Inhibition of Conidial Production of *Verticillium dahliae* with Ammonium Sulfate

D. R. Duncan and E. B. Himelick

Assistant biologist, Department of Agronomy, University of Illinois, Urbana 61801, and plant pathologist, Illinois Natural History Survey, Champaign 61820.
Accepted for publication 5 February 1986.

ABSTRACT

Duncan, D. R., and Himelick, E. B. 1986. Inhibition of conidial production of *Verticillium dahliae* with ammonium sulfate. Phytopathology 76: 788-792.

Dry weight and conidial production were reduced and hyphal morphology was changed when *Verticillium dahliae* was grown on Czapek-Dox broth amended with ammonium ion compared with its growth on unamended Czapek-Dox broth. Dry weight was not reduced if the ammonium ion-modified medium was buffered or amended with asparagine, alanine, threonine, or glutamine. Conidial production, however, was reduced on all ammonium ion-modified media compared with that on the unamended Czapek-Dox broth. These ammonium ion-induced growth alterations also were obtained when the medium's solute potential was lowered from −0.45 to −3.0 MPa. Conidial production on sugar maple sap amended with ammonium ion was similar to that on buffered, ammonium ion-amended Czapek-Dox broth. The results are discussed in relation to the use of fertilizers as a means of controlling Verticillium wilt.

Many attempts have been made to control or minimize Verticillium wilt of various plant species with nitrogen fertilizers. Disease symptoms have been reduced by applying various types of nitrogen fertilizers (6,15–17,22,31,34), especially ammonium sulfate (6,15,17,34); however, fertilization has also been reported to enhance or have no effect on disease development (11,13,19–21,28,32). One report indicated that severity of Verticillium wilt of cotton was reduced at low and high rates of fertilization but was increased at moderate rates (25).

There are no satisfactory explanations for the conflicting reports on the use of fertilizers to control Verticillium wilt. Our study investigates the possible role of ammonium ion in Verticillium wilt by examining the growth of *Verticillium dahliae* Kleb. on media modified by ammonium ion and other nitrogen sources.

MATERIALS AND METHODS

The primary isolate used in this study was V-105 of *V. dahliae*, obtained from a diseased sugar maple (*Acer saccharum* Marsh.). Seven other isolates from the mycological collection of E. B. Himelick also were used. These isolates were in storage from 5 to 23 yr, and they originated from seven species of woody trees or shrubs in either Champaign, Urbana, or Wheaton, IL. Gross morphology, microscopic appearance in culture, and culture growth at 30 C were used to confirm pathogen identity. Single-spored cultures of each isolate were grown on Czapek-Dox broth agar (CDA) for 15 days at 24 C in the dark and stored at 10 C for 13–17 days before use. Unamended CDA consisted of 35.3 mM $NaNO_3$, 5.7 mM K_2HPO_4, 2.0 mM $MgSO_4 \cdot 7H_2O$, 6.8 mM KCl, 0.04 mM $FeSO_4 \cdot 7H_2O$, and 20 g/L Bacto agar.

Experiments with the various fungal isolates were carried out on CDA test-tube slants with different nitrogen sources, water potentials, and buffered pH. The concentrations of nitrogen tested in CDA were 30.0 mM ammonium sulfate, 75.4 mM ammonium chloride, 47.1 mM sodium nitrate, 30.3 mM asparagine, 33.6 mM threonine, 27.4 mM glutamine, 44.9 mM alanine, 30.5 mM leucine, and 34.5 mM valine. Also, 50:50 (w/w) mixtures of ammonium sulfate and each amino acid listed were tested. The water potential was lowered to −3.0 MPa by adding sucrose (1.26 M) to the water used to make the medium. A Wescor Dew Point Microvoltmeter (Wescor, Inc., Logan, UT) was used to measure the water potential. Medium pH was buffered at 7.0 with 100 mM KH_2PO_4 + 77.5 mM NaOH. Changes in the pH of nonbuffered media of various nitrogen compositions were also studied either by acid titration of media without *V. dahliae* or by daily sampling of replicated flasks of media with *V. dahliae*. Initial pH of all media was about 7.0 after autoclaving.

Growth of *V. dahliae* in both unamended sugar maple sap and sap amended with 60, 180, or 540 μM ammonium sulfate was also tested. The sap was extracted by vacuum from nursery-grown maple trees in March, stored at −20 C until needed, and filter-sterilized into oven-sterilized flasks. Flasks of the unamended and amended sap were seeded with *V. dahliae*, shaken at 160 rpm, and incubated at 24 C in the dark for 15 days.

A spore suspension of $5–7 \times 10^4$ conidia per tube was used to establish *V. dahliae* in each tube or flask, and each culture was incubated in the dark for 15 days at 24 C. Dry weights of the fungus grown on solid agar media were obtained by the method of Chaudhuri (8). Dry weights from liquid cultures were obtained by vacuum-filtration onto dried and preweighed Whatman No. 1 filter paper; samples were then dried at 70 C for 48 hr before reweighing. A hemacytometer was used to measure conidial production. Each experimental treatment was replicated five times, and each experiment was repeated two to six times.

RESULTS

Hyphal growth on all ammonium ion-containing media tested, except those ammonium ion-containing media that also contained asparagine, alanine, threonine, or glutamine, was bulbous, disjointed, and easily fragmented compared with hyphal growth on other nitrogen sources (Fig. 1A,B). Microsclerotial production was inhibited on ammonium ion-amended media unless the amino acids were also present.

When ammonium ion was present in CDA, dry weight of *V. dahliae* was reduced unless alanine, asparagine, threonine, or glutamine was added to the medium. Leucine and valine increased dry weight the most; however, when these amino acids were used with ammonium sulfate, growth was reduced. Conidial production was reduced in all media containing ammonium sulfate (Table 1, Fig. 2).

Increased dry weight comparable to that induced by nitrate-containing CDA was achieved with ammonium ion-amended media buffered to pH 7.0 (Fig. 2). Typical hyphal structure and microsclerotial production were maintained in all buffered media; however, conidial production was always reduced when ammonium ion was present. Nonbuffered media containing

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

asparagine, ammonium ion, or asparagine + ammonium ion (50:50, w/w) showed similar titration curves when titrated with hydrochloric acid (Fig. 3). Daily monitoring of pH changes during the growth of *V. dahliae* in these three media showed a rapid drop in pH to about 2.8 when ammonium ion was the sole nitrogen source and a fluctuation around pH 6.5 in the other two media (Fig. 4).

With the same treatments as listed in Figure 2, the solute potential of each medium was reduced from −0.45 to −3.0 MPa with sucrose. All media with a lowered solute potential induced lower dry weight and greater conidial production than CDA of −0.45 MPa (Table 2). There were also fewer microsclerotia produced on media of low solute potential than on CDA of −0.45 MPa. A comparison of treatments with lowered solute potential showed that ammonium ion had the same effect on dry weight and conidial production as it had at the normal solute potential of −0.45 MPa (Table 2).

The effects of ammonium ion on dry weight and conidial and microsclerotial production were consistent with all isolates tested except V-83 and V-91 (the oldest ones) (Table 3). With isolates V-83 and V-91, the ammonium ion + asparagine treatment induced greater conidial production than did asparagine alone. With isolate V-83, media containing nitrate induced the greatest conidial production, whereas with all other isolates tested, asparagine induced the greatest conidial production.

Ammonium added to sugar maple sap increased dry weight at the highest concentration tested but did not reduce dry weight at the other two concentrations. Production of conidia was always less with ammonium ion-amended sap than with unamended sap (Fig. 5).

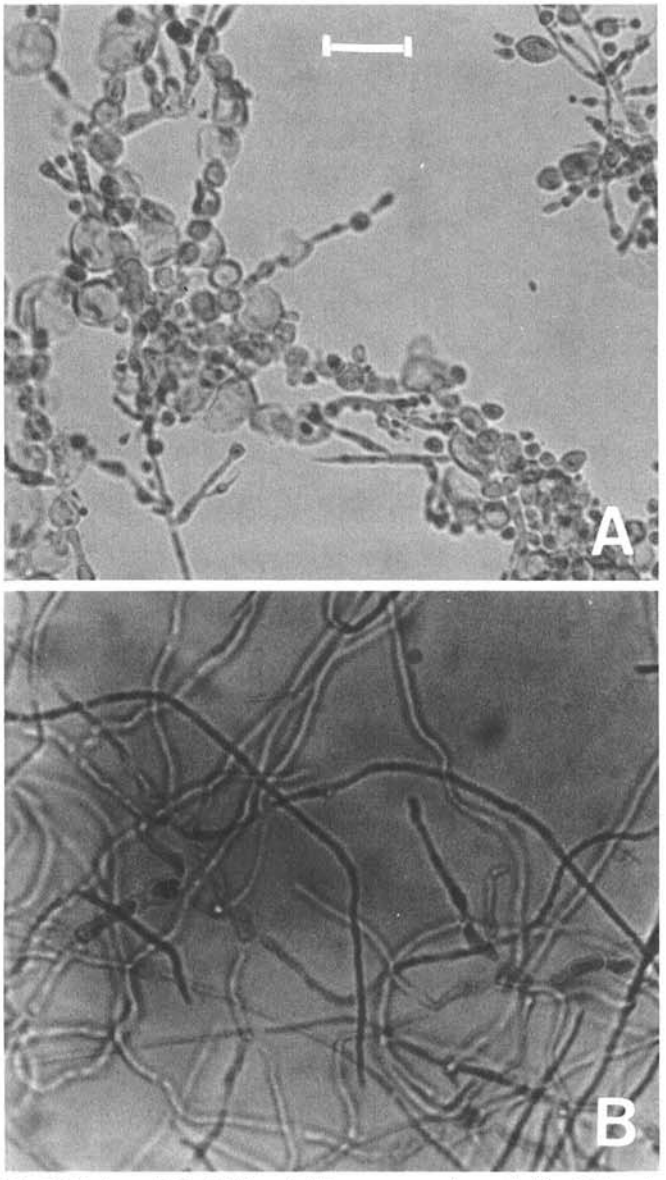

Fig. 1. Hyphae of *Verticillium dahliae* grown on **A,** nonbuffered Czapek-Dox broth containing ammonium sulfate and on **B,** Czapek-Dox broth without an ammonium ion source. Scale bar = 10 μm.

DISCUSSION

Several reports have indicated that the reduced growth of *Verticillium* species with ammonium ion is related to a lowered pH in the culture medium (9,12,18,23). Inhibition of growth when ammonium ion and nitrate are present has also been demonstrated (12). The effects of buffering the CDA medium (Fig. 2) and the pH measurements of inoculated media (Fig. 3) support these previous studies. More importantly, however, the present data show that the reduction of dry weight and hyphal production of *V. dahliae* by

TABLE 1. Dry weight and conidial production of *Verticillium dahliae* (isolate V-105) on Czapek-Dox broth amended with several amino acids or 50:50 (w/w) mixtures of amino acids and ammonium sulfate[x]

Amino acid	Dry weight (g) ($\times 10^{-2}$)		Conidia/ml of medium ($\times 10^{7}$)	
	Alone	+ $(NH_4)_2SO_4$	Alone	+ $(NH_4)_2SO_4$
Alanine	5.48 b[y]	5.34 b	3.11 a	1.12 c
Asparagine	4.69 c	5.06 c	9.98 a	2.04 abc
Glutamine	4.50 c	4.15 d	6.78 a	0.61 d
Leucine	6.87 a	3.89 d	1.10 bc	0.36 e
Threonine	5.13 bc	4.17 d	0.47 d	0.28 e
Valine	6.55 a	2.80 e	1.37 c	0.39 e
$(NH_4)_2SO_4$ control[z]	···	1.20 f	···	0.45 d

[x] Amino acid concentration alone was 4 g/L.
[y] All values are averages of five replicates per treatment; each experiment was repeated three times. Different letters indicate significantly different values for FLSD at $P \leq 0.05$.
[z] Control was Czapek-Dox broth with 30 mM ammonium sulfate.

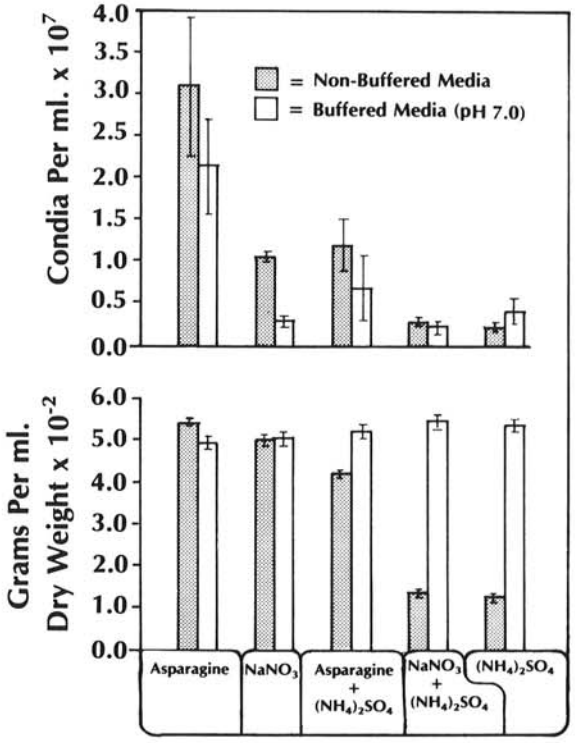

Fig. 2. Dry weight and conidial production of *Verticillium dahliae* on Czapek-Dox broth modified by several nitrogen sources.

ammonium ion can be prevented by adding one of several amino acids to the medium. The effect of these amino acids seems to be pH-related and comparable to the effect of buffering the media (Figs. 3 and 4).

In this study, separate growth measurements were made of both dry weight and conidial production. This is the first attempt to quantify the effects of ammonium ion on conidial production of *V. dahliae*. Any treatment that contained ammonium ion had a significantly reduced ($P \leq 0.05$) conidial production compared with the appropriate controls (Tables 1–3; Figs. 2 and 5).

Most tree saps are highly buffered, dilute nutrient solutions containing amino acids (2,4,5). Consequently, the lack of dry weight reduction by ammonium ion added to sap, which was observed in this study, would be expected (Fig. 3). The stimulated growth at the highest ammonium ion concentration suggests that ammonium ion in sap within a tree might stimulate, not reduce, fungal growth and that it possibly may increase disease development (10). This would be true if only the increased dry weight is considered; however, the rapid spread of the fungus within the host is by the translocation of conidia through xylem vessels (1,3,14,24). Upward spread by hyphal growth is slow (29,30).

Disease severity has been correlated with inoculum potential (26,27) and suggests that factors altering conidial production by the fungus could have a more pronounced effect on disease development than those affecting hyphal production. The presence of ammonium ion in the medium greatly inhibited conidial production (Tables 2–4; Figs. 3 and 6). Thus, if the presence of ammonium inhibits conidial production in the sap of a host plant, severity of Verticillium wilt could be reduced and account for the reduction in disease development with ammonium fertilizers that has been reported previously (6,15,17,34).

The theory that soilborne nitrifying bacteria prevent the accumulation, and possibly the existence, of ammonium in soils (7) has perhaps discouraged the use of ammonium fertilizers to control Verticillium wilt. The effects of nitrifying bacteria are widespread

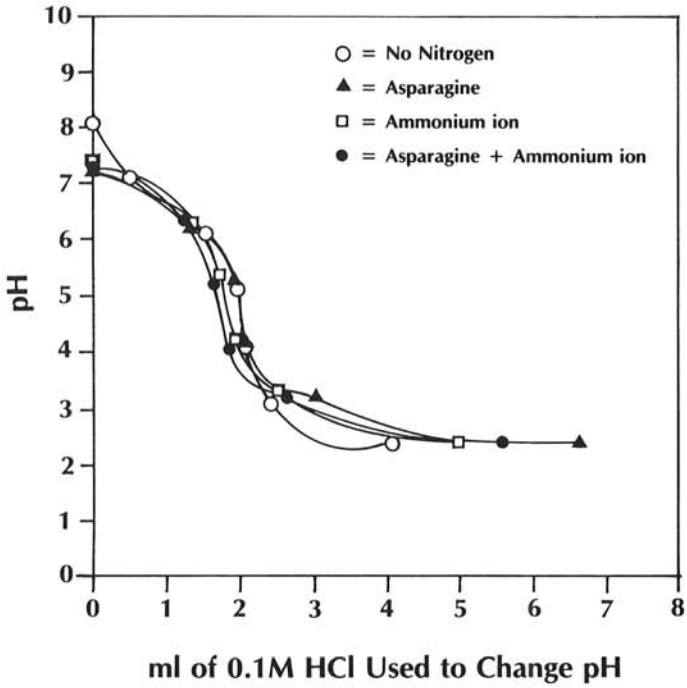

Fig. 3. Titration curves of Czapek-Dox broth modified with ammonium ion and/or asparagine as nitrogen sources. Media titrated with 0.1 M hydrochloric acid.

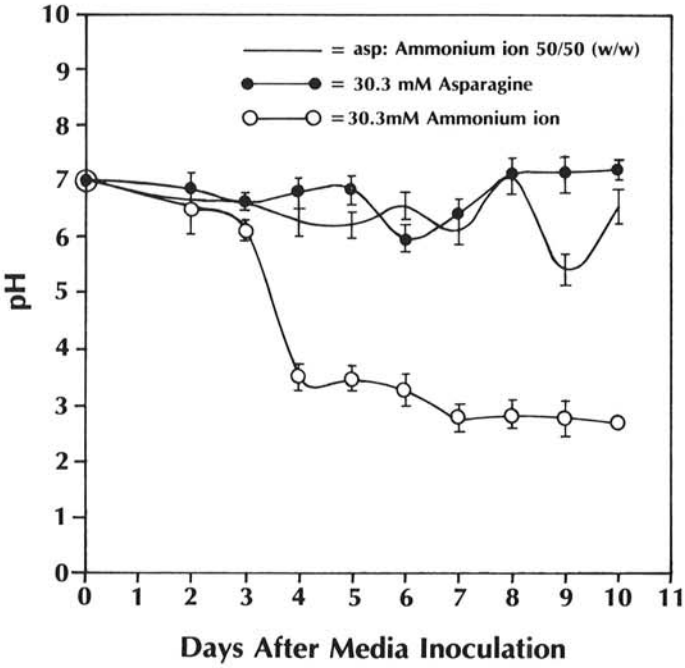

Fig. 4. Medium pH change as *Verticillium dahliae* grew in Czapek-Dox broth modified with ammonium ion and/or asparagine as nitrogen sources.

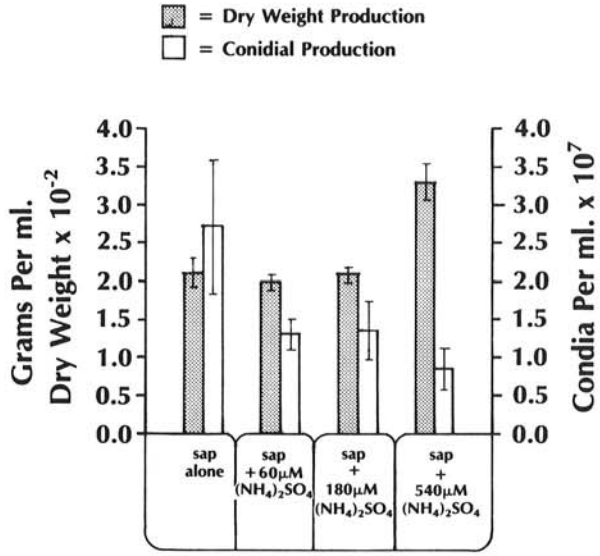

Fig. 5. Dry weight and conidial production of *Verticillium dahliae* grown on sugar maple sap amended with ammonium sulfate.

TABLE 2. Dry weight and conidial production of *Verticillium dahliae* grown on Czapek-Dox broth amended with several nitrogen sources and adjusted from −0.45 MPa to −3.0 MPa water potential[x]

Nitrogen source	Dry weight (g) ($\times 10^{-2}$)	Conidia/ml of medium ($\times 10^{7}$)
Asparagine (30 mM)	2.08 a[y]	65.42 a
Asparagine (15 mM) + $(NH_4)_2SO_4$ (15 mM)	1.69 bc	17.91 b
$NaNO_3$ (47 mM)	1.58 c	4.80 c
$NaNO_3$ (23.5 mM) + $(NH_4)_2SO_4$ (15 mM)	1.03 c	1.77 c
$(NH_4)_2SO_4$ (30 mM)	0.99 d	0.95 c
Control[z]	5.00 e	1.00 c

[x] Water potential was adjusted with sucrose and measured with a Wescor Dew Point Microvoltmeter.

[y] All values are averages of five replicates per treatment; each experiment was repeated six times. Different letters indicate significantly different values for FLSD at $P \leq 0.05$.

[z] Control was Czapek-Dox broth with 47.1 mM nitrate nitrogen at −0.45 MPa water potential.

TABLE 3. Dry weight and conidial production of isolates of *Verticillium dahliae* on Czapek-Dox broth amended with different nitrogen sources

Nitrogen source	Isolates of *V. dahliae*						
	V-83	V-91	V-106	V-108	V-109	V-110	V-111
$NaNO_3$ (47 mM)							
Conidia[a]	3.68[b]	1.06	0.65	0.70	0.85	0.49	0.55
Dry weight[c]	4.60	5.06	5.29	4.17	4.08	4.18	5.28
$(NH_4)_2SO_4$ (30 mM)							
Conidia	0.30	0.24	0.07	0.18	0.24	0.17	0.14
Dry weight	1.27	1.22	1.45	0.93	0.87	1.15	1.37
NH_4Cl (75 mM)							
Conidia	0.25	0.32	0.28	0.38	0.45	0.21	0.33
Dry weight	1.29	1.24	1.19	1.09	0.69	0.96	1.18
$(NH_4)_2SO_4$ (15 mM) + asparagine (15 mM)							
Conidia	1.91	2.08	0.48	1.12	0.80	0.87	0.70
Dry weight	4.12	5.57	4.37	3.76	3.61	2.74	4.41
$NaNO_3$ (15 mM) + $(NH_4)_2SO_4$ (15 mM)							
Conidia	0.27	0.12	0.08	0.29	0.22	0.70	0.17
Dry weight	1.47	1.36	1.71	1.15	1.03	1.85	1.65
Asparagine (30 mM)							
Conidia	0.92	1.23	2.42	6.37	4.47	1.71	3.03
Dry weight	4.69	5.26	5.24	4.32	4.44	5.06	5.20

[a] Conidial production, conidia per milliliter in suspension medium ($\times 10^7$).
[b] All values are averages of five replicates per treatment; each experiment was repeated three times.
[c] Grams dry weight ($\times 10^{-2}$).

and predominate in most agronomic soils; however, grassland, forest, and orchard soils sometimes have low levels of nitrifying bacteria (7). These observations have been correlated with high phosphate levels in the soil and/or low soil pH.

Soils that have been autoclaved, or which contain large amounts of peat moss, and potting mixes of washed sand and vermiculite (materials used in greenhouse experiments) may not contain nitrifying bacteria. Weisman (33) demonstrated that 25% of the nitrogen in the sap of sunflowers grown in washed sand was ammonium ion. Consequently, if the plant species tested transports ammonium ion in its xylary sap, and if the soil in which that species is growing lacks nitrifying bacteria, ammonium fertilizers might reduce Verticillium wilt by inhibiting conidial production of *V. dahliae* and subsequent rapid colonization of the host.

The potential of ammonium fertilizers to control *V. dahliae* should be reexamined. The chemical form of nitrogen within the host and the fate of nitrogen in the soil have not been considered in previous studies concerning the control or reduction of Verticillium wilt. These two factors may help to interpret such studies and to develop effective fertilization regimes. The effects of nitrogen-stabilizing compounds also need to be examined to determine if they can enhance the effectiveness of ammonium fertilizers.

LITERATURE CITED

1. Banfield, W. M. 1941. Distribution by the sap stream of spores of three fungi that induce vascular wilt diseases of elm. J. Agric. Res. 62:637-681.
2. Barnes, R. L. 1963. Organic nitrogen compounds in tree xylem sap. For. Sci. 9:98-102.
3. Beckman, C. H., VanderMolen, G. E., and Mueller, A. C. 1976. Vascular structure and distribution of vascular pathogens in cotton. Physiol. Plant Pathol. 9:87-94.
4. Bennett, J. P., Anderson, F. G., and Milad, Y. 1927. Methods of obtaining tracheal sap from woody plants. New Phytol. 26:316-323.
5. Bollard, E. G. 1956. Nitrogenous compounds in plant xylem sap. Nature 178:1189-1190.
6. Caroselli, N. E. 1956. The effect of various soil amendments on maple wilt incited by *Verticillium* sp. (Abstr.) Phytopathology 46:240.
7. Chase, F. E., Corke, C. T., and Robinson, J. B. 1968. Nitrifying bacteria in soil. Pages 583-622 in: The Ecology of Soil Bacteria, T. R. G. Gray and D. Parkinson, eds. University of Toronto Press, Toronto.
8. Chaudhuri, H. 1923. A study of the growth in culture of *Verticillium albo-atrum* B. et Br. Ann. Bot. 37:519-539.
9. Cochrane, V. W. 1958. Physiology of Fungi. John Wiley & Sons, New York. 524 pp.
10. Duncan, D. R., and Himelick, E. B. 1981. Effect of ammonium sulfate on *Verticillium dahliae* growth. (Abstr.) Phytopathology 71:871.
11. Dwinell, L. D., and Sinclair, W. A. 1965. Effects of N, P, K and inoculum density on Verticillium wilt of American elm and sugar maple. (Abstr.) Phytopathology 55:1056.
12. Foster, J. W. 1949. Chemical Activities of Fungi. Academic Press, New York. 648 pp.
13. Gallegly, M. E. 1949. Host nutrition in relation to development of Verticillium wilt of tomato. (Abstr.) Phytopathology 39:7.
14. Garber, R. H., and Houston, B. R. 1966. Penetration and development of *Verticillium albo-atrum* in the cotton plant. Phytopathology 56:1121-1126.
15. Huber, D. M., and Watson, R. D. 1972. Nitrogen form and plant disease. Down Earth 27:14-15.
16. Huber, D. M., and Watson, R. D. 1974. Nitrogen form and plant disease. Annu. Rev. Phytopathol. 12:139-165.
17. Huber, D. M., Watson, R. D., and Steiner, G. W. 1965. Crop residues, nitrogen, and plant disease. Soil Sci. 100:302-308.
18. Isaac, I. 1949. A comparative study of pathogenic isolates of *Verticillium*. Trans. Br. Mycol. Soc. 32:137-157.
19. Isaac, I. 1956. Some soil factors affecting Verticillium wilt of *Antirrhinum*. Ann. Appl. Biol. 44:105-112.
20. Isaac, I. 1957. The effects of nitrogen supply upon the Verticillium wilt of *Antirrhinum*. Ann. Appl. Biol. 45:512-515.
21. Keyworth, W. G., and Hewitt, E. J. 1948. Verticillium wilt of the hop (*Humulus luppulus*). V. The influence of nutrition on the reaction of the hop plant to infection with *Verticillium albo-atrum*. J. Hortic. Sci. 24:219-227.
22. Miller, P. N., and Edgington, L. V. 1962. Effect of paper and sawdust soil amendments on meadow nematodes and subsequent Verticillium wilt of tomato. Plant Dis. Rep. 46:745-757.
23. Pateman, J. H., and Kingborn, J. R. 1976. Nitrogen metabolism in the filamentous fungi. Pages 159-237 in: The Filamentous Fungi. J. E. Smith and D. R. Berry, eds. John Wiley & Sons, New York.
24. Presley, J. T., and Taylor, E. E. 1969. Ontogeny of vessels influence the development of *Verticillium* in young cotton plants. Phytopathology 59:253-254.
25. Ranney, C. D. 1962. Effects of nitrogen source and rate on the development of Verticillium wilt of cotton. Phytopathology 52:38-41.
26. Schnathorst, W. C., and Mathre, D. E. 1966. Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology 56:1155-1161.
27. Schnathorst, W. C., Presley, J. T., and Carnes, H. R. 1967. Determination of the internal inoculum potential of *Verticillium albo-atrum* in cotton plants. (Abstr.) Phytopathology 57:101-102.
28. Singh, D., Brinkerhoff, L. A., and Guinn, G. 1971. Effect of alanine on development of Verticillium wilt in cotton cultivars with different levels of resistance. Phytopathology 61:881-882.

29. Smith, K. L. 1976. Verticillium wilt of maples: stem colonization by the pathogen and prospects for chemotherapy. M.S. thesis. Cornell University, Ithaca, New York. 50 pp.
30. Smith, L. D. 1978. Colonization of herbaceous and woody plants by *Verticillium dahliae* isolates. Ph.D. thesis. University of Illinois, Urbana. 94 pp.
31. Tolmsoff, W. J., and Young, R. A. 1959. The influence of crop residues and fertilizers on the development and severity of Verticillium wilt in potato. (Abstr.) Phytopathology 49:114.
32. Walker, J. C., Gallegly, M. E., Jr., Bloom, J. R., and Scheffer, R. P. 1954. Relation of plant nutrition to disease development. VIII. Verticillium wilt of tomato. Am. J. Bot. 41:760-762.
33. Weissman, G. S. 1964. Effect of ammonium and nitate nutrition on protein level and exudate composition. Plant Physiol. 39:947-952.
34. Wilhelm, S. 1951. Effect of various soil amendments on the inoculum potential of the Verticillium wilt fungus. Phytopathology 41:684-690.