TEMPERATURE EFFECTS ON GROWTH, SURVIVAL, AND PATHOGENICITY OF PHYTOPHTHORA MEGASPERMA

by

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ABSTRACT

Stand decline of alfalfa fields in Arizona has been partially attributed to the root rotting fungus, *Phytophthora megasperma*. The absence of seedling damping-off in alfalfa during late summer and early fall in the low elevation areas of Arizona where *P. megasperma* is known to occur may be temperature related. The optimum temperature for growth in culture of the fungus was 25 C, while no growth occurred at 35 C. There was no significant difference in the *in vitro* growth at various temperatures (15 to 40 C) of six isolates of the fungus obtained from either the cool, high elevation areas, or the hot, low elevation areas of Arizona. The fungus was highly pathogenic on both alfalfa seedlings and two-week old plants at twelve hour cycles of 18 and 30 C, whereas it caused little or no damage at alternating temperatures of 24 and 35 C.

Oospores of *P. megasperma* held in a dessicator at 35 C survived for at least eight weeks. The fungus was recovered from infected alfalfa roots containing oospores after being incubated in air dry, non-sterile soil at 17.4% and 21.9% moisture. Moist soil infested with oospores of *P. megasperma* was held at 50% moisture and incubated for 72 hours at 25, 35, and 40 C. The soil was dispersed in 0.05% water agar and spread over a selective medium, and incubated at 25 C for 48 hours. An average of 25 and 11 colonies of *P. megasperma* were recovered at 25 and 35 C, respectively, but the fungus was not recovered at 40 C.
INTRODUCTION

Alfalfa is the most important forage crop grown in Arizona. It accounts for about twenty percent of the total irrigated cropland. At lower elevations in Arizona, where non-dormant alfalfa varieties are predominantly grown, the major problem is stand and yield decline. While many factors undoubtedly contribute to this problem, studies by Hine et al. (20) indicate that root rot caused by the soil fungus Phytophthora megasperma (Drechs) is a major contributory factor. Phytophthora root rot (PRR) in alfalfa is widespread throughout the principal alfalfa producing areas in the state: Salt River Valley, Pinal County, Mohawk-Wellton, Yuma, Parker, Safford, Benson, and in the Sulphur Springs Valley in Cochise County (20). PRR has also been found in the high elevation areas of Snowflake and Show-low in Navajo and Apache counties, respectively.

The influence of temperature on Phytophthora species has been studied in some instances. Growth in culture at different temperatures is an important criterion for the identification of Phytophthora species (36). The occurrence of Phytophthora cinnamomi at high elevations and of P. parasitica at low elevations in the pineapple producing areas of Hawaii is apparently regulated by soil temperature (18). Phytophthora parasitica is known to parasitize fuchsia, parsley, papaya, and tobacco most effectively at temperatures between 28 and 31 C (19). A temperature of 25 C was optimum for growth of Phytophthora citrophthora in culture, while the optimum for P. parasitica was 32 C (15). Phytophthora
**Phytophthora megasperma** is most virulent on safflower at temperatures between 25 and 30°C (9).

Seedling damping-off has not been reported as a major problem of alfalfa in Arizona. Greenhouse studies, however, have shown that *Phytophthora megasperma* does cause damping-off of alfalfa seedlings (20). This discrepancy might be due to the high temperatures in Arizona during the months when alfalfa is planted in the field. This study was undertaken to elucidate the effect of temperature on the growth, survival and pathogenicity of *Phytophthora megasperma*, the causal agent of alfalfa root rot.
LITERATURE REVIEW

Root rot of alfalfa, *Medicago sativa*, caused by *Phytophthora megasperma* drechs., was first reported by Erwin in 1954 (10). The fungus was originally described as *Phytophthora cryptogea* Pethybridge and Lafferty (10). It was later reclassified as *P. megasperma* (11). Since then, root rot in alfalfa has been reported in several states in the U.S. (4, 10, 16, 17, 23), Canada (6), and Australia (27). Initial studies, showed the fungus to be highly virulent on alfalfa (32), and that the fungus was pathogenic only on hosts in the genus *Medicago* (12). But, *P. megasperma* has been reported to cause root rots of cauliflower (34), sugar cane (37), clover (22), broccoli and swede (7).

*Phytophthora* root rot is more common in poorly drained, heavy soils (25). The optimum temperature for growth in culture is between 25 and 30 C (12) with little or no growth above 33 C (12). Many alfalfa seedlings infected with *P. megasperma* survived in the greenhouse at 32 C. Optimum temperature for disease in two month old plants ranged from 17 to 27 C (12).

Oospores are generally considered to be involved in the long term survival of *Phytophthora* spp. in soil (14). All *Phytophthora* structures except chlamydospores and oospores die under dry conditions (33). Mycelia, hyphal swellings and sporangia of *P. megasperma* lysed within twelve days in moist, non-sterile, field soil at 20 to 25 C (12). Formation of oospores and/or chlamydospores in soil has been reported for some *Phytophthora* spp. (39). Reports on the duration of survival of
Phytophthora spp. in soil range from "indefinitely" for *P. erythroseptica* (5) to 77 days for *P. infestans* (38). Legge has noted that oospores of *P. cactorum* and *P. megasperma* survived for a year in soil (24). Zentmyer and Mircetich recovered *P. cinnamomi* from dead avocado roots stored in a sandy loam soil for six years (40).

Oospores of *Phytophthora* spp. usually exhibit low and variable levels of germination. Oospores of homothallic species such as *P. cactorum*, *P. erythroseptica*, and *P. megasperma var sojae* germinate faster and at higher levels than those of heterothallic species (39). The following factors have been studied with regard to their effect on oospore germination: dormancy (2), light (1,30), temperature (3), chemicals (31), and genetic proneness (28,29). Forty to seventy percent of the oospores of *P. megasperma var sojae* formed in V-8 juice agar germinated when incubated in water (14). Oospores of *P. megasperma* from V-8 agar when plated on water agar plates and incubated at 25 to 28 C did not germinate after 9 days. When these plates were irrigated with 5 ml of double-distilled water, 10% of the oospores germinated after a further twelve days. When oospores from alfalfa root tips were similarly plated on water agar, about 10% germinated after incubation for only six days (12).
MATERIALS AND METHODS

General Techniques

Isolates used in this study, were obtained from diseased, mature alfalfa roots from Buckeye, Laveen, Gilbert, Parker, Yuma, and Snowflake, Arizona. The isolates are named according to the geographical area from which they were recovered. They were maintained on a selective medium (8): 1.7% corn meal agar, 10 ppm myprozine, 100 ppm pentachloronitrobenzene, and 200 ppm vancomycin. The isolates were cultured in 200 ml of V-8 juice, 2.0 g calcium carbonate, and distilled water made up to one liter.

Inoculum for pathogenicity studies was prepared by washing two-week-old mycelial mats in tap water and blending it in a Waring blender for approximately fifteen seconds. The fungus was grown in 16 oz bottles, and four bottles of each isolate were used. The broken mycelium from the isolates was suspended in 1200 ml of distilled water.

Plants grown in perlite were watered with a nutrient solution containing Rapid Grow (Rapid Grow Corporation, Dansville, N.Y.) at 0.75 g/litre, plus an iron supplement containing 0.93 g of 75% Fe₂(SO₄)₃.nH₂O, 0.66 g EDTA, Na₂ in 100 ml of water. Ten ml of the iron supplement was added to a liter of Rapid Grow.

Survival studies in non-sterile soil were carried out with infected alfalfa roots containing oospores and oospores separated from roots. The infected alfalfa roots were obtained by inoculating five to seven day old Hayden alfalfa plants growing in perlite with a suspension
of blended mycelium and ooospores. A week later damped-off seedlings infected with ooospores were harvested.

Free ooospores were collected by placing two-day old Hayden alfalfa seedlings on V-8 agar in which *P. megasperma* was growing. When the seedlings had become infected after two days, they were floated in distilled water for three days. At the end of this period the seedlings contained numerous ooospores (Fig. 1). The ooospores formed principally in the root tips, which were cut off and subsequently blended in a Sorvall omnimixer for about five minutes. The suspension thus produced contained both ooospores and fragments of root tissue.

The non-sterile soil used for survival studies was Gila silt loam with the following characteristics: pH 7.8, 46.7% sand, 36.5% silt, 16.8% clay, 0.95% organic matter, 305 ppm NO₃, 0.025% N (Kjeldahl).

**Influence of Temperature on Growth and Pathogenicity**

The response to temperature of six Arizona isolates of *P. megasperma* was tested. A five mm disc cut from the advancing margin of a one week-old agar culture of the fungus was aseptically transferred to the centre of a petri plate containing the same medium. The plates were incubated for nine days at: 15, 20, 30, 35, and 40 C. Colony diameters were measured at 24 hour intervals. Each experiment was repeated five times.

The pathogenicity of the fungus as affected by temperature was determined by inoculating at time of seeding and when alfalfa plants were two weeks old. In all studies the variety Hayden was used. Seeds were planted in a sterilized mixture of one part sand and one part red mesa
soil (v/v). Two week old plants were grown in sterilized perlite and watered with the previously described nutrient solution. Ten pots were placed in each of four growth chambers and exposed to a twelve hour photo-period and the following temperatures alternating every twelve hours: 24 and 13 C, 30 and 18 C, 35 and 24 C, and 40 and 30 C. All plants were examined daily. The surviving seedlings were counted eleven days after inoculation. The 40 and 30 C temperature cycle was omitted in the experiment with the older plants. A reading of the fresh weight of the plants was made two weeks after inoculation.

Temperature and Moisture in Relation to Survival of Phytophthora megasperma

Several experiments were designed to determine if oospore survival was related to soil temperature and/or moisture. Initially the effect of temperature under dry conditions on survival of oospores was determined. Water-soaked Hayden alfalfa seeds were surface sterilized in 0.2 percent mercuric chloride solution, washed in sterile distilled water and aseptically transferred to bottles containing corn meal agar. When the seeds had germinated and the radical was about two cm long, a ten mm plug from a two week old culture of P. megasperma was placed in each bottle.

At twenty-one days, twenty-five infected seedlings containing oospores were placed in each of six petri dishes which were placed in a dessicator and incubated at 35 C. Samplings were made after 1, 2, 3, 4, 6, and 8 weeks. Upon removal, the seedlings were rehydrated in sterile distilled water, and then placed on a corn meal agar medium containing
10 ppm myprozine, 200 ppm vancomycin, and 200 ppm streptomycin sulphate. Results were recorded in terms of the number of seedlings from which *P. megasperma* mycelium grew expressed as a percentage of the total plated.

The ability of *P. megasperma* oospores present in alfalfa roots to withstand high temperatures in moist soil was elucidated with the following procedure. Five to seven day old Hayden alfalfa plants growing in perlite were inoculated with a suspension of blended mycelium and oospores. A week later damped-off seedlings infected with oospores were harvested in groups of 25, and placed in fibre glass mesh which was buried in soil contained in 250 ml beakers. The soil used was non-sterile Gila silt loam. Soil moisture was adjusted to three levels: air dry - 2.7%, field capacity - 17.4%, and saturation - 21.9% moisture. The incubation temperatures were 25, 35, 40, 45, and 50°C. Survival of *P. megasperma* was determined by recovering the roots, washing and plating on the previously described selective medium. Unaffected oospores germinated and the fungal mycelium grew out of the roots (Fig. 2).

In further studies roots containing oospores were incubated at 35 and 40°C in soil at the three moisture levels to determine the influence of different moisture levels on survival of the fungus at the two temperatures. Exposure periods necessary for inactivation of *P. megasperma* were studied by incubating infected roots of alfalfa for 2, 4, 6, 8, 12, 24, 48, and 72 hours at 40°C in a soil held at field capacity.

Since previous studies had shown that *P. megasperma* was inactivated by a constant exposure at 40°C for 72 hours in soil at field
Figure 1  Oospores of *P. megasperma* in four-day old alfalfa root tissue.

Figure 2  Germinating oospore of *P. megasperma* in alfalfa root.
capacity, the effect of alternating temperatures on inactivation of the oospores was determined. Infected roots were buried in soil at field capacity and incubated in a growth chamber that was maintained at 40 C for eight hours and at 26.5 C for sixteen hours. These conditions were set up to approximate temperature patterns during the summer in low elevation areas of Arizona. The roots were recovered and plated on the selective medium after nine days.

The influence of temperature on oospores in non-sterile soil was also determined. A suspension containing 20 X 10^3 oospores per ml was prepared as previously described. One gram of Gila silt loam soil was infested with 1/2 ml of the oospore suspension and incubated at 25, 35, and 40 C for 72 hours. The soil moisture was maintained at about 50%. There were five replications at each temperature. At the end of the incubation period each gram of soil was suspended in ten ml of 0.05% water agar, and one ml of this suspension was dispensed on the selective medium contained in petri plates. Five plates were similarly treated for each gram of soil. The plates were incubated in the dark for about 48 hours at 25 C, after which time the water agar was removed from the plates with a rubber policeman. The number of *P. megasperma* colonies that developed on each plate were then identified and recorded.
RESULTS

Influence of Temperature on Growth and Pathogenicity

Experimental studies in growth chambers showed that the pathogenicity of P. megasperma was markedly affected by temperature. Seedling damping-off was negligible at alternating twelve hour cycles of 40 C and 30 C and 35 C and 24 C. While over ninety percent of the seedlings survived at these high temperatures less than twenty percent of the plants survived at the lower temperature cycles of 30 and 18 C, and 24 and 13 C. (Table 1).

The pathogenicity of the fungus to two-week old plants under identical conditions was similar. While the total fresh weight of both inoculated and non-inoculated plants remained the same at a temperature regime of 35 and 24 C, it was reduced by a half to a third (Figs. 3, 4) at temperatures of 30 and 18 C, and 24 and 13 C, respectively (Table 1).

There was no significant difference in mycelial growth of six isolates of P. megasperma when grown at temperatures ranging from 15 to 40 C, with an interval of 5 C among the test temperatures. The radius of fungal growth on the selective medium in a petri plate was least at 15 C and greatest at 25 C after nine days. There was no growth at 35 and 40 C. In the temperature range of 20 to 30 C, all the isolates of the fungus grew optimally with no significant difference among the growth rates.
Figure 3  Effect of soil temperature on pathogenicity of *P. megasperma* to roots of four-week old alfalfa plants.

Figure 4  Effect of soil temperature on pathogenicity of *P. megasperma* to foliar growth.
### TABLE 1  Effect of Temperature on the Pathogenicity of *P. megasperma* on Alfalfa Seedlings and Two-Week Old Plants

<table>
<thead>
<tr>
<th>Temperature C</th>
<th>% seedlings surviving</th>
<th>% reduction in weight of two-week old plants</th>
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<tr>
<td>40 - 30(^{\circ})C</td>
<td>96</td>
<td>.-</td>
</tr>
<tr>
<td>35 - 24</td>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td>30 - 18</td>
<td>15</td>
<td>70</td>
</tr>
<tr>
<td>24 - 13</td>
<td>19</td>
<td>57</td>
</tr>
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</table>

\(^{a}\)Readings made 11 days after seeding and expressed as a percentage of the control plants. Results are the average five replications.

\(^{b}\)Readings taken two weeks after inoculation, calculated as percent of control. Results are the average of five replications.

\(^{c}\)12 hours (light) at 40\(^{\circ}\)C and 12 hours (dark) at 30\(^{\circ}\)C.

Temperature and Moisture in Relation to Survival of Phytophthora megasperma

Approximately fifty percent of seedlings infected with oospores of *P. megasperma* survived for at least eight weeks at 35\(^{\circ}\)C in a desiccator. The range of recovery varied from 20 percent of the total seedlings plated on the selective medium (8), to 91.5 percent (Table 2).

Several experiments demonstrated that oospores of *P. megasperma* were insensitive to high temperatures when infected alfalfa roots were buried in dry soil, but were inactivated at lower temperatures in moist soil. The fungus was isolated from infected alfalfa roots subjected to 50\(^{\circ}\)C in air dry soil at 2.7% moisture for 72 hours, and it was also isolated from soil at 17.4% moisture and incubated at 40\(^{\circ}\)C after 48 hours,
TABLE 2 Survival of *P. megasperma* Held in a Dessicator for Varying Time Periods at 35 C^a^  

<table>
<thead>
<tr>
<th>Length of Dessication (Weeks)</th>
<th>Infected Seedlings Plated on a Selective Medium</th>
<th>Seedlings From Which the Fungus Was Recovered</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>15^b^</td>
<td>62.5</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>22</td>
<td>91.5</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>14</td>
<td>70.0</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>11</td>
<td>47.8</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>4</td>
<td>20.0</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>12</td>
<td>48.0</td>
</tr>
</tbody>
</table>

^a^Infected alfalfa seedlings containing oospores were subjected to 35 C in a dessicator for different lengths of time.  

^b^The fungus was recovered from 15 out of 24 seedlings plated on a selective medium.
but it was killed at this temperature after 72 hours in soils at 17.4% and 21.9% moisture (Table 3).

Simulated summer temperatures of 40 C for eight hours and 26.5 C for sixteen hours approximating field soil conditions at 17.4% moisture failed to kill the fungus after nine days. The fungus was recovered from 42 out of 50 roots when the alfalfa roots were placed on the selective medium.

When moist soil infested with oospores of _P. megasperma_ was incubated for 72 hours at 25, 35, and 40 C, and subsequently dispersed in water agar which was spread over the selective medium, an average of 25 and 11 fungal colonies identified as those of _P. megasperma_ were recovered at 25 and 35 C respectively, but the fungus was not recovered at 40 C.
### TABLE 3 Interaction of Soil Temperature and Moisture on Inactivation of *P. megasperma.*

<table>
<thead>
<tr>
<th>Temperature C</th>
<th>Percent recovery of fungus from infected roots held at three moisture levels for 72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.7%</td>
</tr>
<tr>
<td>35</td>
<td>76</td>
</tr>
<tr>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

Roots of alfalfa seedlings containing oospores were placed in soil at three moisture levels and two different temperatures. Twenty-five infected roots were used per trial.
DISCUSSION

*Phytophthora megasperma* was highly pathogenic on alfalfa plants grown under controlled temperatures in growth chambers. The pathogenicity of the fungus was most pronounced at twelve hour cycles of light and dark at 30 and 18 °C, and 24 and 13 °C. Severity of root rot was negligible at temperature cycles of 40 and 30 °C, and 35 and 24 °C. The optimum temperature for growth in culture was 25 °C. Isolates of the fungus from California, Illinois, and Mississippi have been shown to have a similar optimum temperature for growth in culture (4, 12, 23). This study reveals a close correlation between the optimum temperature for *in vitro* growth and that for pathogenicity. Such a correlation has been noted earlier by Erwin (12). These results suggest that the reason for the absence of seedling damping-off of alfalfa (Fig. 5) in Arizona during the hot summer months and early fall may be temperature related.

There was no significant variation in the *in vitro* growth of isolates of the fungus from high (5000 ft elevation) and low (1000 to 1500 ft. elevation) areas of Arizona at temperatures between 15 and 40 °C. Presumably the fungus is more active during the summer months at high elevations such as Snowflake and Showlow. Such a situation is reported to occur in the hot desert valleys of southern California where *Phytophthora* root rot is not a problem in the summer, while the disease does occur in the San Joaquin valley where summer temperatures are moderate (12).
Figure 5 Damping-off of alfalfa seedlings caused by *P. megasperma* in the greenhouse.
Lack of pathogenicity of *P. megasperma* on seedlings and two week old plants at temperatures above 35 C may be related to failure of recovery of the fungus from infected alfalfa roots that were incubated for 72 hours in moist soil at 40 C. Fungus colonies were not recovered when wet soil infested with oospores of *P. megasperma*, was incubated for 72 hours at 40 C. *Phytophthora parasitica* was rarely recovered from moist soils maintained at 39 C for 30 days (21). *Phytophthora parasitica* survives in soil as chlamydospores which are known to be very sensitive to moisture and temperature changes (35). Survival of the fungus was drastically reduced at temperatures above 35 C and moisture levels less than 10% (35). Oospores and chlamydospores of *P. cinnamomi* survived for 12 months in soil at 60% moisture holding capacity, but the fungus was not recovered after three months from soil which was allowed to dry to 3% moisture (26). In the present study oospores of *P. megasperma* in alfalfa roots survived in dry soil at temperatures up to 50 C for 72 hours, while the fungus was not recovered from infected alfalfa roots in moist soil at 40 C after the same time period.

Failure to kill the fungus by exposure to 40 C in moist soil for 8 hours a day precludes consideration of a cultural control of the fungus in the hot areas of Arizona. Furthermore, alfalfa roots are known to collapse from conditions of high soil temperatures and water-saturated soil (13).
LITERATURE CITED


