Activity of Juniper Diffusates on Spores of Phomopsis juniperovora

Ronald W. Pero and Frank L. Howard

Research Assistant and Professor of Plant Pathology, respectively, University of Rhode Island, Kingston 02881. Present address of senior author: Postdoctorate Appointee, Department of Plant Pathology, North Carolina State University, Raleigh 27607.

Contribution No. 1318 of the Rhode Island Agricultural Experiment Station, based on a portion of a Ph.D. thesis by the senior author.

Accepted for publication 17 October 1969.

ABSTRACT

Juniperus virginiana and *J. chinensis* 'Keteleeri' foliage immersed in water yield diffusible substances that affect spore germination. Concentrations similar to those that occur in drops of water on *J. virginiana* leaves stimulate germination and germ tube elongation of *Phomopsis juniperovora* spores. The solutes from Keteleeri foliage exposed to water for an equal period have no significant stimulatory effect on *P. juniperovora* spores, but if the diffusate from Keteleeri is concentrated 10 to 40 times, then *P. juniperovora* spores are stimulated. Keteleeri is resistant to *P. juniperovora* infection; *J. virginiana* is susceptible. Two active components were isolated, potassium as K_2SO_4 and an unsaturated carboxylic acid. The carboxylic acid had ultraviolet absorption maxima of 212 and 262 mμ in distilled water, an equivalent wt of 105.69, a melting point of 164-166 C (unc.), and an elemental composition of carbon 47%, hydrogen 9%, and oxygen 44%. Potassium above 1,250 ppm in a 5:1 ratio with sodium stimulated *J. juniperovora* spores to germinate. The carboxylic acid was approximately 16 times more active, causing stimulation of spores above 77 ppm. Potassium was found in all juniper diffusates analyzed, whereas the carboxylic acid was found only in foliar diffusate from actively growing, 7-year-old *J. virginiana*. Phytopathology 60:491-495.

Phomopsis juniperovora Hahn, the cause of juniper blight, is a destructive pathogen of many conifers. Previous investigation of this pathogen centered around chemical control and mycological studies (1, 2, 3, 9). The life cycle of *P. juniperovora* is not fully understood. For example, the spores can initiate disease in a susceptible host, but germinate either poorly or not at all in distilled water (8). This fact suggests that host diffusates stimulate germination of the spores. The purpose of this study was to (i) determine the effects of diffusate from juniper leaves on spore germination of *P. juniperovora;* (ii) characterize the nature of any spore stimulants found; and (iii) correlate the effects of foliar diffusate on *P. juniperovora* spores with their potential for host invasion.

MATERIALS AND METHODS.—Junipers naturally infected with *P. juniperovora* served as the source of the isolates used. The fungus cultures were maintained in the dark on cornmeal agar at 25 C; spore masses were harvested randomly after 30 days.

Juvenile leaves were excised from *Juniperus virginiana* L. and *J. chinensis* L. 'Keteleeri' Cornman, which are susceptible and resistant to *P. juniperovora,* respectively. The leaves were arranged separately on parloidin coated slides. A drop of spore suspension (25 spores/×450 microscopic field) covered each leaf on the slide, which was then incubated in a petri dish moist chamber for 24 hr at 25 C. After incubation the leaves were removed, and per cent spore germination for 200 randomly selected spores was recorded and compared to germination in drops of spore suspension only. Spores were considered germinated if the germ tubes were as long as the width of the spore. Germ tube length was measured for 25 randomly selected spores that germinated in diffusate from *J. virginiana* and Keteleeri juniper and in distilled water. Under the conditions obtained, the concentration of diffusible substances from the leaves was considered comparable to that which occurs in water drops on juniper leaves in nature.

Concentrated diffusates were obtained by suspending the foliage of intact plants in distilled water at room temperature for 24 hr, then reducing the extracting solution under vacuum at 43 C to one-fortieth its original volume (×40). The ×40 diffusates were serially diluted 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 with distilled water, and bioassayed for stimulation of *P. juniperovora* spore germination and germ tube elongation by adding an equal volume of spore suspension to each dilution of the diffusate. Spore germination and germ tube growth were measured as previously described.

The crude ×40 diffusates were fractionated and purified according to the scheme in Fig. 1. Purification was evaluated by comparing the extent to which a crude ×40 diffusate and each of the resulting fractions could be diluted before losing its ability to stimulate *P. juniperovora* spores.

Spore germination stimulants in diffusates were isolated, using thin-layer and paper chromatography. Thin-layer plates were made using Silica Gel G (E. Merck A. G., Darmstadt, Germany) according to Randerhath (10). The solvent system was methanol:acetic acid (9:1, v/v). Whatman No. 1 filter paper was used for paper chromatography. The solvent system was *n*-butanol:acetic acid:water (4:1:5, v/v, organic phase). The detection reagents were 0.1 N $AgNO_3$ in 5 N NH_4OH (1:1) heated to 110 C; concentrated H_2SO_4 heated to 110 C; 1% ethanolic $FeCl_3$; and 1% $KMnO_4$ in 2% Na_2CO_3. Paper chromatograms were spotted with approximately equal amounts of a purified diffusate, irrigated in the solvent system, air dried, and divided longitudinally into equal halves. One-half was developed with one of the detection reagents. The other half was

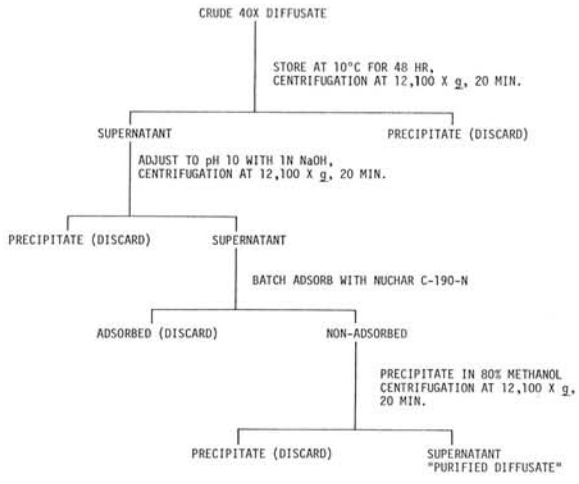

Fig. 1. Scheme used to fractionally purify foliar diffusate from dormant and actively growing 7-yr-old *Juniperus virginiana* and from actively growing 1-yr-old *J. chinensis* 'Keteleeri' grafts.

divided into segments from the baseline to the solvent front. These segments were bracketed to correspond with the developed and undeveloped areas found on the reagent-sprayed chromatograms. Each segment was cut into smaller sections, eluted with distilled water, and bioassayed for the presence of a germination stimulator. Thin-layer chromatograms were handled similarly, except that development and elution of the chromatograms were carried out on separate thin-layer plates.

The infrared spectra for isolated germination stimulants were obtained in KBr pellets using a Model IR-8 Infrared spectrophotometer, Beckman Instruments, Inc.

RESULTS.—*Activity of juniper diffusates on P. juniperovora spores.*—Diffusate from *J. virginiana*, evaluated at the concentrations created by immersion of single leaves in drops of spore suspension, stimulated spore germination to 95% and germ tube length to 30.1 μ. Spore germination and germ tube length in leaf diffusate from Keteleeri juniper were 28% and 7.3 μ, respectively, which were not significantly different from spores incubated in distilled water alone. However, when foliar diffusates were concentrated, both *J. virginiana* and Keteleeri juniper diffusates stimulated *P. juniperovora* spore germination and germ tube growth (Table 1). The stimulatory substances present in concentrated *J. virginiana* diffusate were 32 times more effective at stimulating germination of spores than were those in the Keteleeri diffusate (Table 1).

Germ tube growth caused by *J. virginiana* diffusate was always greater than that incited by Keteleeri juniper diffusate regardless of the concentration (Table 1). Germ tube length in distilled water rarely increased appreciably with an increased incubation period. When spores were stimulated to germinate by *J. virginiana* diffusate, germ tube branching was so prolific that a 48-hr incubation period resulted in the further development of a mycelial mat. Some spores exposed to the higher concentrations of *J. virginiana* diffusate exhibited an abnormal type of germination; germ tubes grew at random from the sides of the spore instead of emerging at the poles.

Fractionation of diffusate from dormant, 7-year-old J. virginiana foliage.—Paper chromatograms of the purified diffusate from dormant, 7-yr-old *J. virginiana* contained just one segment at $R_F = 0.09$ that stimulated *P. juniperovora* spore germination (Table 2). Similar results were obtained when the purified diffusate was chromatographed on thin-layer plates (Table 2). The spore stimulant was not detected with concentrated H_2SO_4, which indicated that the active spot was an inorganic compound. Crystallization of the inorganic germination stimulant was accomplished by further concentrating the purified diffusate on a hot water bath at 60 C and adding absolute methanol dropwise until the solution neared cloudiness. Crystals formed within 24 hr at 5 C.

The IR spectrum for this germination stimulant showed only one broad peak at 9.1 μ. This peak is indicative of unconjugated sulphate ions (6). A qualitative test for sulphate ion (16) was also positive. Knowledge of the positive ion associated with the sul-

TABLE 1. The effect of concentration of foliar diffusate from *Juniperus virginiana* and *J. chinensis* 'Keteleeri' on spore germination and germ tube length of *Phomopsis juniperovora*

Dilution of ×40 diffusate	*J. virginiana*			*J. chinensis* 'Keteleeri'		
	% Spore germination[a]	Germ tube length in μ[b]		% Spore germination[a]	Germ tube length in μ[b]	
		24 hr	48 hr		24 hr	48 hr
Distilled water	32	5.1	5.6	29	4.8	5.1
1:2	85[c]	26.1	[d]	53[c]	11.7	16.7
1:4	84[c]	24.3	[d]	37	9.8	15.1
1:8	70[c]	22.1	[d]	22	7.1	9.1
1:16	64[c]	17.8	[d]	23	7.4	9.1
1:32	50[c]	14.7	20.1	26	8.1	10.3
1:64	42[c]	15.1	22.1	25	7.6	10.0
1:128	36	8.2	10.4	27	7.8	8.7

[a] Based on 200 spores/treatment.
[b] Based on 25 randomly selected germinated spores/treatment.
[c] Significantly different from spore germination in distilled water at the 5% level.
[d] Germ-tube growth was so prolific that individual germ tubes could not be distinguished.

TABLE 2. The isolation on paper and thin-layer chromatograms of the spore stimulants in purified foliar diffusate from actively growing (AG-7JV) and dormant (D-7JV) 7-yr-old *Juniperus virginiana* and from actively growing *J. chinensis* 'Keteleeri' (JCK)

Detection reagent	Paper chromatograms						Thin-layer chromatograms					
	R_F values of eluted segments causing spore stimulation[a]			R_F value of spots detected[b]			R_F values of eluted segments causing spore stimulation[a]			R_F value of spots detected[c]		
	AG-7JV	D-7JV	JCK	AG-7JV	D-7JV	JCK	AG-7JV	D-7JV	JCK	AG-7JV	D-7JV	JCK
Ammoniacal $AgNO_3$	0.09	0.09	0.09	0.09[d]	0.09[d]	0.09[d]						
	0.78			0.34[e]	0.34[e]	0.34[e]						
Concd H_2SO_4							0.00	0.00	0.00	0.79	0.79	0.79
							0.88			0.88		
$FeCl_3$	0.09	0.09	0.09	0.78	none	none	0.00	0.00	0.00	0.88	none	none
	0.78						0.88					
$KMnO_4$	0.09	0.09	0.09	0.34	0.34	0.34	0.00	0.00	0.00	0.79	0.79	0.79
	0.78			0.78			0.88			0.88		

[a] Chromatograms were divided into segments from baseline to solvent front, eluted with distilled water, and each segment was then tested for stimulation of *P. juniperovora* spores.
[b] The R_F values were from chromatograms developed in *n*-butanol:acetic acid:water (4:1:5).
[c] The R_F values were from chromatograms developed in absolute methanol:acetic acid (9:1).
[d] The spot was distinct, but an atypical white color developed that turned purple with time.
[e] This was not a distinct spot, but a streak that developed.

phate ion was important, since either ion or both could be the active agent. The presence of sodium and potassium ions was determined by use of a Model 52A Perkin-Elmer flame photometer, and the direct intensity method was used to evaluate the unknown amounts. Potassium and sodium ions were found present in the purified diffusate at levels of 6,200 and 1,200 ppm, respectively. Hence, K_2SO_4 and Na_2SO_4 appeared to be present in a ratio of 5:1.

In order to determine which ion or ions were responsible for increasing *P. juniperovora* spore germination, 40,000-ppm stock solutions of analytical grade NaCl, KCl, Na_2SO_4, and K_2SO_4 were prepared. The concentration of the NaCl and KCl solutions was based on the sodium and potassium ions, but the concentration of Na_2SO_4 and K_2SO_4 solutions was based on the sulphate ion. All the stock solutions and 5:1 ratios of KCl/NaCl and K_2SO_4/Na_2SO_4 were serially diluted 1:2, 1:4, 1:8, 1:16, and an equal volume of *P. juniperovora* spore suspension was added to test for stimulation of germination.

When potassium was present at 2,500 ppm or more, spores of *P. juniperovora* were stimulated to germinate (Tables 3, 4). Sodium as NaCl and sulphate as Na_2SO_4 at comparable concentrations had no significant effect on spore germination. Potassium sulphate had no greater effect than KCl. However, potassium with sodium in a 5:1 ratio, or as K_2SO_4/Na_2SO_4, enhanced the activity of potassium.

Fractionation of diffusate from actively growing, 7-yr-old J. virginiana foliage.—Two different segments of paper chromatograms, spotted with purified diffusate from actively growing *J. virginiana* foliage, contained spore germination stimulants (Table 2). These results were confirmed by chromatography of the diffusate on thin-layer plates (Table 2). One of the germination stimulants was inorganic, and found to be potassium as K_2SO_4, according to the methods previously discussed. The other germination stimulant was crystallized by adjusting the purified diffusate with 1 N HCl to approximately pH 1.0, and extracting with an equal volume of diethyl ether. The ether extract produced crystals after concentration under a N atmosphere. The IR spectrum for this spore germination stimulant showed a broad band of absorption from 2.9 to 3.6 μ, with other absorption maxima at 5.9, 7.1, 7.65, 8.35, and 10.9 μ. These areas of absorption are typical of organic acids (12). The germination stimulant also evolved CO_2 in 5% $NaHCO_3$ solution, a characteristic of carboxylic acids.

TABLE 3. Germination of *Phomopsis juniperovora* spores in water containing different amounts and ratios of sodium and potassium sulphate salts

Compound	Anion in ppm	% Spore germination[a]
Distilled water		0
Na_2SO_4	10,000	0
Na_2SO_4	5,000	1
Na_2SO_4	2,500	0
Na_2SO_4	1,250	0
Na_2SO_4	625	0
K_2SO_4	10,000	67[b]
K_2SO_4	5,000	55[b]
K_2SO_4	2,500	0
K_2SO_4	1,250	0
K_2SO_4	625	0
K_2SO_4/Na_2SO_4	10,000/2,000	80[b]
K_2SO_4/Na_2SO_4	5,000/1,000	71[b]
K_2SO_4/Na_2SO_4	2,500/500	32[b]
K_2SO_4/Na_2SO_4	1,250/250	5
K_2SO_4/Na_2SO_4	625/125	0

[a] Based on 200 spores/treatment.
[b] Significantly different from spore germination in distilled water at the 5% level.

TABLE 4. Germination of *Phomopsis juniperovora* spores in water containing different amounts and ratios of sodium and potassium chloride salts

Compound	Cation in ppm	% Spore germination
Distilled water		0
NaCl	10,000	1
NaCl	5,000	1
NaCl	2,500	1
NaCl	1,250	0
NaCl	625	0
KCl	10,000	63[b]
KCl	5,000	56[b]
KCl	2,500	2
KCl	1,250	0
KCl	625	0
KCl/NaCl	10,000/2,000	77[b]
KCl/NaCl	5,000/1,000	66[b]
KCl/NaCl	2,500/500	30[b]
KCl/NaCl	1,250/250	3
KCl/NaCl	625/125	1

[a] Based on 200 spores/treatment.
[b] Significantly different from spore germination in distilled water at the 5% level.

Sulphur was not detected in the stimulant using the method of Vogel (15). Carbon, hydrogen, and N were determined on a Model 185 F & M carbon-hydrogen-N analyzer. The results were carbon 47.2%, hydrogen 8.6%, and N 0.3%. Since an insignificant amount of N and no sulphur were detected, oxygen, the organic element unaccounted for, would equal about 44% of the elemental composition. The equivalent wt was determined as 105.7 following the procedure of Vogel (15), except that sample sizes ranged from 0.617 to 2.466 mg. A pH titration curve using standard 0.001 N NaOH was calculated, and no leveled-off areas were observed within the titrated range of pH 1.5-10.5. The bromine and Baeyer's tests for unsaturation both indicated the presence of a double bond. The ultraviolet spectrum was taken on a Model 15 Cary recording spectrophotometer, and revealed absorption peaks at 212 and 262 mμ in distilled water. The melting point, determined on a Thomas-Hoover capillary melting point apparatus, was 164-166 C (unc.). These results suggest that the unknown spore germination stimulant can be tentatively identified as an unsaturated carboxylic acid. The carboxylic acid was bioassayed for stimulation of *P. juniperovora* spore germination at concentrations of 616, 308, 154, 77, and 38.5 ppm. The spores were incited to germinate at concentrations as low as 77 ppm. This activity was approximately 16 times greater than that of potassium, since potassium was found to be active at a minimum concentration of 1,250 ppm.

Fractionation of diffusate from actively growing, 1-yr-old Keteleeri juniper foliage.—Paper and thin-layer chromatograms of purified diffusate from Keteleeri juniper developed one spot which had a spore-germination stimulant present (Table 2). The IR spectrum and flame photometric analysis of this spot, carried out as described, indicated the stimulant to be potassium as K_2SO_4.

DISCUSSION.—Resistance may be contingent upon the shortage or absence of one or more plant metabolites essential for successful colonization of a host by a parasite (17). The presence or absence of spore stimulators found in water leachates from castor bean capsules (7) and grape berries (5) has been correlated with the success or failure of infection by *Botrytis cinerea*. The factors responsible for successful infection were identified as glucose and fructose. Keitt et al. (4) also implicated the role of host metabolites in determining susceptibility or resistance in their studies on the host-parasite interactions in apple scab disease. The resistance of the Keteleeri juniper to *Phomopsis* blight may also be explained on this basis. When the diffusible substances were tested at concentrations that exist in water drops on leaves, spores of *P. juniperovora* germinated and grew profusely in diffusate from a susceptible juniper, but not in diffusate from the resistant Keteleeri juniper.

Spores of many fungi have been stimulated to germinate by minerals as well as by a variety of organic materials. Among the many reported inorganic and organic fungal spore stimulants are potassium salts and organic acids (13). The occurrence of large amounts of potassium in foliar diffusate is not uncommon. Tukey & Morgan (14) reported losses by leaching in 24 hr as high as 80-90% of the total potassium content of aboveground plant parts. Organic acids present in biologically important concentrations have also been shown to be a primary constituent of foliar leachates (11). Both a potassium salt and an apparent carboxylic acid have been found in diffusates of *J. virginiana*. Although the Keteleeri juniper exhibits resistance to *P. juniperovora* in the field, foliar diffusate from this plant contains potassium as a spore germination stimulator. The potassium concentration is apparently too low, however, to cause any spore germination in natural leaf-surface water films.

The physiological condition of junipers affects the composition of foliar diffusate. The foliar diffusate from 7-yr-old, dormant *J. virginiana* plants has only potassium as a spore stimulatory component, but if plants of the same age are actively growing, potassium and a spore-stimulating carboxylic acid are present.

In Rhode Island, juniper-blight symptoms usually appear at the beginning of August. Since blight symptoms develop within 4 to 6 weeks after inoculation, field infection of junipers must have occurred when the plants were developing new growth. The presence of the more active carboxylic acid in the actively growing juniper, and the absence of it in the dormant juniper, presumably determine the relative chances for infection, and might explain the pattern of disease development under natural conditions.

The age of junipers may also be an important factor in altering diffusate composition qualitatively. Preliminary experiments have shown that foliar diffusate from actively growing, 2-yr-old *J. virginiana* has at least three distinct *P. juniperovora* spore stimulants: an inorganic substance, a $FeCl_3$-positive substance, and a substance with properties of a glycoside (8).

The possible role of microbial metabolites themselves in determining diffusate composition has also been considered, but was believed to be unimportant (8).

The spore stimulant, tentatively identified as a carboxylic acid, was never isolated from diffusates of the resistant Keteleeri juniper. Diffusate from *J. virginiana* contained the carboxylic acid, but only when the foliage was actively growing and in its most susceptible state for infection by *P. juniperovora*. These facts, together with the fact that the carboxylic acid was 16 times more active on *P. juniperovora* spores than potassium, suggest that this substance is of particular interest in the incidence of blight in junipers.

LITERATURE CITED

1. Caroselli, N. E. 1957. Juniper blight and progress on its control. Plant Dis. Reptr. 41:216-218.
2. Hahn, G. G. 1926. *Phomopsis juniperovora* and closely related strains on conifers. Phytopathology 16:899-914.
3. Hahn, G. G. 1943. Taxonomy, distribution, and pathology of *Phomopsis occulta* and *P. juniperovora*. Mycologia 35:112-129.
4. Keitt, G. W., D. M. Boone, & J. R. Shay. 1959. Genetic and nutritional controls of host parasite interactions in apple scab, p. 157-168. *In* C. W. Holton et al. [ed.]. Plant Pathology, Problems and Progress, 1908-1958. Univ. Wis. Press, Madison.
5. Kosuge, T., & W. B. Hewitt. 1964. Exudates of grape berries and their effect on germination of conidia of *Botrytis cinerea*. Phytopathology 54:167-172.
6. Nakamato, K. 1963. Infrared spectra of inorganic and coordination compounds. John Wiley & Sons, N. Y. 328 p.
7. Orellana, R. G., & C. A. Thomas. 1962. Nature of predisposition of castor beans to *Botrytis*. I. Relation of leachable sugar and certain other biochemical constituents of the capsule to varietal susceptibility. Phytopathology 52:533-538.
8. Pero, R. W. 1968. Juniper diffusates; their composition and effect on *Phomopsis juniperovora* spores. Ph.D. Thesis, Univ. Rhode Island Libr., Kingston. 92 p.
9. Peterson, G. W., D. R. Sumner, & C. Norman. 1965. Control of *Phomopsis* blight of eastern redcedar seedlings. Plant Dis. Reptr. 49:529-531.
10. Randerhath, K. 1963. Thin-layer chromatography. Academic Press, N. Y. 250 p.
11. Ruinen, J. 1961. The phyllosphere. I. An ecologically neglected milieu. Plant Soil 15:81-109.
12. Shriner, R. L., R. C. Fuson, & D. Y. Curtin. 1964. The systematic identification of organic compounds [5th ed.] John Wiley and Sons, N. Y. 458 p.
13. Sussman, A. S. 1965. Physiology of dormancy and germination of the propagules in cryptogamic plants, p. 931-1025. *In* A. Lang [ed.]. Encyclopedia of Plant Physiology. Vol. 15(2). Springer-Verläg, Berlin.
14. Tukey, H. B., & J. V. Morgan. 1964. The occurrence of leaching from aboveground plant parts and the nature of the materials leached. XVI. Int. Hort. Cong. Proc., Belgium 4:146-153.
15. Vogel, A. I. 1956. A textbook of practical organic chemistry [3rd ed.] John Wiley & Sons, N. Y. 1188 p.
16. Welcher, F. J., & R. B. Hahn. 1955. Semimicro-qualitative analysis. D. Van Nostrand Co., Inc., Princeton, N. J. 497 p.
17. Wood, R. K. S. 1967. Physiological plant pathology. Blackwell Scientific Publication, Oxford & Edinburgh, Great Britain. 570 p.