A New High-Temperature Phytophthora Pathogenic to Roots of Alfalfa

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Accepted for publication 15 July 1977.

ABSTRACT

RIBEIRO, O. K., D. C. ERWIN, and R. A. KHAN. 1978. A new high-temperature Phytophthora pathogenic to roots of alfalfa. Phytopathology 68: 155-161.

A *Phytophthora* sp. was isolated from alfalfa (*Medicago sativa*) with root rot in the Imperial Valley of Southern California during the summer. The pathogen grew optimally at 33-36 C and actively at 36 and 39 C but not at 42 C. Severe root rot under experimental conditions was obtained only at temperatures of 29-32 C and above. Inoculated plants incubated at greenhouse temperatures between 24-27 C were stunted but did not succumb to the pathogen. Although the fungus did not produce oospores under any experimental treatment tested, it was classified on the basis of the morphology of sporangia and hyphal swellings as a high-temperature variant of *Phytophthora megasperma* and designated as cultivar HTI (high-temperature isolate).

Additional key words: root rot, proliferation of sporangia, zoospores.

Since the first reports (7, 8) that *Phytophthora megasperma* Drechs. was the causal agent of a root rot of alfalfa (*Medicago sativa* L.), it has become apparent that the pathogen is prevalent in most alfalfa-growing regions of the United States (2, 12, 13, 17, 20, 21, 22, 28, 35), Canada (4), and Australia (18, 25).

The disease caused by *P. megasperma* does not occur during the summer months (June - August) in the irrigated desert regions of the Imperial or Palo Verde Valleys in California because the high soil temperatures exceed the maximum for growth of the fungus (33 C); however, during the other months Phytophthora root rot is one of the most important components contributing to the problem of stand decline. In the summer, flooding injury (scald), a physiological disease caused by excessive soil water when temperatures are high (approximately 40 C) (10) is one of the most devastating factors. To determine whether pathogens also are involved in the decline in stands of alfalfa in summer, isolations were made from alfalfa plants with root rot in the Imperial and Palo Verde Valleys of Southern California in the summer. From these isolations a *Phytophthora* sp. was obtained which grew in culture at 39 C, a temperature much higher than that reported for *P. megasperma* from alfalfa (7, 8).

In this paper we report evidence for pathogenicity, the effects of temperature on growth and virulence, and describe the morphology of the high-temperature *Phytophthora* isolate from alfalfa. Some of the results were presented previously in an abstract (11).

MATERIALS AND METHODS

The high-temperature *Phytophthora* sp. was first isolated from diseased alfalfa roots in the Imperial Valley near El Centro, California in the summer of 1963. In subsequent years about 20 similar isolates were obtained from both the Imperial and Palo Verde Valleys. A single-zoospore isolate (P240-1) of the original isolate was used in these studies except where otherwise designated. The isolate, P240-1, was selected from among 30 single-zoospore isolates on the basis of its similarity to the parent isolate in morphology, production of sporangia, and pathogenicity to alfalfa seedlings.

In some experiments for inoculation of potted alfalfa (cultivar Moapa), inoculum of the high temperature *Phytophthora* was grown on V-8 juice agar in petri dishes (90 mm diameter) and blended for 15 sec in a Waring Blendor. Alfalfa plants (cultivar Moapa) (about 1-mo old) were inoculated by adding 50 ml of the inoculum in a trench near the plants (10 plants per 15 cm^2 plastic pot of soil). After inoculation, the saucer under each pot was kept full of water for 2 days, and for 5 days the saucers were inverted and the plants were watered from the top as needed. This cycle was repeated for 8 wk after which plants were examined for root rot. There were two-to-four replicates per inoculant and three isolates were tested. The experiment was repeated several times. In these experiments, the greenhouse temperature varied from 24-27 C day and 20-21 C night. In the Sherer-Gillett growth chamber, temperatures were 29-32 C day and 25 C night.

In some experiments for which zoospore inoculum was used, seeds of alfalfa were surface-sterilized in a 1:1 (v/v) mixture of 1.5% sodium hypochlorite and ethanol for 30 min, rinsed in sterile distilled water, and placed on moist sterile filter papers in deep petri dishes, or in clear plastic Beckman (Beckman, 2500 Harbor Blvd., Fullerton, CA 92634) germination flasks (Fig. 1-B). The petri dishes and Beckman flasks contained a modified Hoagland's nutrient solution (19). In the Beckman flasks the solution was drawn from the base of the flasks by a filter paper wick to the filter paper platform on which the alfalfa seeds were germinated. The pathogenicity of the *Phytophthora*

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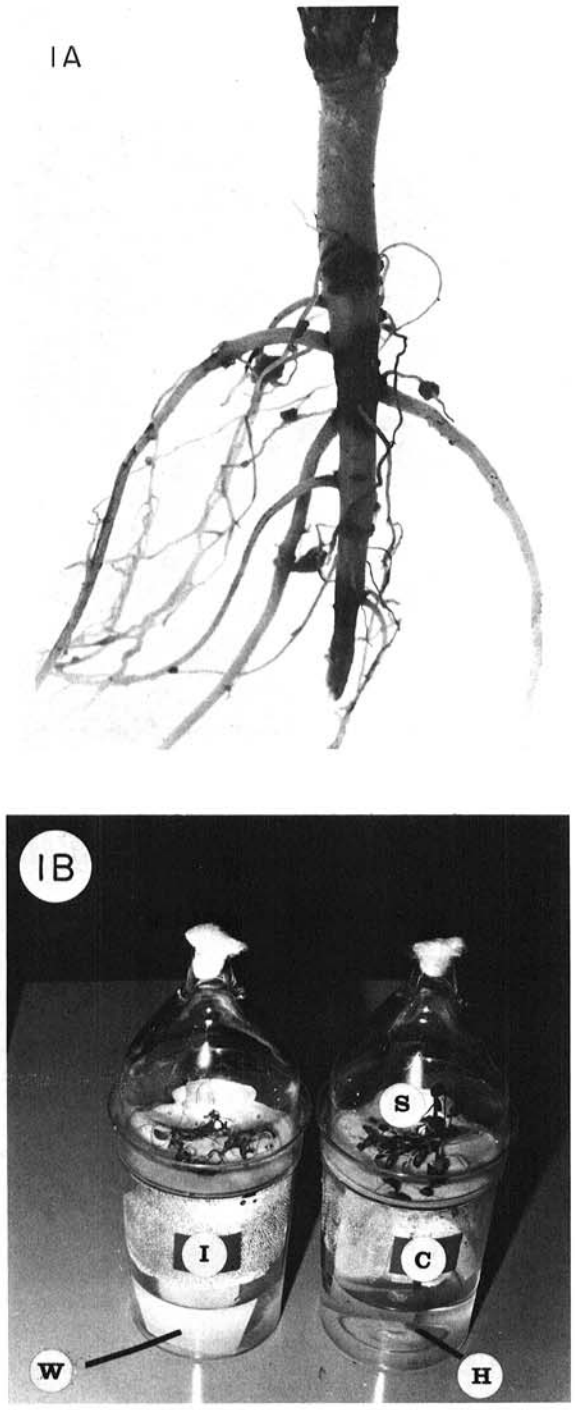

Fig. 1-(A, B). A) Root rot symptoms on Moapa alfalfa caused by inoculation of roots with blender-macerated mycelium of the high-temperature strain of *Phytophthora megasperma* 50 ml of inoculum (4 plates/liter) per 10.2 cm diameter pot of soil. **B)** The effect of inoculation of Moapa alfalfa seedlings with a suspension of zoospores from the high-temperature isolate of *Phytophthora megasperma* in Beckman germination flasks. I, inoculated; C, control; S, seedlings; W, sterile filter paper wick; H, modified Hoagland's nutrient solution (19).

isolates was tested on 10-day-old seedlings. Pathogenicity tests were carried out at moderate temperatures in the greenhouse (24-27 C day; 19-21 C night) and also in a Sherer-Gillett growth chamber at high temperatures (36.0-38.5 C day; 20-21 C night). Plants were inoculated by spraying the crowns and foliage with suspensions of zoospores (approximately 2×10^4/ml) followed by covering the plants with a plastic bag for 4 days. Zoospores were produced as follows: mycelial plugs (5-mm diameter) from 5-day-old cultures of isolate P240-1 growing on clarified V-8 juice agar were transferred aseptically to petri dishes containing clarified V-8 juice broth diluted 1:5 with sterile distilled water. The petri dishes then were placed under a bank of two 40-W fluorescent Indorsun® (Verd-A-Ray Corp., San Bernardino, CA 92412) lamps at 800 μW cm^{-2} using a 12-hr light/12-hr dark cycle at 24 ± 1 C. After 24 hr, the medium was decanted and sterile deionized water was added. After the dishes were again placed under the lights for an additional 24 hr, zoospores were abundant in each dish (about 2×10^4 zoospores/ml). Replacement of the water for an additional 24 hr greatly increased the yield of zoospores. Zoospore production continued for several days. Zoospores were collected for use as inoculum 48 hr after sporangia first were observed. *Phytophthora megasperma* cultures (isolate P410, an appressed isolate obtained from alfalfa in Minnesota by F. Frosheiser) and P844 from alfalfa in Salinas, California, both of which caused typical Phytophthora root rot of alfalfa, were used for comparison to the high-temperature isolate in some experiments.

All observations and measurements of fungal structures were made using a Zeiss Photomicroscope III equipped with Nomarski differential phase contrast optics.

RESULTS

Inoculation experiments.—Although pathogenesis by the high-temperature isolates always was greatest at high temperatures, in general they were relatively less virulent than the *P. megasperma* isolates with lower cardinal temperatures for growth (9). In conducting pathogenicity experiments at high temperatures, there was a problem of confusing pathogenicity of the fungus with high-temperature flooding injury (scald) (10). When control plants in an experiment showed any of the xylem necrosis symptoms typical of scald, the data in that experiment were not used.

When plants about 1 mo or more of age were inoculated with blended mycelium at moderate temperatures in the greenhouse, little actual root rot occurred and the only perceptible symptom was a slight stunting and lateral root necrosis. When half of the pots were transferred to a growth chamber maintained at the high temperature (29-32 C) root rot occurred on about 75% of the plants compared to only a trace of root rot at the moderate temperature. In another experiment when the pathogenicity of isolates P240 and 931-33 was tested in the high-temperature chamber, the root rot score (0-5) (9) was 1.9 for cultivar Lahontan and 3.5 for cultivar Moapa. There was no difference in the virulence of the two isolates. A plant with root rot symptoms is shown in Fig. 1-A.

When 10-day-old seedlings in the Beckman germination flasks (Fig. 1-B) were inoculated by spraying foliage and crowns with zoospores, extensive necrosis of the roots occurred within 5 days after inoculation. Inoculated seedlings eventually died, but noninoculated seedlings continued to grow. Symptom expression was more pronounced when the lower chamber of the germination flask contained a modified sterile Hoagland's nutrient solution (19), rather than sterile distilled water.

When plants were inoculated with encysted zoospores (approximately 2×10^4/ml) sprayed over crowns and foliage, symptoms of the disease (both foliar and root) were not apparent at moderate temperatures with plants 10 to 42 days old at time of inoculation in UC mix (50% sand:50% peat moss) (1), sandy loam, or vermiculite. After the plants were mowed, however, growth of inoculated plants recovered more slowly than that of noninoculated plants. The high-temperature *Phytophthora* sp. was reisolated on antibiotic medium (31) from the tap- and lateral roots of these plants 3 mo after inoculation.

When foliage and crowns of 16-, 33-, and 42-day-old plants were sprayed with suspensions of zoospores in a mist chamber and transferred 48 hr later to a Sherer-Gillett growth chamber maintained at 36.0-38.5 C day and 20-21 C night, a root and crown rot developed. The 16-day-old plants were the first to develop root rot symptoms regardless of whether the plants were growing in UC mix, sandy loam, or vermiculite. Older plants (33 and 42 days old) were stunted compared to the controls, but severe root rot was not observed although the fungus could be isolated readily from the tap-and lateral roots. However, in this experiment when plants were stressed by raising the temperature of the growth chamber to a constant 38 C for 48 hr, typical root rot occurred on plants of all ages. Control plants were unaffected.

The pathogen.—In culture on clarified V-8 juice agar the high-temperature *Phytophthora* exhibited a more radiating, petallate, chrysanthemum-type of growth (Fig. 2) than other isolates of *P. megasperma* (6, 8, 16, 33, 35). Freely branching hyphae varied in width from 4.2 μm to 10 μm. Hyphal swellings were intercalary (average diameter 31.9 μm), single, and clustered (average diameter 21.9 μm), in several media [Fig. 3-(G to I)]. The contents of the terminal hyphal swellings became dense

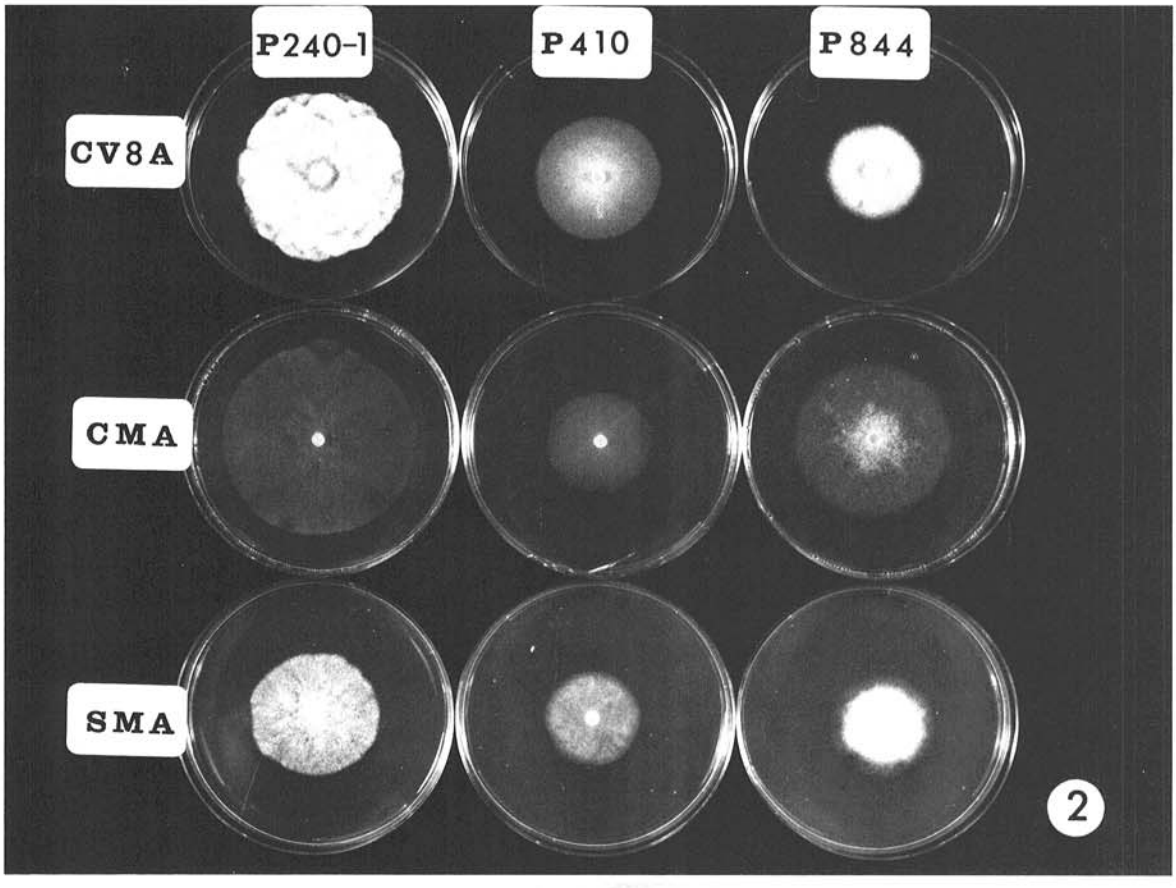

Fig. 2. Morphology of 5-day-old colonies of *Phytophthora megasperma* from alfalfa (P410 and P844) and of the high-temperature isolate of *P. megasperma* (P240-1) at 30 C on different media. CV8A, clarified V-8 juice agar; CMA, cornmeal agar; SMA, synthetic agar medium.

with age and often resembled chlamydospores. On corn meal agar (15 ml/petri dish) the average radial growth of 5-day-old cultures of 20 single-zoospore isolates was as follows: 6.0 mm at 9 C, almost 80 mm at 27-33 C, and 55.5 mm at 39 C. The temperature of 42 C was lethal since no growth occurred and cultures did not regrow when subsequently returned to room temperature (24 C). The high-temperature isolate, P240, made considerably more growth on agar at the higher temperature than *P. megasperma* (P844), the known pathogen of alfalfa (Fig. 4).

Ovoid-nondeciduous sporangia of the high-

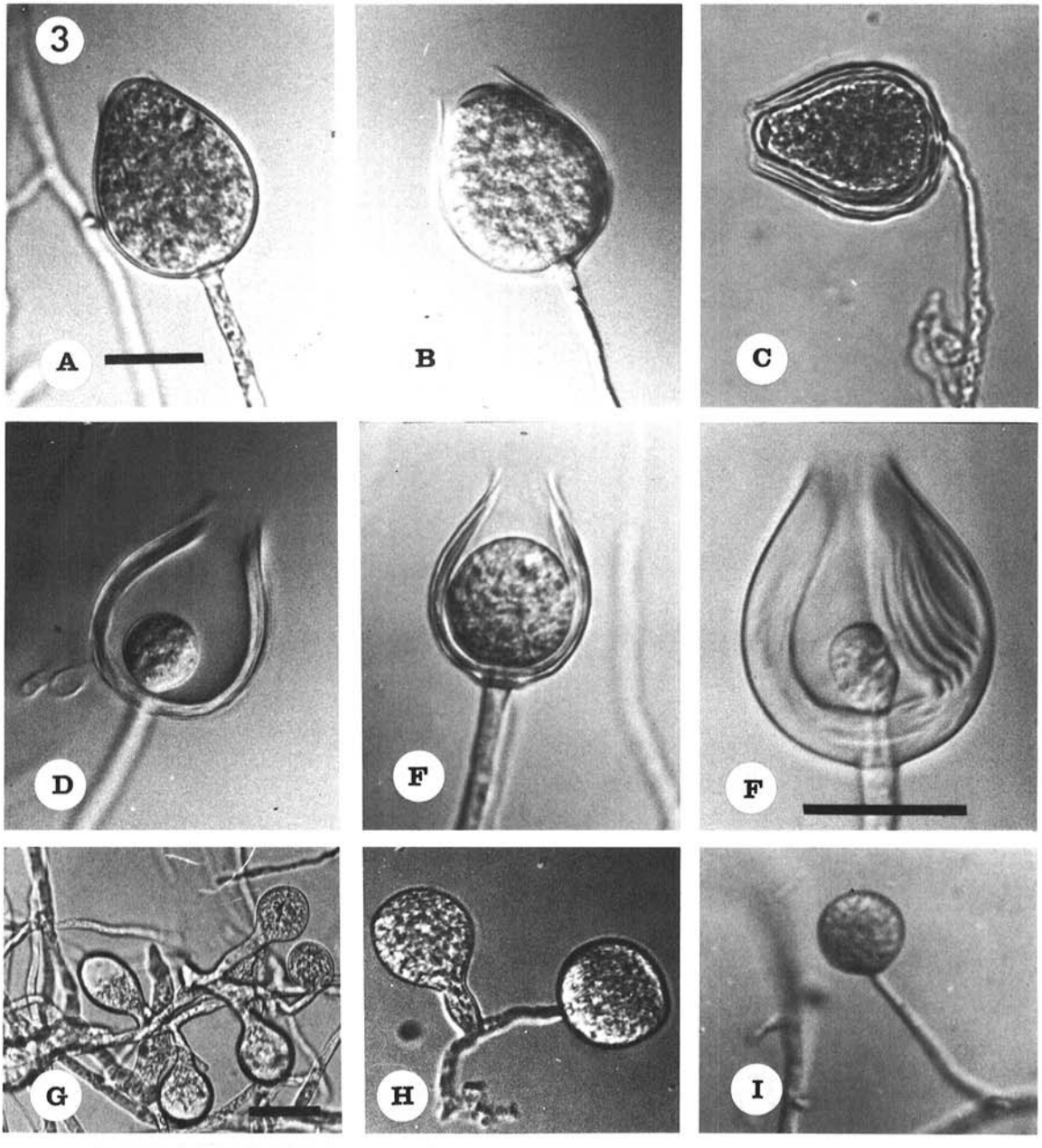

Fig. 3-(A to I). Sporangia and hyphal swellings of the high-temperature isolate of *Phytophthora megasperma* (P240-1) isolated from alfalfa. **A** and **B)** Nonpapillate young sporangium; **C)** proliferating sporangium; **D** and **E)** sporangia exhibiting the proliferating "nesting" phenomenon; **F)** postproliferation sporangium showing the remains of at least eight sporangial walls; **G)** lateral hyphal swellings; **H)** lateral and terminal hyphal swellings; **I)** terminal hyphal swelling. Bar = 20 μm. Photographs are of unfixed material mounted in water and viewed with Nomarski differential phase contrast microscopy using a Zeiss Photomicroscope III.

temperature *Phytophthora* formed readily in sterile deionized water. These were borne on unbranched filaments generally greater than 200 μm in length. Sporangia measured 37.44-54.2 × 29.0-41.6 μm (average 46.9 × 33.0 μm with a length:breadth ratio of 1:1.42. Shortly after formation, the sporangia released zoospores at room temperature, and new sessile "nesting" (proliferating) sporangia were produced continuously within the previously evacuated sporangium [Fig. 3-(D to F)]. Sporangia produced externally by growth of the filament through the evacuated sporangium (6) were rarely observed. Zoospores exited through a broad pore (8.7 μm average diameter) at the apex of the sporangium without the prior formation of a noticeable papilla [Fig. 3-(A to C)]. The number of zoospores released after washing 48-hr cultures grown in cleared V-8 broth increased rapidly to a maximum after 24 hr at 24 C, but at 27 C and 30 C, considerably fewer zoospores were produced (Fig. 5). Zoospores (average diameter 10.1 μm) were biflagellate and germinated by formation of germ tubes soon after encystment.

Oospores were not observed. Efforts to produce them included single culture and dual culture with both the A^1 or A^2 mating types of *P. capsici, P. cinnamomi, P. cryptogea,* and *P. palmivora* grown on clarified V-8 juice agar, cornmeal agar, oatmeal agar, sterile oat grains, carrot agar, lima bean agar, Ribeiro's synthetic medium (26), V-8 juice agar supplemented with beta-sitosterol and tryptophan (3), alfalfa extract medium, soil extract medium, or in V-8 juice agar medium amended with 3-5 μg/ml of chloroneb (24). Also, oospores were not observed in any plant material following natural or artificial inoculation. In some pairings of an isolate with A^1 and A^2 mating types of *P. capsici,* several oospores were observed, but these were of the *P. capsici* type and were believed to be induced by stimuli from the high-temperature isolate. This phenomenon was not observed in pairings with any other heterothallic species tested.

DISCUSSION

High temperatures are required for this newly recognized *Phytophthora* strain to cause severe root disease and subsequent plant death. The day- and night temperatures used in the growth chamber were similar to those recorded in the field from May to September in the Imperial Valley [average maximum and minimum air temperatures recorded for a 13-yr period were 39.4 C and 20.0 C, respectively (32)]. The maximum soil temperatures recorded at a depth of 20 cm (May to September) in the imperial Valley over a 13-yr period was 36.5 C (32). This soil temperature is only slightly above the optimum for growth of the high-temperature *Phytophthora* isolate. This correlation of optimum temperature for mycelial growth with that required for pathogenicity by the high-temperature strain indicates that the fungus is ecologically capable of causing root rot in the summer in the desert valleys of California. Previous observations indicate that other alfalfa isolates of *P.*

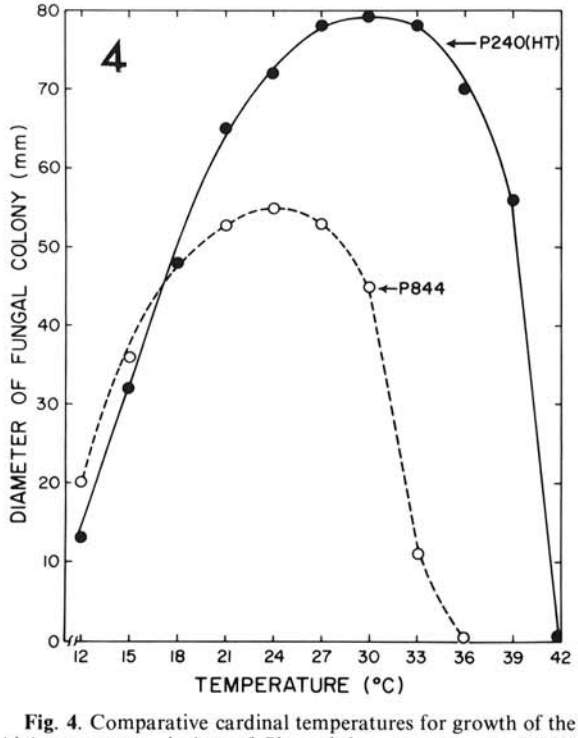

Fig. 4. Comparative cardinal temperatures for growth of the high-temperature isolate of *Phytophthora megasperma* (P240) and *P. megasperma* (P844) from alfalfa on corn meal agar.

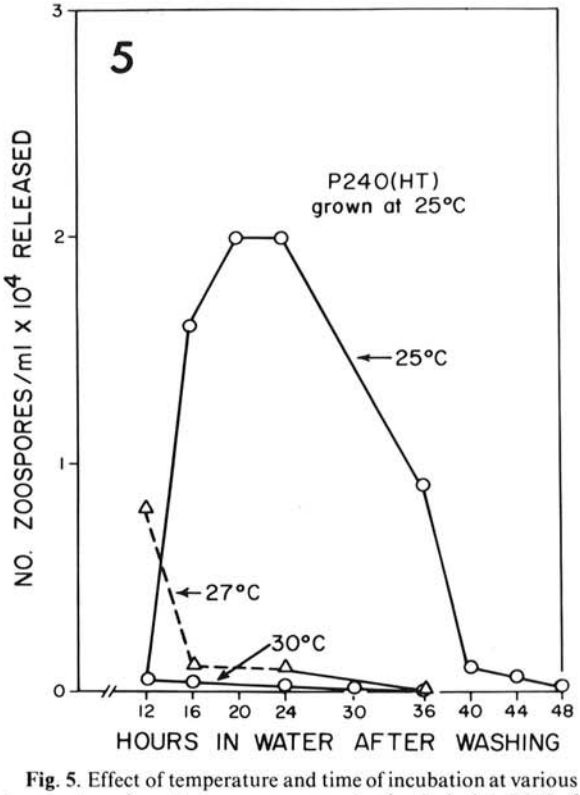

Fig. 5. Effect of temperature and time of incubation at various temperatures in water on zoospore production by isolate P240 of the high-temperature cultivar of *Phytophthora megasperma.*

megasperma are only active there in the fall, winter, and spring (7, 8, 9). Also, since it has been shown that approximately 85% of Phytophthora root rot lesions occur in the upper 20 cm of soil (14), it is conceivable that most of the damage could occur within that zone.

Plants inoculated at moderate temperatures in the greenhouse had few lateral feeder roots which may partially explain the observed stunting. Stunting of plants with Phytophthora root rot has been noted by others (5, 15, 21, 23). Although the high-temperature strain is confirmed as a root rot pathogen of alfalfa, its importance to alfalfa stand loss is difficult to assess due to compounding factors such as the physiological high-temperature flooding injury disease also known as scald (11). However, the presence of the pathogen in several areas of Southern California, indicate that potentially serious losses could occur in alfalfa if prevailing edaphic conditions such as waterlogging and/or extreme summer temperatures place additional stress on plants already weakened by the fungus. Although our cultivar-screening program is in the preliminary stages, it appeared that the cultivar Lahontan was less susceptible than Moapa. This resembles the responses of those cultivars to *Phytophthora megasperma* (9).

Classification of an isolate of a *Phytophthora* sp. that does not produce oospores presents problems that are not readily resolved. We have tried all the methods we know to induce the oospore stage with no success. Although there is reason to believe that an oospore stage could occur, it does not seem to be useful to delay classification of the high-temperature isolate until it is found. Although we can readily distinguish the high-temperature isolates from the previously described *P. megasperma* from alfalfa, the differences do not appear to us to be great enough to warrant creation of a new species.

Intercalary, clustered, and terminal hyphal swellings found in other *P. megasperma* isolates (7, 18, 33) also are present in the high-temperature isolate. However, some of the spherical hyphal swellings produced by the high-temperature isolate differ from those of the typical *P. megasperma* isolates from alfalfa in that many are produced terminally on long hyphal filaments and resemble chlamydospores [Fig. 3-(H, I)]; however, no septation or delimitation of the swollen area from the hypha could be discerned by microscopic examination. The high-temperature *Phytophthora* isolates grow more rapidly and have a more petallate colony type than the previously described *P. megasperma* isolates from alfalfa (8). The "nesting" proliferating nature of the sporangia also is different. However, despite these differences, in general, the similarity of shape of sporangia and the numerous hyphal swellings justify considering the high-temperature *Phytophthora* to be closely related to the previously described isolates of *P. megasperma* from alfalfa. Snyder et al. (29) suggested that isolates of certain variants might be designated as cultivars to differentiate certain cultures. Since the term "cultivar" does not have botanical status, it can be used to designate a certain class of isolates that has unique characteristics which are not great enough to warrant creation of a new species. We would suggest that the high-temperature variant be designated as *P. megasperma* 'HTI' with the abbreviation HTI indicating *high-temperature isolate.*

Since a variety classification should be used only for a morphological variant (International Code of Botanical Nomenclature), Waterhouse utilized the name *P. megasperma* var. *sojae* in Hildebrand's key (16) to designate isolates with smaller oogonia (less than 40 μm from those with larger oogonia which were designated *P. megasperma* var. *megasperma* (34). Although some accept this classification for alfalfa isolates (18), it could be a source of confusion especially to those who still look upon isolates named *P. megasperma* var. *sojae* as specific to soybean. Also Erwin (8) was concerned whether isolates of *P. megasperma* eventually would be found with oogonium sizes that bridged both varieties. Data now exist which indicate that some isolates of *P. megasperma* are borderline between the two oogonium size variations (Kuan and Erwin, *unpublished*).

Descriptions of *P. megasperma* (6, 7, 8, 18, 21, 30) have led to some confusion on the status of the epithet, *P. megasperma* var. *sojae* (16). Savage et al. (27) have shown that some isolates described as *P. megasperma* could be considered to be *P. megasperma* var. *sojae* based on their smaller oogonia, but that these same isolates had lower maximum temperatures (30 C) for growth compared to the maximum growth temperature (33-35 C) listed for *P. megasperma* var. *sojae* by Waterhouse (34). Oogonium sizes of *P. megasperma* pathogenic on alfalfa (7, 8, 18, 21, 35) are within the size range listed for *P. megasperma* var. *sojae* (16, 34). Also, our data and those of others (7, 8, 18, 21) indicate that sporangium sizes of *P. megasperma* from alfalfa overlap those of *P. megasperma* var. *sojae*. The use of varietal nomenclature based on oogonium sizes to classify *P. megasperma* isolates thus appears to confuse rather than clarify the situation.

Based on morphology of hyphal swellings, noncaducous, nonpapillate, proliferating sporangia, and the capability to cause a root disease similar to that caused by *P. megasperma* (7, 8, 9), the high-temperature *Phytophthora* from alfalfa is proposed to be classified as a variant of *Phytophthora megasperma* designated as the cultivar HTI. If further research reveals that the fungus produces a sexual stage that differs from that of *P. megasperma*, its classification will have to be reconsidered.

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