Identification of Four Phytophthora Isolates Previously Unreported from Arizona

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ABSTRACT

Arizona isolates of *Phytophthora* from *Rosmarinus officinalis, Leucophyllum frutescens, Washingtonia* spp., and *Petunia* spp. were identified as *P. parasitica* on the basis of morphological and physiological similarity to citrus isolates of *P. parasitica* from Arizona and California. Colony characteristics and distinguishing characters of sporangia were similar to those of an Arizona isolate of *P. parasitica* from citrus. Ooospores seldom developed in pure cultures or when the five were paired in all possible combinations. Malachite green reduced growth of all isolates at concentrations above 0.10 μg/ml. Optimum growth of all isolates occurred at 30 C, and was entirely inhibited at 38 C. All isolates used mannose, xylose, maltose, fructose, dextrose, and sucrose as a carbon source except the rosemary isolate, which did not use xylose. Asparagine was the most favorable source of nitrogen for all isolates. All isolates infected rosemary and sweet orange stems and fruits, but differed in virulence. Only the petunia isolate was pathogenic to petunia. Phytopathology 61:1293-1296

Additional key words: rosemary, Texas Ranger, palm.

A species of *Phytophthora* was isolated frequently from rosemary (*Rosmarinus officinalis* L.) and from Texas Ranger (*Leucophyllum frutescens* Johnston), two unreported hosts extensively grown in Arizona as ornamentals. *Phytophthora* sp. is frequently found in Arizona causing a bud rot of palm (*Washingtonia* spp.) and a crown rot of petunia (*Petunia spp.*). *Phytophthora parasitica* causes these diseases in California and Colorado (3, 10), but specific identification of the Arizona isolates has not been verified previously. We sought, therefore, to identify the pathogens from Arizona using morphological and physiological characteristics of isolates and host range.

MATERIALS AND METHODS.—Isolates of *Phytophthora* were obtained from host plants by placing infected tissue on water agar containing 125 μg/ml chloramphenicol (Parke-Davis & Co.) and 100 μg/ml pimaricin (Royal Netherlands Fermentation Co.). They were maintained on commercial cornmeal agar plus 5 g dextrose and 5 g yeast extract/liter (CMA+). Cultures of *P. parasitica* and *P. citophthora,* obtained from George Zentmyer, University of California, Riverside, were used to verify the identity of the Arizona isolate from citrus as *P. parasitica.*

Host ranges were determined in the greenhouse. Experiments using root inoculations were conducted in water baths at 28-30 C. Plants were inoculated while growing in sterile soil composed of sand, peat, and clay loam (1:1:1) unless otherwise stated. Containers usually used in the temperature tanks were 1-gal cans with rubber inner liners having drainage holes in the bottom. Mycelium from 10-day-old cultures on CMA+ was used as inoculum. The fungi were reisolated at the termination of the experiment using the selective medium.

RESULTS.—*Morphology.*—Morphological and cultural characteristics of the five isolates were determined according to the criteria of Waterhouse (14). Pairings of the isolates in all possible combinations were made on cornmeal agar (CMA) and on rape seed extract agar (RSA) (11). Each cross was replicated 3 times.

Isolates from Texas Ranger, rosemary, petunia, and palm were morphologically similar to the isolates from citrus, all having irregular hyphae with maximum widths of 8 to 10 μ. Sporangiophores of the five isolates had a maximum width of 5 μ, and were irregularly branched except for occasional sympodial branching by the isolates from citrus and palm. Sporangia varied in shape within each isolate, but were similar to the isolate from citrus (Fig. 1), being markedly papillate and pyriform to broadly ovoid, with some slightly elongated. Sporangia of all isolates were terminal, not usually deciduous, but with few dropping off with short pedicels, and yellow to light brown in color. Sporangia of the citrus isolate averaged slightly smaller than those of the other four, but had nearly the same maximum size. Sizes of sporangia for all isolates are as follows: citrus, average 35 × 28 μ, maximum 56 × 44 μ; Texas Ranger, average 42 × 38 μ, maximum 61 × 55 μ; rosemary, average 39 × 34 μ, maximum 55 × 46 μ; palm, average 40 × 30 μ, maximum 57 × 46 μ; petunia, average 35 × 29 μ, maximum 49 × 40 μ.

The isolate from citrus produced abundant chlamydospores up to 40 μ in diam, but chlamydospores exceeding 30 μ in diam were scarce in cultures of the other four isolates. Chlamydospores were predominantly terminal in cultures of the five isolates.

All isolates were similar to the one from citrus in producing few oogonia in 8 weeks in single (CMA) and paired cultures (CMA, RSA). Only the isolates from citrus and rosemary produced an occasional oogonium on CMA. Pairings in all possible combinations on RSA and CMA failed to increase the incidence of oogonia up to 56 days.

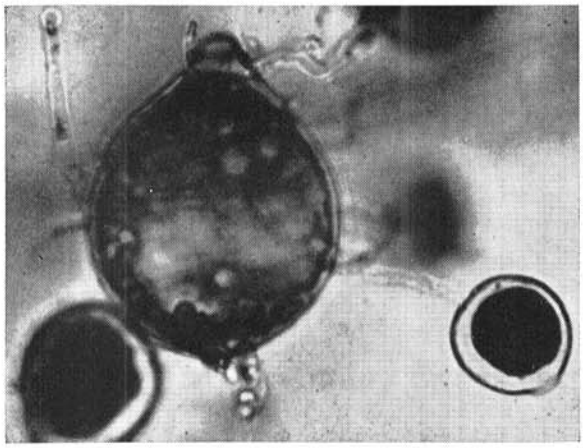

Fig. 1. Sporangia of the citrus isolate typical of the five isolates of *Phytophthora parasitica* (×780).

Colonies of the isolate from Texas Ranger were indistinguishable from the citrus isolate, being glossy and white when young and becoming light tan and slightly fluffy with age. The isolates from rosemary, palm, and petunia differed in appearance from young cultures of the citrus isolate, resembling older colonies of that isolate.

Tolerance to malachite green.—One, 0.50, 0.25, and 0.10 μg/ml malachite green in CMA+ were used to compare tolerance of the five isolates to the dye (7). A 5-mm plug cut from a culture of each isolate was placed in the center of each plate. Controls consisted of cultures on CMA+ minus malachite green. Growth was recorded every 2 days by averaging measurements of diameter of each colony.

Growth rates of the five *Phytophthora* isolates were similar at all concentrations. Maximum growth occurred only in the absence of the dye and at the lowest concentration (0.10 μg/ml).

Effect of temperature.—Growth response to temperature was studied by two methods: (i) The fungi were grown in liquid medium containing 10 g dextrose, 2 g asparagine, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g Fe^{++}, 0.2 mg Zn^{++}, 0.1 mg Mn^{++}, 5 μg thiamine, and distilled H_2O to equal 1 liter. The pH was adjusted to 6.2-6.4 with HCl. A plug, 5 mm in diam, cut from CMA+ cultures was placed in each 125-ml flask containing 75 ml nutrient solution, and the fungi were grown for 7 days in water bath shakers at 15, 20, 25, 28, 30, 35, or 38 C. The mycelial mats were harvested on a tightly woven cloth over a Büchner funnel using suction. Mycelium from each of the three replicates was placed on a preweighed piece of tared aluminum foil, oven-dried at 60 C for 48 hr, and weighed after standing 2 hr at room temperature. Weights of replicates were averaged. (ii) Three replications of CMA+ petri dish cultures were used to measure linear growth of the five *Phytophthora* isolates at 16, 20, 24, 27, 30, 35, and 38 C. Measurements were taken every 2 days over a 10-day period, and averages computed as in the study with malachite green.

Optimum and maximum temperature for growth of all isolates in liquid and on agar media were 30 C and 35-38 C. There were differences in growth rate among the five isolates. Isolates from palm and from Texas Ranger grew fastest and slowest, respectively.

Carbon utilization.—Carbon utilization was determined using the same materials and methods as in the first procedure outlined in the temperature experiment except for varying the carbon source. Mannose, xylose, maltose, fructose, dextrose, lactose, and sucrose were used with three replications for each isolate. The pH of the media after autoclaving ranged from 5.42 to 5.50. The isolates were grown in shake culture at room temperature (26-28 C).

Measurements of dry weight of mycelium after 7 days indicated that isolates from Texas Ranger and palm grew best on dextrose, although the palm isolate made equal growth on fructose. The rosemary isolate grew best on maltose, while optimum growth of the citrus and petunia isolates occurred on mannose and sucrose, respectively. Each isolate was capable of utilizing all carbon sources except the rosemary isolate, which failed to use xylose (Table 1).

Utilization of nitrogen.—In the nitrogen study, the carbon source found optimum for each isolate was used, and the nitrogen source (urea, L-arginine, asparagine, and $[NH_4]_2SO_4$) was varied. It has been noted that carbon source has a direct relationship to nitrogen usage (2). Total nitrogen for each nitrogen source was calculated on the basis of total nitrogen in asparagine. The pH of the media after autoclaving ranged between 5.0 and 5.3.

All isolates grew best, as measured by dry weight of mycelium, when asparagine served as the source of nitrogen. None grew well with $(NH_4)_2SO_4$ as the nitrogen source. L-arginine supported good growth of the isolate from rosemary, and the isolate from citrus grew well on urea (Table 2).

Host range.—Rosemary cuttings rooted in vermiculite and transferred to 1-gal containers after 14 days

TABLE 1. Average dry weight in milligrams of mycelium of five isolates of *Phytophthora parasitica* grown in liquid media with various sources of carbon for 7 days on a reciprocal shaker

Source of isolate	Mannose	Xylose	Maltose	Fructose	Dextrose	Lactose	Sucrose
Texas Ranger	47[a]	46	22	22	49	20	18
Rosemary	6	0	61	40	8	11	26
Palm	64	15	59	145	145	41	112
Petunia	43	26	27	12	12	12	52
Citrus	45	25	42	10	10	12	26

[a] Average weight of three or four replicates.

TABLE 2. Average dry weight in milligrams of mycelium of five *Phytophthora parasitica* isolates grown in liquid media with various sources of organic and inorganic nitrogen for 7 days on a reciprocal shaker

Isolate	Urea	L-Agrinine	Asparagine	$(NH_4)_2SO_4$
Texas Ranger	0[a]	11	39	0
Rosemary	0	26	37	1
Palm	0	0	18	0
Petunia	5	6	20	0
Citrus	43	1	69	1

[a] Average weight of five replicates.

were inoculated in one of three ways: (i) 200 ml inoculum, obtained from blending 10 CMA+ 7-day-old cultures of each isolate in 1 liter of water, were mixed with potting soil; (ii) small bits of CMA+ cultures were incorporated into the soil; and (iii) pieces of inoculum (10 × 5 mm) cut from CMA+ cultures were inserted under flaps of bark, free end down in the stem 0.5 inch above the soil surface.

All isolates infected rosemary. The rosemary isolate was most virulent, and those from petunia and citrus least virulent. Inserting a piece of inoculum under the bark of the plant or adding homogenized inoculum to the soil were effective inoculation techniques.

Petunias grown from seed in vermiculite and transplanted into a standard soil mix in 1-pint plastic pots were inoculated by adding to the soil in each of five pots one plate of the appropriate isolate. CMA+ was added to five other pots as controls.

The petunia isolate completely destroyed the root systems of the petunia plants within 10 days, and was easily recovered from decayed roots of wilted and dead plants. Forty days after inoculation, no symptoms appeared in petunia plants inoculated with the other four isolates, and none of the four isolates could be recovered from the inoculated plants.

Stem inoculations of citrus were made using 1-year-old sweet orange (*Citrus sinensis* L. 'Olivelands') seedlings. There were four plants in each 1-gal pot. A flap, open end down, was cut in the bark of the stem about 3 inches above the soil surface. A small piece of mycelium and adhering agar was inserted under each flap. The bark was pressed back into place and a small pad, saturated with sterile distilled water, was fastened to the flap with masking tape. Controls were handled in the same way, except that a sterile piece of CMA+ was placed under each flap. One cotton pad was removed from a plant in each treatment after 26 days, and observations were made at various intervals thereafter. Attempts to reisolate the fungi were made after 136 days.

The same plants used in this experiment were subsequently inoculated ca. 3 inches above the first inoculation point. A 2-inch square of paraffin paper was wrapped around each wound and bound tightly at the ends with masking tape to form a humid chamber. Observations were made through the partially transparent paper. Attempts to reisolate the fungi were made after 120 days.

All five isolates were recovered from bark lesions on inoculated citrus. The isolate from citrus caused gummosis, and no callus formed. The other four isolates failed to induce distinct symptoms, although the stem tissues surrounding the incisions were sometimes slightly discolored. Isolates from citrus and Texas Ranger were reisolated from the greatest number of inoculated stems.

Comparisons of the effect of *P. parasitica* from citrus and of the other four isolates on citrus fruits were considered to be useful in elucidating relationships. Valencia orange fruits washed 3 times with sterile distilled water were placed in two plastic pans on water-saturated paper towels. Plugs of inoculum 5 mm in diam were placed, mycelium side down, on the uninjured orange epidermis. In a second group, the plug was placed in the cavity left by the removal of the fruit stem. The pans were covered tightly with aluminum foil and incubated at 30 C for 7 days. Sterile agar plugs on orange fruits were used as controls. Three replications of each treatment were made. Average sizes of lesions produced after 7 days were recorded and symptoms were noted.

Ability of the five isolates to grow through orange fruit tissue was similar when the plug of inoculum was placed in the pedicellular cavity. Average lesion size produced by the isolates from Texas Ranger, rosemary, palm, petunia, and citrus was 47, 35, 58, 52, and 54 mm, respectively. Placement of the plug of inoculum on the uninjured epidermis of the fruits did not result in infection by any isolate.

DISCUSSION.—Reaction to malachite green, optimum and maximum temperatures for vegetative growth on CMA, and presence or absence of sporangial papillae and similar color of sporangia are generally accepted as useful taxonomic criteria for studying *P. parasitica* (13) and progenies from intraspecific matings of *Phytophthora capsici* (12).

The five isolates used in these studies reacted identically to the various concentrations of malachite green, as would be expected were they strains of the same species (7).

The optimum and maximum temperature for growth (30 C and 35-38 C, respectively) are within the range to be expected for isolates from Arizona soils, and are in agreement with temperature ranges for *P. parasitica* reported by Waterhouse (14). Value of growth response to temperature as a taxonomic criterion is controversial (7, 9, 13, 14).

Isolates differed in size of sporangia but that characteristic is considered less important than form and color, which emphasize the close relationship of the five isolates (7, 13, 14).

In addition, production of oogonia and oospores was nearly identical in cultures of the five isolates, and agrees well with the description of *P. parasitica* (14). Lack of increase in oospore production in any of the crosses as compared with single isolate cultures supports the belief that *P. parasitica* is heterothallic (4,

6) and that the isolates studied were all of the same mating type.

Results of host range studies give the most convincing evidence of close relationship between the five *Phytophthora* isolates. All isolates were capable of infecting rosemary and citrus, and were similar in ability to invade orange fruits. The fact that only the isolate from petunia was capable of infecting petunia represents physiological specialization, but the ability of this isolate to infect rosemary and citrus shows the Arizona isolate to be less specific in host range than isolates from petunia in other areas (5, 10).

Differences in carbon utilization are interpreted as indication of physiological variation among the five isolates. According to Cameron & Milbrath (1), however, it is difficult to draw conclusions from such data. The isolate from rosemary was the only one demonstrating physiological specialization by failure to utilize all sources of carbon. The isolates from Texas Ranger and citrus showed similar specialization in that they grew best on dextrose. Physiological differences were indicated also by the ability of isolates from rosemary, palm, and petunia to utilize best maltose, fructose, and sucrose, respectively.

Asparagine is the most favorable source of nitrogen for several isolates of *P. parasitica* (1, 8). It was best in our studies for all five isolates, and $(NH_4)_2SO_4$ and urea were the poorest.

On the bases of their identical tolerance to malachite green, similar sporangial characteristics, uniformity of optimal and maximum temperature at which vegetative growth occurs, and host range we conclude that the isolates from rosemary, Texas Ranger, petunia, and palm are all strains of *P. parasitica.*

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