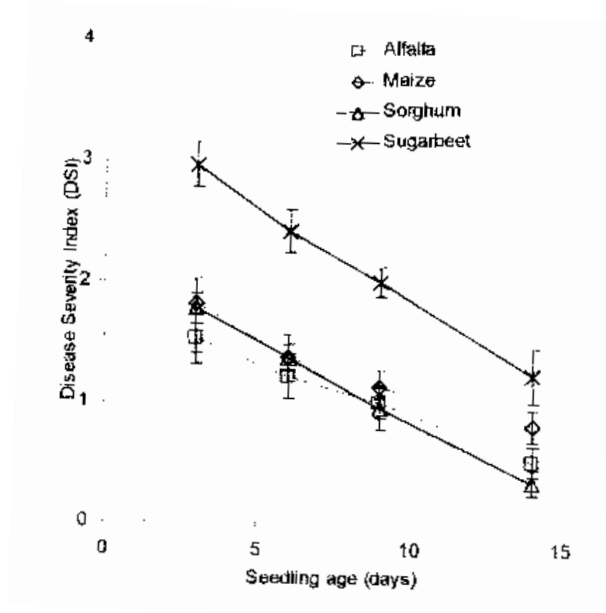


Fig. 2. Effect of *Pythium aphanidermatum* zoospores on roots of alfalfa, maize, sorghum and sugarbeet seedlings of different age, measured as root discoloration and expressed as disease severity index. Each point represents the mean \pm 1 SD of 30 observations.

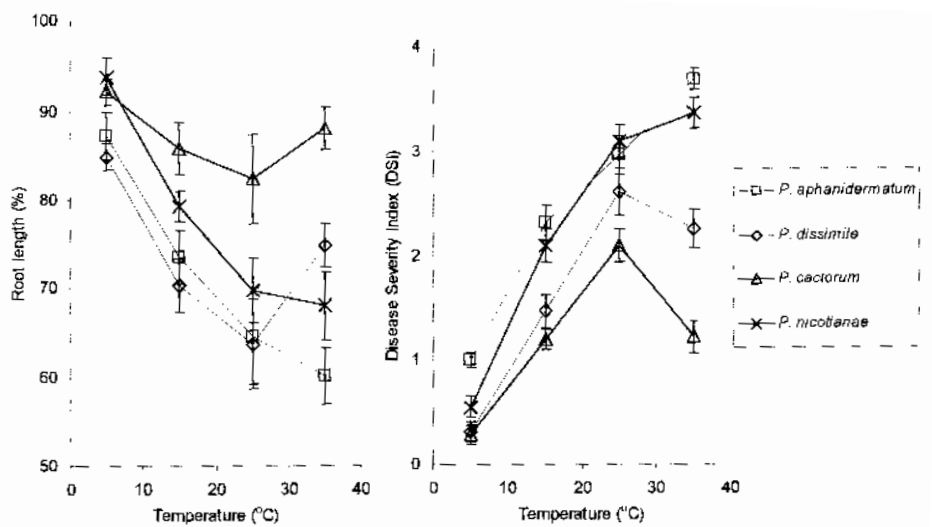


Fig. 3. Effect of *Pythium aphanidermatum*, *Pythium dissimile*, *Phytophthora cactorum* and *Phytophthora nicotianae* on roots of sugarbeet plants grown under various temperature regimes, measured as root length expressed as percentage of control (left) and root discoloration expressed as disease severity index (right). Each point represents the mean \pm 1 SD of 30 observations.

Effect of plant age Analysis of variance revealed a significant effect of plant age on root discoloration caused by *P. aphanidermatum*. All plants showed a reduction in DSI with increasing plant age (Fig. 2). Root discoloration of 3-day-old alfalfa and maize seedlings was significantly different from the 14-day-old plants. Three-day-old sorghum and sugarbeet plants showed a significantly higher degree of root discoloration compared with plants inoculated at 9 days of age, and both age levels differed significantly from the 14-day-old seedlings.

Effect of temperature Analysis of variance showed a significant effect of temperature and pathogen species upon disease severity and RL, and also a highly significant temperature \times pathogen interaction (Fig. 3). The incremental RL decreased as temperature increased up to 25°C. *P. aphanidermatum* and *P. nicotianae* reduced the RL of sugarbeet from 25 to 35°C, although the values did not differ significantly. On the other hand, *P. dissimile* and *P. cactorum* caused less root damage at 35°C than at 25°C. Root discoloration caused by *P. aphanidermatum* and *P. nicotianae* rising with increasing temperatures up to 35°C, whereas that caused by *P. dissimile* and *P. cactorum* increased up to 25°C and then decreased at 35°C.

DISCUSSION

Our study showed that the incremental RL of alfalfa and sorghum plants decreased and root discoloration increased with increasing inoculum concentration; however, the rate of

change depended on the plant – pathogen combination. Similar results were observed by other workers on pythiaceus pathogens (15,20,22). This relationship between zoospore concentration and disease severity can be interpreted as infection without interaction between zoospores but with competition for susceptible sites on the host (30). Zoospores respond to chemoattractants released from roots and accumulate at certain areas, such as the zone of elongation or points of emergence of lateral roots. These ‘windows of opportunity’ for infection are small in seedlings and might be filled at zoospore concentrations up to 10^4 , so addition of more zoospores does not affect disease development significantly. Infection and disease for pythiaceus species may result from very low zoospore numbers (21). It is also probable that the lack of direct proportionality of infection to disease is attributable to factors that reduce dissemination, duration of motility or survival of zoospores. The choice of high levels of inoculum will make all varieties seem susceptible to the same strain of pathogen, so it is important to choose an inoculum density that most closely characterizes the relationship and corresponds to inoculum density values found under field conditions. The use of dissimilar concentrations might constitute a serious cause for variation in results reported on zoospore encystment and the need for further studies in this topic is imperative. It is also not known whether the observed disease severity and root length reduction resulted from the initial amount of inoculum added to the tubes or if a secondary inoculum was produced during the incubation period.

In all tested plant species of this study, disease severity was negatively related to plant age. Many other workers have reported similar results (18,28,29,31). In contrast, adult pepper plants infested with *P. capsici* zoospores in a recirculating hydroponic system resulted in 100% mortality, whereas in young plants mortality was only 20% (27). This might be the outcome of increased secondary inoculum in the hydroponic system or altered plant morphology or physiology. As plants grow, morphological and physiological changes take place. Zoospores respond in changes of root morphology and exuded chemicals, and as a result one should expect variation in zoospore encystment and subsequent disease development. At a young age, root systems are small but offer many highly sensitive areas to infection. As seedlings grow, the root morphology is differentiated, offering additional protection against pathogens.

Our results showed that disease development on sugarbeet seedlings infected with *Pythium* and *Phytophthora* species was affected by temperature, but the pattern of response was determined by the pathogen’s temperature preferences. *Phytophthora cinnamomi* was pathogenic to eucalyptus seedlings in the range of 10–30 °C, with the optimum at 18–22 °C (11,25). It was also clear that at low temperature, susceptibility gives way to resistance (9). Disease severity on chickpea caused by *Pythium ultimum* increased as the temperature rose from 20 to 30 °C (1). Winter-grown tomato plants maintained at 15 °C developed severe wilt symptoms when infected with *Phytophthora cryptogea*, whereas the majority of infected plants grown at 25 °C remained symptomless during the 14-week experimental period (14). Zoospores respond to chemicals released by the roots, and every effect of temperature on zoospore vigor or plant physiological condition, will affect the plant–pathogen relationship. Temperature can be seen as an environmental factor, which affects the zoospore responses toward roots, especially at sub-optimal levels for plant roots and zoospores. At low temperatures the zoospores accumulate in only small numbers, perhaps because of the low exudations from roots and the low rate of movement of zoospores. As the temperature increases, high amounts of chemicals are exuded from the roots and the

speed of zoospores increases. At median temperatures there would be a positive correlation between rise of temperature, root exudation and zoospore motility. At higher temperatures zoospore motility would become limited, with a resultant decrease in zoospore encystment density on the roots.

The relationship between zoospore encystment and temperature was determined by the responses toward temperature of plants and pathogens. The adaptation of *P. aphanidermatum* and *P. nicotianae* to relatively high temperatures might have resulted in satisfactory levels of zoospore vigor and high levels of encystment at such temperatures. The effect of *P. dissimile* and *P. cactorum* on RL was significantly lower at the higher temperatures. *P. dissimile* and *P. cactorum* are recorded from temperate areas. This might be the reason why their activity or pathogenicity was reduced at high temperatures and their effect on RL at 35°C was significantly different from that at 25°C. The differential virulence between warm and cool fungal species at temperatures outside their optima is well documented. In root rot of spinach there was a transitional temperature (23°C), above or below which *P. aphanidermatum* and *Pythium dissotocum*, respectively, predominated (8). Temperatures higher than 30°C could greatly increase infection severity of *P. nicotianae* on *Hibiscus* plants (16). *Pythium* species are not especially temperature-specific for infection of adventitious roots of *Agrostis palustris*, but pathogenicity of some isolates is temperature-dependent following infection. One *Pythium* isolate which inhibited growth of *Agrostis* at high temperature, stimulated growth at low temperature (13).

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