Effects of Five Acids that Occur in Pine Needles on Fusarium chlamydospore Germination in Nonsterile Soil

Freddi Hammerschlag and R. G. Linderman

Plant Pathologists, Ornamentals Laboratory, Plant Genetics and Germplasm Institute, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland 20705; and Ornamental Plants Research Laboratory, Oregon State University, Corvallis 97330, respectively.

The authors thank P. B. Adams and E. H. Allen for helpful suggestions, and E. J. Koch, Biometrical Services, for the statistical analysis.

Accepted for publication 28 April 1975.

ABSTRACT

Five acids that occur in pine needles, shikimic, quinic, malic, citric, and phosphoric, both separately and in combination stimulated chlamydospore germination of two isolates of *Fusarium oxysporum* f. sp. *lilii* (HF and 319) in nonsterile soil. Germination in the presence of the five acids was followed by germ-tube lysis without formation of replacement chlamydospores. Germination of the HF isolate in the five acids at pH 2.8, 4.5, 6.5, and 8.0 was 54, 81, 41, and 37%, respectively. Germination of the 319 isolate was 70, 70, 59, and 46%, respectively. A tenfold dilution series of the five acids revealed that germination was proportional to the concentration of the total acids. When combinations of acids were tested, shikimic and quinic proved to be the most important germination stimulants.

The presence of these acids in pine forest soil (pH 4.0–5.5) may be partly responsible for the absence of *Fusarium* spp. from these soils.

Phytopathology 65:1120-1124

Additional key words: weak organic acids, phenolic acids, secondary chlamydospore formation.

The absence of *Fusarium* spp. from forest soils has been reported by Thornton (30) and Park (24). Smith (27) showed the decline of *Fusarium* in roots of sugar pines (*Pinus lambertiana* Dougl.) transplanted from nursery to forest soil. When chlamydospores in forest soil were exposed to pine duff extracts by Toussoun et al. (32), 95-98% germinated, and lysed. Replacement chlamydospores did not form. When chlamydospores were exposed under the same conditions to either 1% glucose or 2.5% asparagine, 62-80% germinated without lysis before replacement chlamydospores had formed. Menzinger (18) analyzed water extracts of pine duff and found that 15 amino acids independently stimulated at least 90% germination of chlamydospores, with germ tube lysis.

The organic acid fraction of an aqueous extract of pine needles was shown (21) to contain shikimic, malic, citric, quinic, and phosphoric acids. These five acids account for 85% of the total acidity of this fraction. The objective of our study was to evaluate the effects of these acids on *F. oxysporum* chlamydospores in nonsterile soil.

MATERIALS AND METHODS.—Two isolates of *Fusarium oxysporum* Schl. f. sp. *lilii* Imle were used in the study. The field isolate (HF) was obtained from field soil containing diseased lily bulbs. The second isolate (319) was isolated from an Easter lily (*Lilium longiflorum* Thunb.) bulb scale. Both isolates produced root and basal plate lesions on *L. longiflorum* seedlings. The isolates were maintained in test tubes of sterile soil at 18.5 C. When needed, the isolates were plated onto water agar, single-spored onto V8 Juice agar plates, and incubated at 24 C in a 12-hour photoperiod under cool-white fluorescent lights. After 7 days, macroconidia were collected, added to Cochrane's citrate buffer medium (7) to give a final spore concentration of 1×10^5 spores/ml, and shaken for 6-10 days at room temperature. Chlamydospores, which formed from macroconidia, were washed and centrifuged three times in distilled water. These chlamydospore preparations were added in high numbers to lily field soil (pH 4.6) that was maintained at 50% moisture-holding capacity. Numbers of chlamydospores per gram of soil containing either isolate 319 or HF were on the order of 4.6×10^6 and 5.0×10^6, respectively.

For chlamydospore germination experiments, 20 mg of 319- or HF-infested soil were placed in the well of a porcelain spot plate. The soil was saturated with 0.10 ml of a test solution and incubated for various time periods in the dark at 24 C. Percentage of germination was determined by making a soil smear on a slide, staining it with a 1:3 dilution of 0.16% acid fuchsin in 50% lactic acid, and counting the spores at a magnification of ×430. Percentage of germination was expressed as the average of six counts, 100 spores per count.

The organic acid fraction of an aqueous extract of pine needles contains shikimic, quinic, citric, malic, and phosphoric acids (21). Synthetic acid solutions containing commercially produced acids were prepared by dissolving 0.2101 g citric acid, 0.0871 g malic acid, 0.4992 g quinic acid (K & K Laboratories, Plainview, NY 11803), 0.7656 g shikimic acid (ICN Nutritional Biochemicals, Cleveland, OH 44128), and 0.3539 g potassium dihydrogen phosphate in 100 ml water. In this way, solutions contained an amount of acid per milliliter equal to that found in 1 g of dried pine needles (20).

In pH studies, the pH of the acid solutions was adjusted with 1.0N HCl or 1.0N NaOH before addition to the test soil.

RESULTS.—*Stimulation of chlamydospore germination by organic acids.*—The effects of the five acids from pine needles on chlamydospore germination tested individually and in combination are shown in Tables 1 and 2. Chlamydospore germination was stimulated more by shikimic (S) and quinic (Q) acids than by any other individual acid. Citric (C) and phosphoric (P) acids were not significantly different from the water control. When the concentration of C was raised from 0.01 M to 0.44 M, germination increased only slightly. Germination of isolates HF and 319 in a mixture of the five acids was 54% and 70%, respectively. Germination of both was significantly reduced when S was removed from the combination. When Q was removed from the combination, germination was reduced significantly only with isolate 319. These data suggest that in chlamydospore germination, S and Q are the most important components of the mixture of acids.

TABLE 1. Effect of individual organic acids from pine needles on the germination of chlamydospores of *Fusarium oxysporum* f. sp. *lilii* in soil

Acid	Germination (%)	
	Isolate HF	Isolate 319
Shikimic (0.044 M)	48 a[z]	53 a
Quinic (0.026 M)	45 a	41 ab
Malic (0.006 M)	25 b	29 bc
Citric (0.010 M)	11 bc	6 d
Citric (0.044 M)	21 b	15 cd
Phosphoric (0.026 M)	11 bc	8 d
Water control	0 c	0 d

[z]Each value represents the mean of six observations, each observation involving the random count of 100 chlamydospores. Any two means in each column not followed by a letter in common are significantly different, $P = 0.05$, according to Duncan's multiple range test.

TABLE 2. Effect of combinations of organic acids from pine needles on the germination of chlamydospores of *Fusarium oxysporum* f. sp. *lilii* in soil

Acid combination	Germination (%)	
	Isolate HF	Isolate 319
Total mixture[y]	54 a[z]	70 a
Mixture minus S	35 b	47 c
Mixture minus Q	46 ab	51 c
Mixture minus M	59 a	61 abc
Mixture minus C	61 a	67 ab
Mixture minus P	62 a	54 bc
Water control	0 c	0 d

[y]Mixture composed of shikimic (S) (0.044 M), quinic (Q) (0.026 M), malic (M) (0.006 M), citric (C) (0.01 M), and KH_2PO_4 (P) (0.026 M).

[z]Each value represents the mean of six observations, each observation involving the random count of 100 chlamydospores. Any two means in each column not followed by a letter in common are significantly different, $P = 0.05$, according to Duncan's multiple range test.

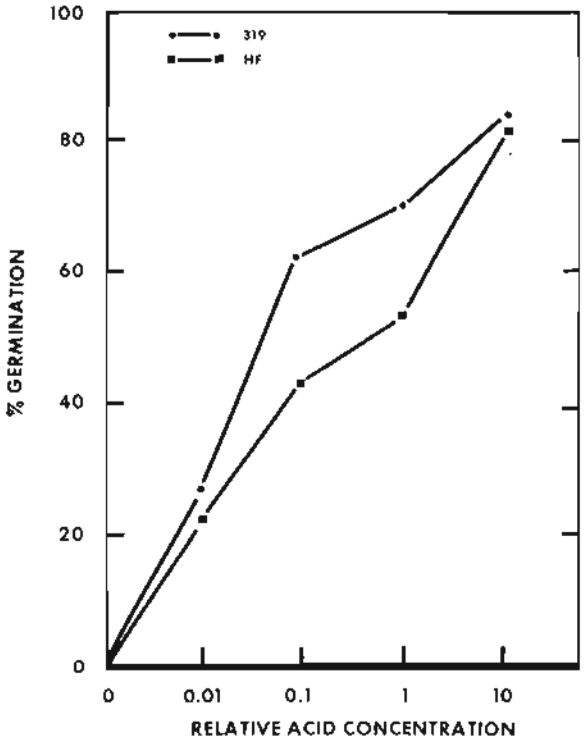

Fig. 1. Effect of relative concentration of a mixture of organic acids from pine needles on chlamydospore germination of isolates HF and 319 of *Fusarium oxysporum* f. sp. *lilii* in nonsterile soil; 1 = 0.044 M shikimic acid, 0.026 M quinic acid, 0.026 M phosphoric acid, 0.01 M citric acid, and 0.006 M malic acid. Each point represents the mean of six counts, 100 spores/count.

Chlamydospore germination and germ tube lysis.—Chlamydospores were observed 2 and 5 days after the test solution containing all five acids was applied. Percentage of germination after 48 hours did not increase over that observed after 24 hours. After 2 days, the beginnings of lysis were observed. After 5 days, germ tubes were void of cytoplasm or fragmented. Replacement chlamydospores did not form. These results, obtained also when the acid mixture was adjusted to pH 4.5 before addition to the soil, are similar to those presented by Toussoun et al. (32).

Influence of acid-mixture concentration on chlamydospore germination.—The total acids were concentrated tenfold in a flash evaporator. Tenfold dilutions of the acids were also made. All acid concentrations were added to HF- and 319-infested soils, and chlamydospore germination was observed after 24 hours (Fig. 1). Percentages of germination of HF chlamydospores in relative acid concentrations of 10, 1, 0.1, and 0.01 were 81, 54, 44, and 22, respectively. Those of 319 were 82, 70, 63, and 27, respectively. Germination of both isolates, therefore, appeared to be directly proportional to total acid concentration.

Influence of pH on chlamydospore germination.— The five acids at pH 2.8, 4.5, 6.5, and 8.0 were added to soil containing either isolate HF or 319. Percentage of germination after 24 hours was 54, 81, 41, and 37 for HF and 70, 70, 59, and 46 for 319 at those respective pH levels (Fig. 2). Clearly, chlamydospore germination was higher at the lower pH levels.

DISCUSSION.—Several workers have reported that *Fusarium* does not exist or survive in coniferous soils (23, 27, 32). The presence of the needle litter layer covering those soils was implicated in this phenomenon by Toussoun et al. (32). They found that leachates of pine needles stimulated germination of *Fusarium* chlamydospores followed by germ tube lysis, without formation of replacement chlamydospores. Menzinger (18) later reported that amino acids in the leachates could induce the germination-lysis reaction. Our study has shown that the organic acids in pine needles (21) can also induce the germination-lysis reaction. If this reaction occurred repeatedly over time, due to any of the components of the total-acid fraction, one could visualize the eventual decline of *Fusarium* in that soil.

Chlamydospore germination in soil is influenced by available nutrients (1, 8, 25) and the chemical (34) and biotic environment around the spore (28). Although sugars and amino acids have been reported to induce chlamydospore germination of fusaria (18, 32) the effect of the organic acids on chlamydospore germination has not yet been reported. However, the two most effective acids, shikimic and quinic acids, are known to be necessary precursors for microbial synthesis of aromatic amino acids (9, 10, 12) that are needed for protein synthesis during spore germination (13).

The organic acids in our study were more effective

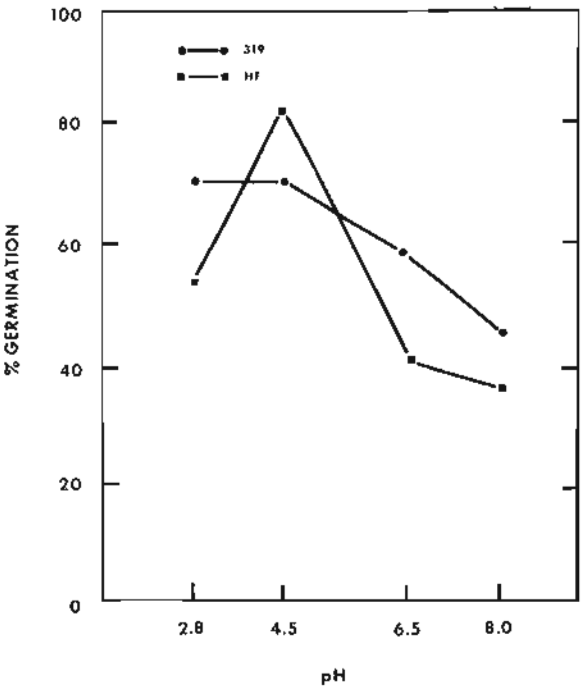

Fig. 2. Effect of pH of mixture of organic acids from pine needles on chlamydospore germination of isolates HF and 319 of *Fusarium oxysporum* f. sp. *lilii* in nonsterile soil. Percentages are based on six counts, 100 spores/count. The pH of the acid mixture was adjusted by addition of 1.0N HCl or 1.0N NaOH.

germination stimulants when the soil pH was low than when it was high. This result is understandable, since organic acids are either neutral or only partly ionized at low pH. According to Albert (1), organic acids in that state are more biologically active than those in the completely ionized state.

We visualize several possible reactions when *Fusarium* chlamydospores are exposed to the organic acids that leach from pine needles. The chlamydospores are stimulated to germinate, but then, in the absence of a susceptible or supportive host plant, must form a replacement chlamydospore or perish (26). Somehow, the pine needle leachates or the organic acids create an unfavorable soil environment in which replacement chlamydospores cannot or do not form. The leachates may lower the soil pH and thereby inhibit chlamydospore formation (5, 14). They may inhibit numbers and types of bacteria needed to produce necessary chlamydospore-inducing substances (11), or they may increase the C/N ratio, which has also been reported to reduce chlamydospore formation (6, 23).

In addition, we suggest that other microorganisms besides *Fusarium* could convert components of the needle leachates to compounds that could inhibit growth or chlamydospore formation by *Fusarium*, or both. Several workers have shown that fungi (3, 4, 15, 16) and bacteria (17) can convert shikimic and quinic acids to phenolic acids such as protocatechuic and gallic acids, which can inhibit fungi (2, 22).

Needle leachates appear to affect directly the survival of fusaria in soils covered with needles. They stimulate chlamydospore germination and also inhibit formation of replacement chlamydospores. However, the leachates may contribute to the decline of fusaria by other indirect means. Several workers have isolated fusaria from pine forest soil with high pH (pH 5.7 - 6.3) (19), or from grassland soil or grass rhizosphere with low pH (4.2 and 3.9, respectively) (29, 34). Thus, vegetational types, soil pH, and the presence or absence of *Fusarium* appear to be related (24). Fusaria appear to survive even in low-pH soils, if supportive grasses are present, or in high-pH soils presumably leached with organic acids from the needle litter. But they cannot survive in low-pH soils leached with the acids that can stimulate chlamydospore germination and lysis. If the supportive plants are eliminated, possibly by growth suppression by inhibitors in the needle leachates (33), the germinated chlamydospores would be deprived of a favorable rhizosphere environment in which new ones could form. The data of R. S. Smith, Jr., as reported by Toussoun (31), supports this idea: *F. oxysporum* declined in nursery soils during the winter, but increased again in the summer, but only in the plots without a pine-needle litter layer in which annual weeds had developed. Thus, his data suggest that fusaria may be rhizosphere inhabitants of grasses or other herbaceous plants (29) to the extent that their populations are maintained by such plants.

Another factor that bears on this phenomenon was pointed out by Park (23): some fusaria may have too low a competitive saprophytic ability to compete in the rhizospheres of woody forest species against specialized root associates such as mycorrhizal fungi.

The net effect of the combination of chemical, physical, and biological factors in a forest or low-pH soil lacking supportive annuals, is the eventual decline and elimination of fusaria. We feel that the organic acids in the needle leachates may be important, because they cause the spores to germinate, expend stored energy, and, in the process, expose themsleves to an extremely inhospitable soil environment.

LITERATURE CITED

1. ALBERT, A. 1965. Selective toxicity. John Wiley & Sons, New York. 233 p.
2. ANGELL, H. R., J. C. WALKER, and K. D. LINK. 1930. The relation of protocatechuic acid to disease resistance in onions. Phytopathology 20:431.
3. BERNHAUER, K., and B. GORLECH. 1935. Transformation of aromatic compounds by microorganisms. II. Transformation of quinic acid and inositol. Biochem. Z. 280:394-395.
4. BERNHAUER, K., and H. H. WAELSCH. 1932. Transformation of aromatic and hydroaromatic compounds by molds. I. The transformation of quinic and hydroxybenzoic acids. Biochem. Z. 249:223-226.
5. BOURRET, J. A. 1965. Physiology of chlamydospore formation and survival in Fusarium. Ph.D. Thesis, University of California, Berkeley. 96 p.
6. CARLILE, M. J. 1956. A study of the factors influencing non-genetic variation in a strain of Fusarium oxysporum. J. Gen. Microbiol. 14:643-654.
7. COCHRANE, V. W., and J. C. COCHRANE. 1971. Chlamydospore induction in pure culture in Fusarium solani. Mycologia 63:462-477.
8. COOK, R. J., and M. N. SCHROTH. 1965. Carbon and nitrogen compounds and germination of chlamydospores of Fusarium solani f. phaseoli. Phytopathology 55:254-256.
9. DAVIS, B. D. 1955. Biosynthesis of the aromatic amino acids. Pages 799-811 *in* D. W. McElroy and H. B. Glass, eds. A symposium on amino acid metabolism. Johns Hopkins Press, Baltimore. 1048 p.
10. DAVIS, B. D., and U. WEISS. 1953. Aromatic biosynthesis. VIII. The roles of 5-dehydroquinic acid and quinic acid. Arch. Exp. Pathol. Pharmakol., Naunyn-Schmiedeberg's 220:1-15.
11. FORD, E. J., A. H. GOLD, and W. C. SNYDER. 1970. Induction of chlamydospore formation in Fusarium solani by soil bacteria. Phytopathology 60:479-484.
12. GORDON, M., F. A. HASKINS, and H. K. MITCHELL. 1950. The growth promoting properties of quinic acid. Proc. Nat. Acad. Sci. U.S.A. 36:427-430.
13. GOTTLIEB, D. 1966. Biosynthetic processes in germinating spores. Pages 217-233 *in* M. F. Madelin, ed. The fungus spore. Butterworths, London. 338 p.
14. GRIFFIN, G. J. 1964. Influence of carbon and nitrogen nutrition on chlamydospore formation by Fusarium solani f. radicicola. Phytopathology 54:894 (Abstr.).
15. GROSS, S. R. 1958. The enzymatic conversion of 5-dehydroshikimic acid to protocatechuic acid. J. Biol. Chem. 233:1146-1151.
16. HASLAM, E., R. D. HAWORTH, and P. F. KNOWLES. 1961. Galiotannins. Part IV. The biosynthesis of gallic acid. J. Chem. Soc. (Lond.) No. 361:1854-1859.
17. ISHIKAWA, H., and T. OKI. 1959. Biological conversion of shikimic acid or quinic acid to protocatechuic acid, gallic acid and aromatic amino acids. Agric. Chem. Soc. Jap. 23:451-453.
18. MENZINGER, W. 1969. Zur Keimungsphysiologie von Fusarium- Chlamydosporen in Boden. Bundesanst. Land Forstwirtsch., Berlin-Dahlem 132:38-39.
19. MORROW, M. B. 1932. The soil fungi of a pine forest. Mycologia 24:393-402.

20. MUIR, J. W., J. LOGAN, and C. J. BOWN. 1964. The mobilization of iron by aqueous extracts of plants. II. Capacities of the amino-acid and organic acid fractions of a pine needle extract to maintain iron in solution. J. Soil Sci. 15:226-237.

21. MUIR, J. W., R. I. MORRISON, C. J. BOWN, and J. LOGAN. 1964. The mobilization of iron by aqueous extracts of plants. I. Composition of the amino-acid and organic-acid fractions of an aqueous extract of pine needles. J. Soil Sci. 15:220-225.

22. MUKHERJEE, N., and B. KUNDU. 1973. Antifungal activities of some phenolics and related compounds to three fungal plant pathogens. Phytopathol. Z. 78:89-92.

23. PARK, D. 1954. Chlamydospores and survival in soil fungi. Nature (Lond.) 173:454-455.

24. PARK, D. 1963. The presence of Fusarium oxysporum in soils. Trans. Br. Mycol. Soc. 46:444-448.

25. SCHROTH, M. N., T. A. TOUSSOUN, and W. C. SNYDER. 1963. Effect of certain constituents of bean exudate on germination of chlamydospores of Fusarium solani f. phaseoli in soil. Phytopathology 53:809-812.

26. SEQUEIRA, L. 1962. Influence of organic amendments on survival of Fusarium oxysporum f. cubense in the soil. Phytopathology 52:976-982.

27. SMITH, R. S. JR. 1967. Decline of Fusarium oxysporum in the roots of Pinus lambertiana seedlings transplanted into forest soils. Phytopathology 57:1265.

28. SMITH, S. N., and W. C. SNYDER. 1972. Germination of Fusarium oxysporum chlamydospores in soils favorable and unfavorable to wilt establishment. Phytopathology 62:273-277.

29. THORNTON, R. H. 1958. Biological studies of some tussock grassland soil. II. Fungi. N.Z. J. Agric. Res. 1:922-938.

30. THORNTON, R. H. 1960. Fungi of some forest and pasture soils. N.Z. J. Agric. Res. 3:699-711.

31. TOUSSOUN, T. A. 1975. Fusarium-suppressive soils. Pages 145-151 *in* G. W. Bruehl, ed. Biology and control of soil-borne pathogens. American Phytopathological Society, St. Paul, Minnesota. 216 p.

32. TOUSSOUN, T. A., W. MENZINGER, and R. S. SMITH, JR. 1969. Role of conifer litter in ecology of Fusarium: Stimulation of germination in soil. Phytopathology 59:1396-1399.

33. WANG, T. S. C., T. YANG, and T. CHUANG. 1967. Soil phenolic acids as plant growth inhibitors. Soil Sci. 103:239-246.

34. WARCUP, J. H. 1951. Ecology of soil fungi. Trans. Br. Mycol. Soc. 34:376-399.