Effect of phenolic and other compounds on growth of *Poria weirii in vitro*

C. Y. Li, and *K. C. Lu, *E. E. Nelson, *W. B. Bollen and *J. M. Trappe

Department of Microbiology, Oregon State University, Corvallis, Oregon, U.S.A., and *Forestry Sciences Laboratory, Pacific Northwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture, Corvallis, Oregon 97330, U.S.A.

Abstract

Twenty-five compounds, widely occurring in nature, were tested individually *in vitro* at 0·5 and 2·0 mM concentrations for effects on two isolates of *Poria weirii* Murr., a serious root pathogen of conifers in western North America. Growth of both isolates was strongly inhibited in media containing coumarin, 4-hydroxycoumarin, or 7-hydroxycoumarin at either concentration, but o-catechol, salicylic acid, benzoic acid, ferulic acid, o-coumaric acid, and phenylacetic acid were inhibitory only at the higher concentration. The remaining compounds either inhibited only one isolate, had no effect, or stimulated growth of the fungus. Presence of significant amounts of the inhibitory compounds in host tissue could account for resistance to *P. weirii* by plants such as *Alnus rubra* Bong.

Introduction

Growing evidence suggests that the presence of red alder (*Alnus rubra* Bong.) in mixture with conifers changes soil chemical and microbial properties to the detriment of *Poria weirii* Murr., a major root pathogen of conifers in western North America (Nelson, 1968; Lu *et al.*, 1968). The present or readily foreseeable lack of pesticides for controlling *P. weirii*, coupled with the urgent need to prevent further environmental pollution by chemicals (Tarrant, 1967), dictates that methods for biological control be intensively pursued. The study reported here is part of a broad investigation on the potential of red alder in biological control of *P. weirii*.

Phenolic compounds and their oxidation products are widely associated with resistance of plants to disease (see reviews by Farkas and Kiraly, 1962; Kuć, 1963). Red alder strongly resists infection by *P. weirii* (Wallis and Reynolds, 1965); the rapid reddening or browning of wounded alder tissue, its high concentration of phenoloxidases (Li *et al.*, 1968), and the inactivation of its resistant properties by heating (Wallis and Reynolds, 1962) all indicate a high phenolic content.

Identification of the specific *Poria*-inhibiting compounds in resistant plants such as red alder would be an important advance toward control of the disease. Not only would the knowledge serve as a basis for defining resistance mechanisms, but also it could reveal some reasons why longevity of *P. weirii* is reduced in soils influenced by plants such as red alder. Accordingly, the experiments described here were initiated as an early step toward these ends.

Little is known about the specific phenolic compounds in *Alnus* tissue. Hörhammer and Scherm (1955) detected caffeic and protocatechuic acids in extracts from unspecified tissues of red alder as well as five other alders. Their tests for chlorogenic and gallic acids were negative for red alder but positive for some of the other species. Leaf extracts of *Alnus cordifolia* Ten. yielded

p-hydroxybenzoic, gentisic, and p-coumaric acids (Tomaszewski, 1960), and those of *A. incana* (L.) Moench had p-coumaric, caffeic and gallic acids (Bate-Smith, 1962). These compounds were included in our experiments with *P. weirii* as reported below. Additional compounds found in various alders but not yet tested by us include quercetin, cyanidin, kaempferol, ellagic acid, myricetin, taraxerol and terzeron (Bate-Smith, 1962; Hegnauer, 1964).

Materials and methods

Twenty-five compounds (Table 1) were tested for ability to inhibit growth of two isolates of *Poria weirii*, T55 and T124. Most had been tested with two *Phytophthora* species by Christie (1965) and were selected to permit comparison with his results.

Trione's (1964, Table 1) synthetic medium was used, modified by incorporating 10 gm glucose instead of 20 gm and by not adjusting the postautoclaving pH of 5·5. The respective compounds were dissolved in 50% ethanol which was then adjusted to pH 5·5 with NaOH.

Sixteen 250 ml Erlenmeyer flasks, each containing 50 ml of basal medium, were used in testing each compound. A 0·5 mM concentration of the compound was attained in eight flasks by adding 0·25 ml of its ethanolic solution; in the other eight, 1·0 ml of solution was added for a 2·0 mM concentration. For controls, 0·25 or 1·0 ml of plain 50% ethanol was added, respectively, to each of eight flasks of medium. Plugs of inoculum 4 mm in diameter, taken from the edges of 14-day-old colonies of a given isolate of *P. weirii* grown on malt agar plates, were inoculated into four flasks of each compound at each concentration, as well as into controls. Flasks were then incubated at 20°C for 22 days for isolate T124 and 29 days for the slower growing T55. After harvest, mycelia were weighed after drying 48 hr at 85°C. Final pH of the medium (Table 1) was determined for each flask.

Three replications of each treatment were planned, the fourth flask being a standby in case of contamination; if no contamination occurred within a treatment, the extra flask was randomly excluded from statistical analysis.

Data were subjected to analysis of variance with application of the Scheffé test for differences between means at the 95% confidence level (Snedecor and Cochran, 1967). As noted in the Results, certain treatment means were excluded from the analysis. Mean weights for all treatments are shown in Table 1.

Results

Interpretation of the effects of the compounds on growth of *P. weirii* must be tempered by the possibility of their transformation in reaction with other components of the medium or enzyme activity of the fungus. For convenience in presenting results, however, we will refer to the named compounds as originally added. Addition of 1 ml ethanol to the controls for the 2·0 mM concentration produced apparent, though not statistically significant, stimulation of both isolates (Table 1). Since this effect could influence fungal growth in the higher concentration of tested compounds, mean mycelial weights are statistically compared

Table 1 Mean mycelial weights of two isolates of *Poria weirii* (T55 and T124) ** grown on media to which phenolic and other compounds have been added at two concentrations

Compound	Mean weight in mg phenolic concentration 0·5 mM T55	T124	2·0 mM T55	T125	Mean final pH of medium phenolic concentration 0·5 mM T55	T124	2·0 mM T55	T124
Control	165	155	264	215	5·5	6·0	3·9	4·1
Catechols								
o-catechol	177	137	*7*	*14*	6·1	5·4	5·8	5·8
hydroquinone	132	149	216	*104*	5·4	4·8	4·0	4·1
phloroglucinol	92	128	*130*	224	6·0	5·2	4·8	3·7
Benzoic acids								
benzoic acid	227	144	*0*	*0*	6·2	4·6	5·4	5·4
salicylic acid	142	141	*0*	*0*	5·2	4·1	5·4	5·4
p-hydroxybenzoic acid	251	169	338	193	6·0	4·2	3·4	4·0
protocatechuic acid	199	177	334	302*	6·1	5·0	4·2	3·9
gentisic acid	108	167	*136*	271	6·1	4·5	6·5	4·5
gallic acid	150	182	217	253	5·8	4·5	6·3	4·3
vanillic acid	120	141	161	*30*	6·4	4·6	4·6	4·5
syringic acid	95	119	*113*	245	6·1	4·0	6·2	3·9
Cinnamic acids								
o-coumaric acid	188	140	*4*	*20*	6·0	5·8	4·3	4·6
p-coumaric acid	175	220	265	196	6·4	4·7	5·0	4·4
caffeic acid	110	158	*144*	231	6·3	4·7	6·5	4·3
ferulic acid	108	136	*0*	*0*	6·2	4·5	5·4	5·4
Phenylacetic acids								
phenylacetic acid	92	132	*0*	*0*	6·2	5·0	5·4	5·4
o-hydroxyphenylacetic acid	131	160	193	166	6·5	5·5	5·0	3·8
p-hydroxyphenylacetic acid	182	146	272	235	6·2	4·6	6·0	4·0
3,4-dihydroxyphenylacetic acid	106	131	*77*	205	6·3	5·4	4·2	3·9
Coumarins								
coumarin	*22*	*48*	*6*	*14*	5·0	4·6	5·5	5·2
4-hydroxycoumarin	95	*34*	*8*	*0*	4·4	4·4	4·4	5·4
7-hydroxycoumarin	*52*	*63*	*9*	*12*	5·4	4·1	5·7	5·6
Others								
chlorogenic acid	113	170	*132*	223	6·7	4·1	6·3	4·2
D-catechin	104	198	*148*	327*	6·1	5·8	5·5	3·6
phloridizin	171	154	224	220	5·8	4·9	6·8	3·7

** Incubated at 20 °C for 29 days and 22 days, respectively.
* Significantly higher than control for same column at the 95% confidence level.
Italicized means are significantly lower than the control for the same column at the 95% confidence level or in case of unequal variance, inhibition was obvious.
In the control, equivalent amounts of 50% ethanol added as for ethanolic solutions of test compounds.

between the control and compounds at a given concentration but no between concentrations of a given compound.

At the 2·0 mM level, both isolates of *P. weirii* were strongly inhibited by nine compounds: o-catechol, benzoic acid, salicylic acid, o-coumaric acid, ferulic acid, phenylacetic acid, coumarin, 4-hydroxycoumarin and 7-hydroxycoumarin. The mean mycelial weights were so exceptionally low in these treatments that they were excluded from the statistical analysis to avoid violating the assumption of equal variances. For convenience of presentation in Table 1, these means are shown as significantly lower than controls in the same way as means that were submitted to statistical analysis.

None of the other compounds significantly affected both isolates of *P. weirii* at the 2·0 mM concentration. Vanillic acid significantly inhibited T124 and appeared to depress growth of T55. D-catechin inhibited T55 but stimulated T124, both at a significant level. Gentisic acid produced a similar response, but only the inhibition of T55 was statistically significant. Protocatechuic acid significantly stimulated T124 and caused a similar but not significant trend for T55.

The only significant effect at the 0·5 mM level was a rather consistent suppression of both isolates by the three coumarins, which were also strongly inhibitory at the 2·0 mM level.

No consistent relationships were apparent between growth of fungi and final pH of the medium. During the course of the experiment, in most treatments pH of the medium was lowered with isolate T124. With T55, however, final pH of many treatments was substantially higher than at the beginning.

Discussion

Our results generally parallel those of Christie (1965) for *Phytophthora cactorum* (Leb. and Cohn) Schroet. and *P. parasitica* Dast. The two studies diverge in that Christie found strong inhibition of both *Phytophthora* species by p-hydroxybenzoic acid, p-coumaric acid and o-hydroxyphenylacetic acid, all of which were noneffective or stimulatory to *P. weirii*.

Structural formulae of all compounds tested except the paradoxical D-catechin are grouped in Figure 1 by their relative effect on *P. weirii*. Although this grouping is obviously an oversimplification, it does facilitate detection of possible relationships between structure and effect of compounds. Clearly, the double-ring coumarins stand out as the strongest inhibitors. Within the catechols, benzoic acids and cinnamic acids, some features are generally associated with increased inhibition: (1) lack of a hydroxyl (-OH) group, or (2) only two positions on the benzene ring occupied, and these in the ortho position, or (3) addition of methoxyl (H_3CO-) groups. The phenylacetic acids conformed to the first of these generalities, not to the second, and had no methoxyl groups to test the third. In general, these observations support the conclusions of Christie (*op. cit.*) about the relationship of structure to inhibition of *Phytophthora* species, but too little is yet known on this topic to permit broad conclusions.

Of the compounds noted earlier in this paper as present in alder species, only caffeic acid, chlorogenic acid and gentisic acid proved inhibitory to *P. weirii*

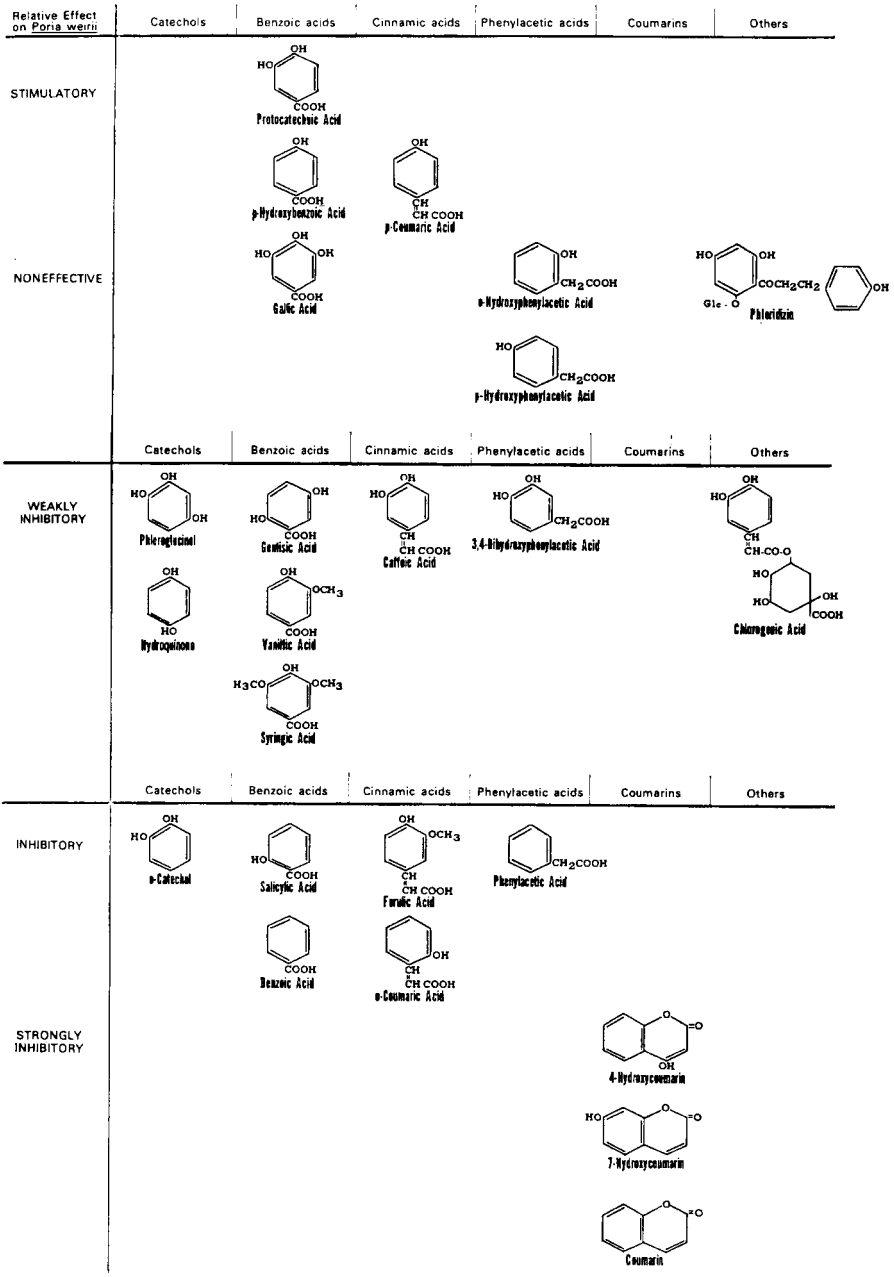

Figure 1 Structural formulae of 24 compounds, grouped by their relative effect on growth of two isolates of *Poria weirii in vitro*.
Stimulatory = significantly stimulating at least one isolate.
Noneffective = no significant effect.
Weakly inhibitory = significantly inhibiting one isolate at 2·0 mM concentration.
Inhibitory = significantly inhibiting both isolates at 2·0 mM concentration.
Strongly inhibitory = significantly inhibiting both isolates at 2·0 mM and one or both isolates at 0·5 mM concentrations.

in our experiment, and then only to one of the two isolates at the ·o mM concentration. Indeed, protocatechuic acid, p-hydroxybenzoic ac d, p-coumaric acid and gallic acid were noneffective or stimulatory. Judge from results of our tests, none of these compounds in themselves seem im ortant in resistance of red alder to *P. weirii* in terms of direct toxicity or oxida ion to toxic compounds as catalyzed by fungus-secreted enzymes. They can ot be discounted as resistance factors, however, because red alder itself may p oduce the enzymes that catalyze their oxidation into fungitoxins. Multiplica on of fungitoxic effects through synergisms between two or more compounds s also possible.

The *Poria*-susceptible conifers, *Pseudotsuga menziesii* (Mirb.) Franc and *Tsuga heterophylla* (Raf.) Sarg., have been reported to contain phlorogl cinol, D-catechin, and gallic, vanillic, caffeic, protocatechuic and ferulic acids (Hergert, 1960; Goldschmid and Hergert, 1961). Of these, only feruli acid markedly inhibited *P. weirii* in our experiments. If *Poria*-resistant individ als of these species are discovered, the presence and concentration of ferulic a id in their roots relative to that in susceptible individuals merit close examinat n in defining the resistance mechanism.

Acknowledgement

This investigation was supported in part by National Science Foundation Grant GB-61 .

[Reprints — purchased by the Forest Service, United States Department of Agricult , for official use.]

References

Bate-Smith E. C. 1962. The phenolic constituents of plants and their taxonomic s gnificance. 1 Dicoty ions. *J. Linn. Soc. London* **58** 95–173.

Christie T. 1965. The effects of some phenolic compounds upon the growth of two species of *Phytop hora*. *New Zeal. J. Agr. Res.* **8** 630–5.

Farkas G. L. and **Kiraly Z.** 1962. Role of phenolic compounds in the physiology of plant diseases and ease resistance. *Phytopathol. Ztschr.* **44** 105–50.

Goldschmid O. and **Hergert H. L.** 1961. Examination of western hemlock for lignin precursors. *Ta , ·* **44** 858–70.

Hegnauer R. 1964. *Chemotaxonomie der Pflanzen, Band 3: Dicotyledoneae: Acanthaceae Cyrillaceae*. 7- pp. Birkhäuser Verlag, Basel and Stuttgart.

Hergert H. L. 1960. Chem cal composition of tannins and polyphenols from conifer wood and bark. *Fores rod. J.* **10** 610–17.

Hörhammer L. and **Scherm A.** 1955. Über das Vorkommen zyklischer Pflanzensäuren bei einigen Polygon een und Betulaceen. *Arch. Pharm.* **288** 441–7.

Kuć J. 1963. The role of phenolic compounds in disease resistance. *Conn. Agr. Exp. Sta. Bull.* **663** 20 30.

Li C. Y., Lu K. C., Trappe J. M. and **Bollen W. B.** 1968. Enzyme systems of red alder and Douglas in relation to infection by *Poria weirii*. *In* Biology of alder. Edited by J. M. Trappe, J. F. Franklin, R. F. Tarran and G. M. Hansen. Northwest Sci. Assoc. Fortieth Ann. Meeting, Symp. Proc. 1967. pp 241–50.

Lu K. C., Chen C. S. and **Bollen W. B.** 1968. Comparison of microbial populations between red alde and conifer soils. *In* Biology of alder. Edited by J. M. Trappe, J. F. Franklin, R. F. Tarrant and G. M. Hansen. North est Sci. Assoc. Fortieth Ann. Meeting, Symp. Proc. 1967. pp. 173 8.

Nelson E. E. 1968. Survival of *Poria weirii* in conifer, alder and mixed conifer-alder stands. Pac. NW. For t & Range Exp. Sta. U.S.D.A. Forest Serv. Res. Note PNW-83, 5 pp.

Snedecor G. W. and **Cochran W. G.** 1967. *Statistical methods*. 592 pp. Iowa State Univ. Press, Ames, Io va, U.S.A.

Tarrant R. F. 1967. Pesticides in forest waters — symptoms of a growing problem. *Soc. Amer. Forest. Proc.* 1966, 19[illegible]63.

Tomaszewski M. 1960. The occurrence of p-hydroxybenzoic acid and some other simple phenols in vascular plants. *Bull. Acad. Polon. Sci. Cl. 2* **8** 61–5.

Trione E. J. 1964. Isolation and in vitro culture of the wheat bunt fungi *Tilletia caries* and *T. controversa*. *Phytopathology* **54** 592–6.

Wallis G. W. and **Reynolds G.** 1962. Inoculation of Douglas fir roots with *Poria weirii*. *Can. J. Bot.* **40** 637–45.

Wallis G. W. and **Reynolds G.** 1965. The initiation and spread of *Poria weirii* root rot of Douglas fir. *Can. J. Bot.* **43** 1[illegible]9.

Accepted 21 July 1969