Effects of Exogenous Nutrients and Inoculum Quantity on the Virulence of *Pythium ultimum* to Cotton Hypocotyls

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ABSTRACT

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Cotton hypocotyls were inoculated with *Pythium ultimum* grown on media prepared with different nutritional levels. Virulence of the fungus decreased with age on potato-dextrose agar (PDA) and as concentrations of nutrients in PDA were decreased. On a basal mineral salts-carbohydrate medium in which concentrations of nutrients were adjusted individually, growth and virulence of the fungus were affected by sucrose, ammonium- and nitrate-nitrogen, and potassium, but not by magnesium, sulfate, chlorine, or phosphate. *P. ultimum* inocula grown on media without sucrose or nitrogen caused only minor symptoms on cotton hypocotyls, but became progressively more virulent when grown on media with increasingly higher concentrations of sucrose or nitrogen. Lower concentrations of those nutrients were required for maximum growth in culture than for maximum virulence. High concentrations of both sucrose and nitrogen were required for maximum disease development following initial infection. The fungus was more virulent when grown on media containing nitrate than on media with ammonium-nitrogen. At all nutritional levels of sucrose or nitrogen, virulence increased when larger quantities of inoculum were applied to hypocotyls. Larger quantities of inoculum grown on low-nutrient media resulted in disease symptoms either similar to or more severe than those produced by smaller quantities grown on high-nutrient media.

Additional key words: cotton seedling disease, postemergence damping-off.

Pythium spp. and *Rhizoctonia solani* Kühn are major pathogens of cotton seedlings (2,4,14,18). In the northern part of the U. S. Cotton Belt, *Pythium* spp. are isolated most frequently from necrotic areas on hypocotyls and roots (14–16). In Tennessee, *Pythium ultimum* Trow is considered to be the major species affecting cotton seedlings (14).

Many soilborne pathogens require exogenous nutrients to infect roots or seedling hypocotyls (5). Effects of exogenous nutrients on virulence have been determined for *R. solani* (6–9,23,24); few such studies have been made for *Pythium*. Germination of spores of *Pythium* is known to be affected by nutrients. Certain nutrients are required for germination of zoospores (11,12), sporangia (20), and oospores (19) of *P. aphanidermatum*, and of sporangia (1,21,22) and oospores (17) of *P. ultimum*. Kraft and Erwin (11) showed that nutrients in mung bean exudate affected virulence of *P. aphanidermatum*. Different amino acids as nitrogen sources influenced virulence of the fungus, especially at low densities of zoospores (12).

The purposes of this study were to determine the effect of external mineral nutrients and sucrose on virulence of *P. ultimum* to cotton hypocotyls, and to determine possible effects on virulence of interactions between quantity of the inoculum, and nutritional status of the fungus.

MATERIALS AND METHODS

An isolate of *P. ultimum* from a diseased cotton seedling grown in a field plot in West Tennessee was used in all experiments. Media used were water agar (2% agar), potato-dextrose agar (PDA), a basal mineral salts-sucrose medium, and variations of the basal medium. The basal medium contained 2 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.015 g $FeSO_4 \cdot 7H_2O$, 10 g sucrose, and 15 g of agar per liter of distilled water. Variations of the basal medium were prepared in which the concentrations of sucrose, ammonium- or nitrate-nitrogen, magnesium, potassium, and phosphorus were adjusted individually. To determine the effect of nutrients on growth and virulence of *P. ultimum*, subcultures of the fungus were grown on these media and used to inoculate cotton hypocotyls.

To minimize differences in seed quality, plants of 27 cotton cultivars were grown to maturity in a greenhouse and self-pollinated. Seeds were bulked and acid-delinted, and those that floated in water were discarded. Seeds were planted in sterilized sand in plastic trays and incubated in continuous light in plant growth chambers at 27 C for 8 days. Chamber light was a combination of fluorescent and incandescent at ~16,140 lux (1,500 ft-c.). Plants were spaced at least 3 cm apart to avoid spreading of hyphae among adjacent plants. To reduce variability in age of seedling, which could affect susceptibility (13), only seedlings that emerged 4–6 days after planting were used; seedlings that emerged on the seventh day or later were removed and discarded.

A depression in the sand adjacent to each hypocotyle was made with a jet of sterile water from a plastic wash bottle. A 5-mm-diameter agar disk, cut with a cork borer from a 5-day-old culture of *P. ultimum*, was placed with mycelium against the hypocotyl in the depression, and sand was pressed gently against it to hold it in place. Inoculated plants were incubated for 7 days at 18 C and then rated for disease severity as follows: 1 = no symptoms; 2 = one to a few pinpoint dark spots or a faint diffuse discolored area on the hypocotyl; 3 = a distinctly necrotic, usually sunken, lesion less than 1.0 cm in length; 4 = a necrotic area more than 1.0 cm in length; 5 = plant wilted with cotyledons drooping; and 6 = plant dead. Thirty plants were inoculated individually with inoculum grown on media with each nutrient concentation. All tests were repeated. Significant differences in disease severity were determined with Duncan's multiple range tests.

Light transmission through blended agar cultures was used to measure inoculum quantity. The media and hyphae of eight 5-day-old cultures were combined with 100 ml of distilled water and blended in a Waring Blendor for 1 min. Samples were placed in cuvets and light transmission was determined at 620 nm with a Bausch and Lomb Spectronic 20 spectrophotometer.

Hypocotyls were also inoculated with mycelium grown in liquid cultures (without agar) of the basal medium containing various concentrations of sucrose or $NaNO_3$. Each culture consisted of the fungus in 100 ml of medium in a 237-ml screw-capped glass bottle. After 5 days of stationary incubation at room temperature, the mycelium was removed from each bottle and spread over a circular area, 5 cm in diameter, on sterile filter paper. To vary the amount of inoculum, mycelial contents from additional bottles were added in layers to the filter paper; all of the mycelial layers on a particular paper were obtained from cultures of identical nutritional levels. Filter papers with mycelial mats were cut into 1-cm^2 segments; each segment was inoculum for a single hypocotyl. To determine the quantity of mycelium used as inoculum, wet mycelium from individual cultures of each nutritional level was weighed.

TABLE 1. Virulence of *Pythium ultimum* to cotton seedlings after growth on potato-dextrose agar (PDA) for different times and at different nutrient concentrations

Age of inoculum		Nutrient concentration of medium	
Days of growth	Disease index[a]	PDA (%)[b]	Disease index[a]
4	4.67 x	100	4.83 x
7	3.67 y	50	4.33 x
14	3.67 y	10	3.25 y
21	3.00 yz	1	2.34 z
28	2.40 z	0	2.13 z

[a] Disease index is a measure of disease severity of cotton hypocotyls based on symptoms, where 1 = no symptoms, and 6 = dead plant. Each index value is the mean of 60 replicate hypocotyls. Index values within each experiment followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

[b] Concentrations of nutrient in PDA were obtained by dilution of PDA with water agar.

TABLE 2. Virulence to cotton seedlings and inoculum quantity of *Pythium ultimum* grown on basal agar media with variable concentrations of nutrients

Nutrient	Concentration	Light transmission[a] (%)	Disease index[b]
Sucrose (g/L)	0	96	1.35 w
	1	81	1.62 w
	10[c]	64	4.02 x
	20	61	4.49 x
	30	61	4.57 x
Sodium nitrate (g/L)	0	90	1.90 w
	0.5	62	4.74 x
	1.0	62	5.15 y
	2.0[c]	62	5.23 yz
	4.0	62	5.60 z
Ammonium chloride (g/L)	0[d]	62	5.22 y
	0	78	1.98 w
	0.5	63	4.03 x
	1.0	63	3.88 x
	2.0	64	4.05 x
Potassium (M)[e]	0	70	3.22 w
	0.003	62	4.77 x
	0.006	62	4.85 x
	0.012[c]	63	4.78 x
	0.024	63	5.05 x

[a] Relative inoculum quantities based on percentage light transmittance through suspension of colonies comminuted in water.

[b] Disease index is a measure of disease severity of cotton hypocotyls based on symptoms, where 1 = no symptoms, and 6 = dead plant. Each index value is the mean of 60 replicate hypocotyls. Index values within each nutrient variable followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

[c] Equivalent to the concentration of the nutrient in the basal medium. For potassium, the concentration in the basal medium was 0.018 M.

[d] For comparison, this medium contained 2 g of $NaNO_3$ per liter. Other NH_4-variable media contained no nitrate.

[e] Potassium was varied by adjusting K_2HPO_4 concentration. To maintain proper levels of chlorine, $CaCl_2$ was substituted for KCl. $Ca(H_2PO_4)_2 \cdot H_2O$ was added in quantities sufficient to maintain proper levels of phosphorus.

RESULTS

Age of inoculum and total nutrient concentration. Cotton hypocotyls were inoculated with *P. ultimum* grown on PDA for 4–28 days. In general, the fungus was progressively less virulent as it aged in culture (Table 1). Agar disks of *P. ultimum* grown on PDA, water agar, and PDA diluted with water agar were used as inocula. As the concentration of nutrients in PDA was decreased by dilution, the fungal inocula were progressively less virulent (Table 1.)

Sucrose nutrition. Growth was sparse and only minor disease symptoms were produced by *P. ultimum* grown on basal media with low sucrose (0 or 1 g/L). The fungus grew equally well and caused severe disease when grown on media with 10, 20, or 30 g of sucrose per liter (Table 2).

Nitrogen nutrition. Hyphae of *P. ultimum* on media without nitrogen grew poorly and caused little or no disease, but the addition of only 0.5 g/L of either $NaNO_3$ or NH_4Cl enabled the fungus to grow profusely and cause severe symptoms (Table 2). The fungus produced progressively more severe disease symptoms when grown on media with higher concentrations of nitrate but not

with ammonium. *P. ultimum* was significantly more virulent when grown on a medium with 2 g/L of $NaNO_3$ than on a medium with 2 g/L of NH_4Cl, despite the fact that the amount of elemental nitrogen in the NH_4Cl medium was larger than in the $NaNO_3$ medium.

Potassium nutrition. *P. ultimum* grown on media prepared without potassium grew sparsely and caused moderate disease symptoms (Table 2). Additions of small or large amounts of potassium (in the form of K_2HPO_4) resulted in profuse growth of the fungus and the production of severe disease symptoms.

Magnesium, sulfate, chlorine, and phosphate nutrition. Magnesium, sulfate, chlorine, or phosphate in the basal medium did not affect growth or virulence of *P. ultimum*. The fungus cultured on media without $MgSO_4$, KCl, or K_2HPO_4 (levels of potassium in phosphate-variable media were maintained by additional KCl) grew well and produced symptoms as severe as those produced by inoculum grown on media containing these nutrients.

Sucrose- and nitrogen-variable liquid media. Growth of *P. ultimum* in the basal liquid medium without sucrose was extremely sparse. Profuse growth occurred in liquid cultures containing 1–30 g sucrose per liter and weights of mycelia did not differ significantly. Virulence, however, varied with sucrose levels. The fungus produced progressively more severe disease as sucrose was increased (Fig. 1A). When the quantity of inoculum placed against hypocotyls was increased by combining mycelia from other cultures of the same level of sucrose, progressively more severe symptoms were produced. This was especially apparent at the low concentration (1 g/L) of sucrose.

P. ultimum responded similarly to nitrate nutrition in liquid culture. Growth was sparse in basal media without $NaNO_3$, but was profuse and not significantly different in media containing 0.1, 0.5, 2.0, or 4.0 g $NaNO_3$/L. Virulence increased progressively as the level of nitrogen was increased (Fig. 1B). When mycelia from cultures with similar levels of nitrate were combined, the fungus produced progressively more severe symptoms as the quantity of inoculum was increased.

DISCUSSION

Present practices for control of seedling disease of cotton consist of fungicide-seed treatment to reduce seed rot, and in-furrow application of fungicides. Because of the occurrence of adverse postplanting weather conditions and the diverse nature of the pathogens involved, fungicide applications often do not result in satisfactory control. Although there are measurable differences in susceptibility to *Pythium* among some cultivars (13), the differences are small and not very useful for making recommendations to growers. Futhermore, differences among commercial cultivars in susceptibility to *R. solani*, the other major pathogen, have not been demonstrated. Losses in yield attributed to seedling disease in Tennessee have been considerable (20, 5, and 15% for 1976, 1977, and 1978, respectively) (3). To significantly reduce losses caused by seedling pathogens, a more integrated approach must be devised, which requires knowledge of environmental and biological factors affecting infection and disease development. Such factors include mineral and carbohydrate nutrition of the pathogens. If certain concentrations of minerals or carbohydrates are required for pathogenesis, then methods should be devised to limit their supply during the highly susceptible seedling stage.

Sporangial germination, growth in soil, and virulence of *P. ultimum* are apparently affected by similar nutrients. Stanghellini and Hancock (21) observed that growth rates of germ tubes from sporangia were independent of the nutrient concentration that induced sporangial germination. Agnihotri and Vaartaja (1) found that amino acids induced sporangial germination but inorganic forms of nitrogen such as $NaNO_3$ did not. In the present study, sucrose, inorganic nitrogen, and potassium affected growth and virulence of hyphae of *P. ultimum*. Growth, however, was at least partly independent of virulence. Maximum growth occurred with as little as 1 g of sucrose per liter of medium. Additions of sucrose in excess of 1 g/L did not increase the total weight of mycelium but did markedly increase virulence. Responses to nitrogen in the medium were similar to those for sucrose. For maximum disease severity, high concentrations of both exogenous nitrogen and sucrose were required.

P. ultimum caused slight symptoms on hypocotyls at very low levels of nutrition. The fungus grew sparsely on water agar from a 5-mm-diameter disk taken from a PDA culture, but did retain virulence. Perhaps residual nutrients in the hyphae or in the PDA disk enabled sparse growth and low virulence, or hypocotyl exudates may have provided a limited supply of nutrients to the fungus.

Virulence of *P. ultimum* increased with increases in the quantity (as measured by weight) of the inoculum applied to hypocotyls. This occurred at all nutritional levels of sucrose and $NaNO_3$ below the maximum levels used. Large increases in virulence with increased inoculum quantity were not expected with mycelia grown in the absence of nitrogen or sucrose. However, when hyphae from four cultures without nitrogen were combined to yield inoculum

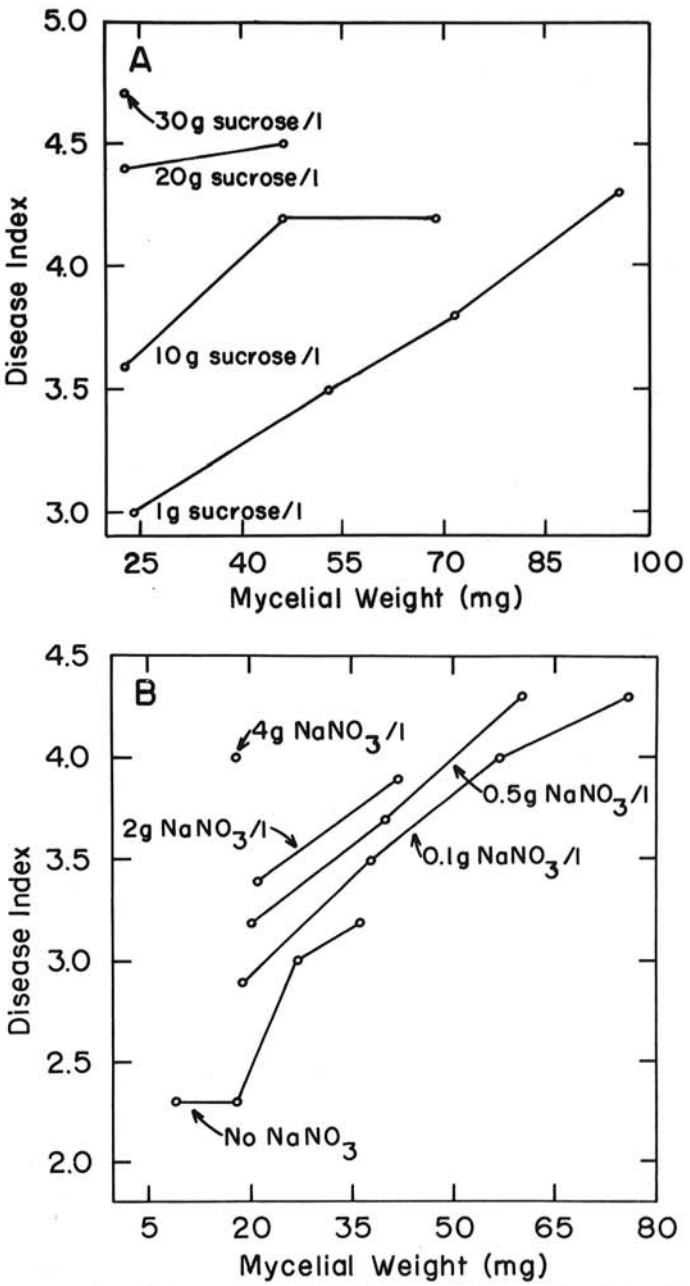

Fig. 1. Effect of inoculum quantity (as measured by mycelial weight) of *Pythium ultimum* on cotton hypocotyl disease severity. Inocula were 1-cm^2 mats of mycelia grown on mineral salts-sucrose liquid media. **A,** sucrose-variable growth media; **B,** sodium nitrate-variable growth media.

disks weighing 36 mg/cm^2, resulting symptoms were equivalent in severity to those produced by inoculum disks weighing 20 mg/cm^2 from a single culture containing 0.5 g of $NaNO_3$ per liter. Distinct mycelial layers did not develop on liquid media without sucrose and therefore we were not able to combine layers uniformly. However, disease severity at low levels of sucrose was similar to that observed with low levels of nitrogen; increasing the quantity of hyphae grown on media with 1 g of sucrose per liter resulted in disease symptoms as severe as those produced by single layers grown on much higher concentrations of sucrose (20 g/L).

These results may be partly explained as follows: increase in the amount of inoculum at any nutritional level increases the number of hypahe capable of infection, and an increase in inoculum quantity at any nutritional level may increase the quantity of enzymes aiding penetration. Although slight infection of hypocotyls was produced by inocula from single cultures containing little or no carbon or nitrogen, increasing the amount of such inocula adjacent to hypocotyls resulted in "infective inocula" as defined by Garrett (5).

It appears that exogenous nutrients are required for *P. ultimum* to incite disease symptoms, and that high concentrations are necessary for maximum disease development. Nutritionally deficient inocula produce less disease following infection. Limiting the supply of nutrient in soil could be an approach to reducing damage to cotton seedlings. Sporangia of *P. ultimum* are major survival structures (21) and amino acids and carbohydrates in seed exudate induce sporangial germination (1,21). Inorganic forms of nitrogen do not induce germination (1), but do affect growth and virulence after germination. Two sources of exogenous nutrients apparently are important for pathogenesis: amino-nitrogen and carbohydrates in cotton seed exudates, and inorganic nitrogen in fertilizers commonly applied to cotton soils prior to planting. The significance of carbohydrates produced during microbial decomposition of soil organic matter is not known. Since cotton seedlings become more resistant to *P. ultimum* as they age (13), delayed application of fertilizers might result in significant disease reduction. Also, as plants differ in the quantity of nutrients in their seed exudate (7,10), a breeding program designed to select lines of cotton with seeds that produce smaller amounts of nitrogen and carbohydrates in exudates could be of value.

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