EFFECT OF HOT-WATER SOLUBLE THUJA PLICATA HEARTWOOD
EXTRACTIVES ON THE GROWTH OF THUJA AND NON-THUJA
ISOLATES OF PORIA WEIRII

by

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Abstract

Poria weirii Murr. isolates cultured from various hosts may be divided into two groups on the basis of their ability to parasitize Thuja plicata Donn and to grow on media containing water-soluble T. plicata heartwood extractives. The role of these extractives in determining whether a Poria weirii isolate can parasitize Thuja plicata was examined. Measurement of linear growth along wood micro-sections confirmed that substances inhibitory to one isolate group are hot-water soluble T. plicata heartwood extractives. The tolerance of isolates from T. plicata (Thuja) and Pseudotsuga menziesii (Mirb.) Franco (non-Thuja) to a hot-water extract of Thuja plicata heartwood and its hexane soluble (principally thujaplicins) and insoluble (thujaplicin-free) fractions was studied in detail. Dry weight of mycelium produced in liquid glucose-asparagine medium was measured. Thuja isolates were more tolerant than non-Thuja of the hot-water extract and its fractions; thujaplicins caused the greatest response differences. Thuja isolates showed greater stimulation than non-Thuja at subinhibitory thujaplicin concentrations. Higher thujaplicin concentrations were required to produce fungistatic and -toxic conditions in Thuja isolates than in non-Thuja. There were highly significant differences between isolate groups in response to 7.5 ppm thujaplicin. Tolerance was not related to date of isolation or geographical location.

With sufficient inoculum potential, a non-Thuja isolate may overcome T. plicata heartwood toxicity. The resulting infection is usually localized. Unlike Thuja isolates, non-Thuja were unable to

increase thujaplicin tolerance during three serial generations on liquid medium containing thujaplicins. Long exposure of non-Thuja isolates to T. plicata heartwood under field conditions did not increase their thujaplicin tolerance. Thuja isolates may be able to adapt the enzyme systems blocked by thujaplicin or to detoxify thujaplicins.

These results have significance for control of Poria weirii. Planting Thuja plicata in and around infection centers caused by non-Thuja isolates could limit their size by reducing the number of root contacts between susceptible species.

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Introduction

The existence of host specific strains of Poria weirii Murr. has been postulated (30,11,13). One strain attacks a number of coniferous species including Thuja plicata Donn, while a second strain attacks the same species except T. plicata.

Limited research has been directed toward clarifying the taxonomy of Poria weirii. Questions remain, such as whether strains comprise a single species or two closely related species. The lack of major differences among cultural and morphological characteristics suggests a single species, whereas significant differences in ability to decay host heartwood, and physiological characteristics, suggest two related species. The small amount of work done on compatibility between strains suggests two species.

This study examines the role of hot-water soluble Thuja plicata heartwood extractives in determining host specificity of Poria weirii isolates. It also investigates the ability of non-Thuja isolates to increase mycelial growth rate during serial transfers to liquid medium containing thujaplicins.

Literature

Poria weirii - Hosts and Biology

Poria weirii was described in 1914 by Murrill (31) from sporophores collected on Thuja plicata in Idaho and Washington. It was reported on Pseudotsuga menziesii (Mirb.) Franco in 1940 (30) and subsequently on Tsuga heterophylla (Raf.) Sarg. (30), Picea sitchensis (Bong.) Carr., Pinus ponderosa Laws., Abies grandis (Dougl.) Lind., Abies amabilis (Dougl.) Forbes, Abies lasiocarpa (Hook.) Nutt. (9), Larix occidentalis Nutt., Pinus monticola Dougl. (45) and Pinus contorta Dougl. (13). Poria weirii is found throughout the north-western United States and southern British Columbia (45). The fungus, described as Fuscoporia weirii (Murr.) Aoshima, has been found on Honshu and Hokkaido Islands, Japan, where it occurs on Tsuga diversifolia (Maxim.) Mast., Picea jezoensis (Sieb. and Zucc.) Carr. and Abies sp. (6).

Based on growth habits, Garrett (23) divided root disease fungi into two categories where: soil-inhabiting fungi are characterized by their ability to survive indefinitely as soil saprophytes, and root-inhabiting fungi are characterized by an expanding parasitic phase on the living host plant and by a declining saprophytic phase after death of the host. Poria weirii typifies root-inhabiting fungi as defined by Garrett. Although viable mycelium can be found in old-growth stumps for at least 50 years after the trees have been cut (12), it is gradually replaced by saprophytic fungi (47,12). It is prevented from spreading through unsterilized soil, probably by antagonistic soil organisms (11). The root and/or butt decay caused by P. weirii is most serious in second-growth stands where infection centers may be an acre or more in size.

Initiation of an infection center occurs when roots of the present stand contact viable mycelium in stumps of the previous stand. Spread from tree to tree occurs where a diseased and healthy root come in contact (47). The ability of basidiospores to initiate infection centers has not been determined.

Poria weirii - Strain Differences

Mounce et al first recognized that there could be host-specific strains of P. weirii (30). Recently, examinations were made of cultural and physiological characteristics (11,13), decay-type (10,12), sporophore-form on hosts (11) and ability to infect host wood (13,44). Differences between isolates from various hosts have suggested classification of P. weirii isolates as Thuja or non-Thuja.

Clark (13) introduced terminology to describe the source and characteristics of Poria weirii isolates. Thuja isolates are obtained from Thuja plicata; Thuja-type isolates are obtained from hosts other than T. plicata but which have the characteristics of Thuja isolates; and non-Thuja isolates are obtained from hosts other than T. plicata which differ in appearance from both Thuja and Thuja-type isolates. In this study, only Thuja and non-Thuja isolates are considered.

On malt extract agar, the mycelium of Thuja isolates is consistently darker brown than that of non-Thuja isolates (30,11,13). Comparison of Poria weirii cultures from Pseudotsuga menziesii and

Thuja plicata showed similarities in growth-rate, margin character, mat appearance, reaction on 0.5 per cent gallic and tannic acid media and microscopic characters (30). However, Buckland et al (11) observed that the margin of the advancing zone of 75 Pseudotsuga menziesii isolates was raised and the mat felty with white aerial hyphae, whereas the margin of 35 Thuja plicata isolates was appressed and the mat cottony with yellow to tan aerial hyphae. In 2- to 4-week old cultures of T. plicata isolates, hyphae on slant surfaces adjacent to test tube walls formed columnar tufts (13).

When two isolates from the same host (Pseudotsuga menziesii or Thuja plicata) were grown in the same culture dish, they showed no signs of antagonism. However, when two isolates from different hosts were grown together, they developed an antagonistic zone line (11).

Clark (13), investigating physiological strains of Poria weirii, examined variation among a large number of isolates from North American hosts under the following headings: oxidase reaction and growth on gallic and tannic acid media; temperature relations; tolerance to host wood extractives; relations of pH; and capacity to survive in sealed cultures.

On 0.5 per cent gallic and tannic acid media, Thuja isolates differed generally from non-Thuja isolates in showing an earlier oxidation discoloration of inoculum plugs and adjacent gallic acid medium, delayed initiation of growth from the inoculum and better mycelial growth (13).

There were slight but consistent and statistically significant average temperature-growth differences for the two groups with respect to both minimum and maximum temperatures permitting growth. Variability of individuals made this difference discernable only with a large number of isolates (13).

There were consistent differences between Thuja and non-Thuja isolates in response to extractives diffused into malt agar from T. plicata heartwood blocks. T. plicata extractives only retarded Thuja isolate growth, but completely inhibited that of non-Thuja isolates. In contrast, extractives diffused into malt agar from Pseudotsuga menziesii, Pinus monticola, Larix occidentalis, Tsuga heterophylla and Abies grandis heartwood blocks generally stimulated growth of non-Thuja isolates, but caused less stimulation or actually inhibited Thuja isolate growth (13).

Usually there was a marked difference between isolate groups in their capacity to survive in sealed tubes. Non-Thuja isolates remained viable up to two and one-half years while most Thuja isolates were dead after two months (13).

Infection by Poria weirii occurs almost exclusively through host roots. Post-infection development of the fungus differs according to the host. In Thuja plicata, heartwood is decayed, frequently for considerable distances up the bole, but trees are seldom killed (10,12). In other hosts roots are invaded and killed, the tree dies as a result of windthrow or destruction of its root system, after which extensive decay of the bole occurs.

Sporophores collected from T. plicata and Pseudotsuga menziesii were identical in their microscopic characters, including size and shape of basidia, basidiospores, hyphae and setae (30). Macroscopic comparison of sporophores from the same hosts yielded the following distinctions. Fruit-bodies collected from P. menziesii were Sudan brown with tones of cinnamon, pink and purple; frequently had a white sterile margin and a rough or coarse surface; and were annual, seldom with a second layer of tubes. In contrast, sporophores obtained from Thuja plicata were more uniformly coloured, being Sudan brown with yellow to tawny tones; had a smooth finely textured surface; and were perennial, commonly forming tube layers for several years (11). The influence of the host in determining sporophore-form is not known.

Weight losses denoting decay were produced in T. plicata heartwood blocks only by Thuja isolates. Thuja isolates also caused considerably more decay in Pseudotsuga menziesii heartwood blocks than non-Thuja isolates (13). Inoculation of Thuja plicata and Pseudotsuga menziesii roots with Thuja and non-Thuja isolates has shown that the former are capable of infecting roots of both hosts and the latter are able to infect P. menziesii roots but not those of Thuja plicata (44).

Thuja plicata Heartwood Extractives and Their Effects on Fungi

Cellulose, hemicellulose and lignin are wood cell wall components and occur in approximately the same proportions in all coniferous woods. Heartwood extractives, the non-structural

components of wood, show considerable qualitative and quantitative variation among coniferous species (16). Extractives, including tannins, dyes, pitch, resins, gums and many other classes of compounds, are removed from wood by inert solvents without impairing wood structure or strength and are responsible for odor, colour, durability and other distinguishing wood properties.

A characteristic of Thuja plicata is its unusually high water-soluble extractive content. In one set of determinations, all T. plicata extractives constituted 10.2 per cent of the wood on a moisture-free basis, whereas total extractive percentages for Tsuga heterophylla and Pseudotsuga menziesii were only 5.3 and 5.9 respectively (19).

Early chemical workers studying Thuja plicata extractives, set down empirical formulae for several related steam-volatile compounds (8,41). Investigations begun in 1948 identified three tropolone isomers which were named α -, β -, and γ -thujaplicin (2,17). A fourth tropolone studied earlier (41) was renamed thujic acid (18). Up to 0.5 per cent pectic substances (5) and 0.27 per cent L-arabinose (3) have been isolated from T. plicata heartwood extracts.

An intensive study of T. plicata heartwood extractives was carried out by Gardner, MacLean and Barton. In addition to identifying several components (20,21,22) they determined percentages of many extractive compounds (20) and intra-tree distribution of thujaplicins and water-soluble phenols (27). Figure 1 summarizes information from these papers that is pertinent to the present study.

Fig. 1. Thuja plicata hot-water soluble heartwood extractives (19)

It should be noted that in addition to water soluble phenols, L-arabinose and pectic substances, the non-volatile fraction contains starch, hemicelluloses and protein. Furthermore, in view of the marked acidity of aqueous extracts (pH 3-4), considerable hydrolytic degradation of the polysaccharide material might occur (19). Table I shows the composition of the steam-volatile oil from T. plicata butt heartwood.

Table I. Composition of steam volatile oil of western red cedar butt heartwood (20)

Component	Percentage of Oil	Percentage of Wood
Methyl Thujate, other Neutrals	21.1	0.17
Thujic Acid	10.4	0.08
Tropolones	68.5	0.56
β -Thujaplicinol	8.0	0.07
γ -Thujaplicin	24.0	0.20
β -Thujaplicin	35.0	0.30
α -Thujaplicin	1.0	0.01
β -Dolabrin	0.01	0.0003

High decay resistance of some T. plicata heartwood is indicated by the lengthy service-life of products made from it. Water-soluble extractives contained in the heartwood are responsible for much of this durability. Cold or hot-water extracted heartwood exposed to decay fungi lost two to three times as much weight as unextracted heartwood (43). Hot-water extracts of heartwood in malt agar were toxic to Lentinus lepideus Fr. (43). A similar extract was fungistatic and fungitoxic at higher concentrations to L. lepideus, Trametes serialis Fr., Fomes roseus (Alb. and Schw. ex Fr.) Cke., Polyporus schweinitzii Fr., Fomes pinicola (Sw.) Cke., Coniophora puteana (Schum. ex Fr.) Karst., Fomes officinalis (Vill. ex Fr.) Faull., Poria xantha (Fr.) Cke., and Polyporus sulphureus Bull. ex Fr. (42).

Data on the effects of several wood tropolones and water-soluble phenols on decay fungus growth on malt agar medium and/or ability to cause weight loss in wood are summarized in Table II (4,35,36,37,38). The water-soluble phenols inhibited Poria monticola

Table II. Selected literature on effects of several Thuja plicata tropolones and water soluble phenols on decay fungus growth on malt agar and/or ability to cause weight loss in wood

Substrate	Extractive Compound	Fungus ¹	Fungistatic Concentration (ppm)	Fungitoxic Concentration (ppm)	Ref.
Malt Agar	Water Soluble Phenols	<u>Poria monticola</u> ²	20,000	-	36
	γ -thujaplicin	<u>P. monticola</u> ²	40	60	37
		<u>Coniophora puteana</u> ²	20	40	37
		<u>Poria weirii</u> ²	-	10	35
		<u>Fomes pinicola</u> ²	80	100	37
		<u>Lentinus lepideus</u> ²	10	20	35
		<u>Polyporus versicolor</u>	-	20	35
		<u>Stereum sanguinolentum</u>	120	160	37
		<u>Stereum sanguinolentum</u>	80	120	37
	β -thujaplicinol	<u>P. monticola</u> ²	20	40	37
		<u>C. puteana</u> ²	20	20	37
		<u>P. versicolor</u>	1500	-	37
		<u>S. sanguinolentum</u>	600	1250	37
		<u>P. weirii</u>	200	400	37
			Extractive Concentration (per cent w/w)	Weight Loss (per cent of control)	
Wood	β -thujaplicin	<u>L. lepideus</u> ²	1.1-1.2	0	4
			1.0	29	38
	γ -thujaplicin		1.1-1.2	3	4
			1.0	9	38
	β -thujaplicinol		1.1-1.2	0	4
			1.0	0	38

1 - hosts unknown

2 - brown-rot

Murr. growth but were not fungitoxic. In malt agar, brown rots were generally more sensitive to thujaplicins than white rots and considerable variation in tolerance to thujaplicins was apparent between isolates of the same species. Similar variation in tolerance was evident with wood substrate. The toxicity of individual thujaplicin isomers to a given fungus appears to vary.

Toxins and Fungi: Effects and Adaptive Tolerance

A given concentration of toxin will stimulate, inhibit or stop irreversibly fungal growth. A concentration causing inhibition that may be reversed by transferring the fungus to toxin-free substrate is fungistatic. One causing irreversible inhibition is fungitoxic.

Stimulation of wood-destroying fungi by subinhibitory concentrations of zinc (24), copper (24), mercury (39), arsenic trioxide (24), sodium fluoride (25,39), phenol (25), pentachlorophenol (25), creosote (25) and other items has been reported. Growth or decay rate stimulation by some of these compounds could result from the addition of required amounts of trace elements. Lyr (25), however, observed stimulation of several fungi when the possibility of a trace element effect was excluded.

In malt agar, low concentrations of a hot-water extract of Thuja plicata heartwood stimulated growth of wood-destroying fungi (42). This accelerated growth rate tended to decrease with time and approach the control rate.

The literature on adaptation of fungi (mostly Ascomycetes) to metal toxicants was reviewed by Ashida (7). There are also reports of adaptation to organic fungicides (15,14,42).

The methods used and observations made during a typical adaptation study were outlined by Partridge and Rich (33). In a series of transfers, Penicillium notatum Westling, Sclerotium fruticola (Wint.) Rehm. and Stemphyllium sarcinaeforme (Cav.) Wilts. were grown on potato dextrose agar containing different concentrations of several fungicides. Adaptations were indicated in three ways: 1) the fungi usually tolerated increased concentrations of a fungicide when grown in its presence for a number of serial transfers; 2) the growth rates approached those of the controls as the fungi were successively transferred on fungicide agar; and 3) sporulation tended to approach that of the controls after successive transfers on fungicide agar.

After continued association with a fungistatic concentration of Thuja plicata heartwood extract, a number of wood-destroying Basidiomycetes became able to tolerate it, with a consequent increase in growth rate (42). Furthermore, when the extract concentration was gradually increased, each fungus became able to grow on these higher concentrations. The extractive concentration causing fungistasis increased by 1.3 to 3.5 times during adaptation (42).

In summary, except for sporophore-form, morphological and cultural differences between Poria weirii isolates from Pseudotsuga menziesii and Thuja plicata are slight or non-discernable. Major differences between Thuja and non-Thuja isolates of the fungus are

found among physiological characteristics, principally reaction on gallic or tannic acid media, ability to decay T. plicata heartwood and response to water-soluble T. plicata heartwood extractives.

Hot-water soluble extractives are known to be responsible for much of the decay resistance of T. plicata heartwood. Two components of these extractives, γ -thujaplicin and B-thujaplicinol, are fungistatic or fungitoxic to Poria weirii.

Experimental Methods and Results

Throughout most of this study four Poria weirii isolates were used. The numbers, hosts, locations and collection dates are given in Table III.

Table III. Collection data for Thuja (T) and non-Thuja (NT) Poria weirii isolates studied

Culture No.	Host	Location	Collection Date
6-2 (T)	<u>Thuja plicata</u>	Revelstoke	August 1962
11-2 (T)	<u>Thuja plicata</u>	Enderby	August 1962
26-4 (NT)	<u>Pseudotsuga menziesii</u>	Cowichan Lake	September 1962
27-3 (NT)	<u>Pseudotsuga menziesii</u>	Cowichan Lake	September 1962

Since their isolation, the cultures have been maintained on malt extract agar at the Forest Research Laboratory, Victoria, B. C. Cultures 6-2 and 11-2 are characteristic of Thuja isolates and 26-4 and 27-3 of non-Thuja isolates.

Growth of Isolates on Wood

Linear growth of Thuja and non-Thuja isolates was measured on sapwood and heartwood micro-sections of Thuja plicata and Pseudotsuga menziesii. A bolt from the lower bole of a 32 year-old P. menziesii was cut on the University of British Columbia endowment lands. Thuja plicata wood for this and subsequent experiments was taken from a

73 year-old tree near Cowichan Lake, B. C. Preparation of micro-sections has been described elsewhere (29). Specimens were not sterilized. One end of a micro-section 1.5 cm by 8 cm by 150 was placed in contact with inoculated wood meal impregnated with one per cent malt extract and one per cent agar contained in 25 mm by 200 mm test tubes. The incubation temperature was 20 C. Extent of growth along micro-sections was observed with a dissecting microscope. Growth measurements after three weeks are shown in Table IV; values are the mean of two replicates.

Table IV. Linear growth of Thuja (T) and non-Thuja (NT) Poria weirii isolates on sapwood and heartwood of Thuja plicata and Pseudotsuga menziesii

Isolate	Growth in Millimeters			
	<u>P. menziesii</u>		<u>T. plicata</u>	
	Sapwood	Heartwood	Sapwood	Heartwood
11-2 (T)	35	12	57	15
27-3 (NT)	23	14	22	1

Several observations may be made from these results. Growth on sapwood was greater than on heartwood for both wood species. Only slight growth of the non-Thuja isolate on Thuja plicata heartwood was observed, whereas the Thuja isolate gave good growth on matched micro-sections. Thuja isolate growth was better on T. plicata than on Pseudotsuga menziesii.

Hot-water extraction of Thuja plicata heartwood meal increased its decay susceptibility (43). To assess the effect of a similar treatment on Poria weirii growth, Thuja plicata heartwood micro-sections were soxhlet-extracted with hot distilled water for 10 hours and placed in contact with inoculum as described previously. Results after three weeks are shown in Table V. Each value is the mean of seven replications.

Table V. Linear growth of Thuja (T) and non-Thuja (NT) Poria weirii isolates on unextracted and extracted Thuja plicata heartwood

Isolate	Growth in Millimeters	
	Unextracted	Extracted
11-2 (T)	21	29
27-3 (NT)	2	12

As in the previous experiment, the non-Thuja isolate grew only slightly on T. plicata heartwood. Hot-water extraction enhanced linear growth, however the effect on the non-Thuja isolate was more pronounced. Although the inoculum potentials and growth rates on malt agar were equal, the Thuja isolate grew more vigorously than the non-Thuja isolate on extracted as well as control micro-sections (Fig. 2).

Fig. 2. Linear growth of Thuja (11-2) and non-Thuja (27-3) isolates on control and hot-water extracted Thuja plicata micro-sections

Growth of Isolates in Liquid Medium Containing Thuja plicata Heartwood Extractives

The compounds inhibitory to non-Thuja isolates occur in Thuja plicata heartwood and may be removed by hot-water extraction. In a series of experiments, mycelial growth of Thuja and non-Thuja isolates was measured at several concentrations of a hot-water extract of T. plicata heartwood and its hexane soluble and insoluble fractions.

Liquid medium with composition as shown in Table VI was used in these experiments. A brief study, described in the appendix, of the vitamin requirements and carbon and nitrogen nutrition of Poria weirii isolates indicated a thiamine requirement and a preference for L-asparagine. Glucose, although inferior to cellobiose or xylose as a carbon source, was more readily available than the latter compounds.

Table VI. Medium composition

Component	Quantity Per Liter
Glucose	10.0g
MgSO ₄ ·7H ₂ O	0.75g
KH ₂ PO ₄	1.75g
L-Asparagine	2.00g
Thiamine	100 g

Effects of the hot-water extract were examined first. Twenty-five ml of a solution containing the minerals and nitrogen source were placed in 250 ml erlenmeyer flasks fitted with cheese cloth covered cotton plugs. These flasks and others of carbon source were autoclaved at 15 pounds pressure for 20 minutes. The required amount of thiamine in 20 per cent ethanol was added to flasks of cool carbon source. Twenty-five ml of carbon source was added to each flask.

To obtain inoculum, Thuja isolates 6-2 and 11-2 and non-Thuja isolates 26-4 and 27-3 were grown on a medium containing two per cent malt extract and two per cent agar. A six mm diameter plug

taken from a colony margin was added to each flask. Three replicates of each isolate were used at extract concentrations of 0, 1, 2, 3, and 4 ml per flask.

A hot-water extract was prepared by refluxing 10.7g (oven-dry weight) of Thuja plicata heartwood meal for three successive two-hour periods. The three portions were combined and made to 500 ml. Three days after inoculation, the appropriate amount of extract was added to each flask.

Flasks were maintained at 20 to 22 C for 27 days. Contaminated flasks were discarded. Replicates of an isolate at each concentration were combined and mycelium was harvested by filtering on tared, 5.5 cm Whatman No. 1 filter papers. Filter papers were dried at 80 C for eight hours and weighed. To remove the effect of variation in isolate growth rate, results, shown in Figure 3, are expressed as the quotient of control mycelium weight and that of each treatment.

Growth of both Thuja isolates was stimulated by extract concentrations of 1, 2, and 3 ml per flask and only slightly inhibited by 4 ml per flask. Non-Thuja isolate behaviour differed significantly. At a concentration of 1 ml per flask, growth was slightly inhibited. Stimulation was observed at 2 ml, growth was again inhibited at 3 ml, and markedly so at 4 ml.

MacLean and Gardner (28) developed a method whereby the thujaplicins are extracted with hexane from a solution of hot-water extractibles. Average weight of hexane extractibles was determined from analysis of three 100 ml aliquots of the hot-water extract used

above. Calculation as parts per million (ppm) thujaplicin at each extract concentration using this value facilitated comparison of results from experiments in the series.

Fig. 3. Growth response of Thuja and non-Thuja isolates to a hot-water extract of Thuja plicata heartwood

In a second experiment, isolate response to a mixture of thujaplicin isomers was examined. The methods used were described in a previous section. Thujaplicins were obtained as follows (28). A hot-water extract of heartwood meal was extracted with hexane. The hexane extract was evaporated to dryness, leaving pale yellow needle-like crystals. Three days after inoculation, stock solution

containing 0.05 g thujaplicin in 200 ml sterile distilled water was added to flasks to give thujaplicin concentrations of 2.5, 5.0 and 10.0 ppm.

Fig. 4. Growth response of Thuja and non-Thuja isolates to a mixture of thujaplicin isomers from Thuja plicata heartwood

Results after incubation at 20 to 22 C for 28 days are shown in Figure 4. No growth stimulation was detected for any isolate. However, in a replication of this experiment, growth stimulation of both Thuja and one non-Thuja isolate occurred at 2.5 ppm thujaplicin; response of isolates to thujaplicin relative to each other was identical

at other concentrations. At no concentration did the ratio to control of a non-Thuja isolate exceed that of a Thuja isolate. Differences between isolate groups in mycelium production were considerable at 5.0 ppm. Strong inhibition occurred at 10 ppm, although that of Thuja isolates was less marked.

The series was completed by examining effects of a thujaplicin-free hot-water extract on isolate growth. The hot-water extract, prepared as before from 10.7 g of heartwood meal (oven-dry weight) was extracted with hexane. Amounts of extract added to flasks were identical to those added in the first experiment.

Fig. 5. Growth response of Thuja and non-Thuja isolates to a thujaplicin-free hot-water extract of Thuja plicata heartwood

Figure 5 shows results after 21 days incubation. Stimulation of Thuja isolates was more pronounced than that of non-Thuja isolates. The ratios to control of the latter group fluctuated around one, whereas those of the former increased to 1.5 to 2.5 at a concentration of 4 ml per flask.

Two aspects of the foregoing experiments were investigated further. Isolate response to thujaplicins was studied in detail to determine if growth stimulation occurs at low concentrations and to establish fungistatic and fungitoxic concentrations. Variation in tolerance to thujaplicins within and between the two isolate groups was also measured.

Following the same procedures as before, three replicates of each isolate were set up at 12 thujaplicin concentrations. For concentrations up to 10 ppm, mycelium was harvested on a per flask basis, instead of the per isolate basis used previously. This permitted statistical analysis of differences between isolate means.

Fig. 6. Detailed growth response of Thuja and non-Thuja isolates to thujaplicins

Results are presented graphically in Figure 6. Growth of one non-Thuja isolate (27-3) was stimulated by 0.25 and 0.50 ppm thujaplicin, but decreased rapidly when the concentration was increased. The other non-Thuja isolate (26-4) was not stimulated and mycelium production decreased gradually with increasing thujaplicin concentration. Response of Thuja isolates differed markedly. Both were stimulated, showed two stimulation peaks, and had a greater thujaplicin tolerance at higher concentrations than the non-Thuja isolates.

At two concentrations, 3.75 and 8.5 ppm, differences between isolate means were tested for significance (Table VII).

Table VII. Results of 't' tests on differences between isolate means at two thujaplicin concentrations

Thujaplicin Concentration	Isolate Means Compared	't' Value
3.75 ppm	6-2 x 11-2	0.33 ^{ns}
	6-2 x 26-4	1.86 ^{ns}
	11-2 x 26-4	1.91 ^{ns}
	6-2 x 27-3	6.77 ^{**}
	11-2 x 27-3	10.54 ^{**}
	26-4 x 27-3	2.91 [*]
8.5 ppm	6-2 x 11-2	2.66 ^{ns}
	6-2 x 26-4	4.75 ^{**}
	11-2 x 26-4	15.12 ^{**}
	6-2 x 27-3	3.50 [*]
	11-2 x 27-3	12.68 ^{**}
	26-4 x 27-3	0.10 ^{ns}

ns - not significant

* - significant at P.05

** - significant at P.01

The means of isolates 6-2, 11-2 and 26-4 were significantly different from that of 27-3 at 3.75 ppm. At 8.5 ppm, means of Thuja isolates 6-2 and 11-2 were significantly greater than those of non-Thuja isolates 26-4 and 27-3. Table VIII shows the fungistatic and fungitoxic concentrations.

Table VIII. Fungistatic and fungitoxic thujaplicin concentrations for isolates 6-2, 11-2, 26-4 and 27-3

Isolate	Thujaplicin Concentration (ppm)	
	Fungistatic	Fungitoxic
6-2 (T)	20.0	22.5
11-2 (T)	17.5	20.0
26-4 (NT)	12.5	15.0
27-3 (NT)	10.0	12.5

Within the Thuja and non-Thuja groups, some variation in isolate response to the hot-water extract and its components was noted. At thujaplicin concentrations above seven ppm, there were considerable differences in mycelium production between isolate groups.

By the use of ten Thuja and ten non-Thuja isolates, intra- and inter-group variation in thujaplicin tolerance was measured. Isolates were obtained from the culture collection at the Forest Research Laboratory, Victoria, B. C. Two treatments, control and 7.5 ppm thujaplicin, were replicated three times for each isolate. Following incubation for 25 days, mycelium was harvested by isolate for each treatment.

Table IX contains the numbers, hosts, locations, collection dates and ratios to control for the isolates tested.

Table IX. Collection data and ratios to control of isolates tested for tolerance to 7.5 ppm thujaplicin

Host and Isolate No.	Location (British Columbia)	Collection Date	Ratio to Control
<u>Pseudotsuga</u>			
<u>menziesii</u>			
Pwp 19-21	Bowser	1957	0.051
Pwp 7-3	Roseberry	1957	no growth
Pwp 7-21	Roseberry	1957	0.232
Pwp 13-2	Hope	1957	0.250
Pw 26-4	Cowichan	1962	0.547
Pw 27-3	Cowichan	1962	0.408
Pw 15-2	Enderby	1962	0.265
Pw 23-4	Sproat Lk.	1962	0.161
Pwm 1-3	Salmon Arm	1967	0.237
Pwm 6-1	Cowichan	1967	0.155
<u>Thuja plicata</u>			
Pwp 18-6	U. B. C.	1957	0.537
Pwp 15-4	Hope	1957	0.732
Pw 18-2	Falkland	1962	0.707
Pw 6-2	Revelstoke	1962	0.637
Pw 11-2	Enderby	1962	0.810
Pw 20-4	Fauquier	1962	1.166
Pw 21-13	Hope	1962	1.421
Pw 7-2	Revelstoke	1962	0.623
Pwm 2-4	Salmon Arm	1967	0.663
Pwm 7-1	Mabel Lk.	1967	0.857

Mean ratio to control of Thuja isolates was 0.815 and of non-Thuja isolates 0.256. These means were significantly different at $P_{.01}$. The most tolerant non-Thuja isolate, Pw 26-4, had a slightly higher ratio to control than the least tolerant Thuja isolate, Pwp 18-6. Standard deviations of 0.275 (Thuja) and 0.147 (non-Thuja) indicated considerable intra-group variation of thujaplicin tolerance. The

isolates tested were collected throughout the British Columbia range of Poria weirii and had been cultured for varying lengths of time on malt extract agar. There appeared to be no relationship between geographical location or collection date and thujaplicin tolerance.

Adaptive Tolerance to Thujaplicins

Thuja and non-Thuja isolates differ significantly in their tolerance of thujaplicins. To determine if tolerance is controlled by adaptive enzymes, growth of isolates 6-2, 11-2, 26-4 and 27-3 in liquid medium containing thujaplicins was measured during three serial generations.

For each isolate, ten flasks were inoculated with six mm diameter malt agar plugs. Three days after inoculation, stock thujaplicin solution was added to one-half the flasks to give a concentration of 5 ppm. The incubation period was 14 days. Mycelium in three control and three treated flasks was harvested and that in the remaining control and treated flasks was used as inoculum for the next generation. Second generation flasks were inoculated with one ml of blended mycelium; control mycelium was used to inoculate control flasks and thujaplicin exposed mycelium to inoculate treated flasks. Three days later five ppm thujaplicin was added to the latter flasks.

The same procedure was followed at the end of the second generation.

Results for three generations are graphed in Figure 7. As

Fig. 7. Ratios to control of Thuja and non-Thuja isolates for three serial generations on liquid medium containing thujaplicins

in previous experiments, isolate 27-3 showed marked sensitivity to ca five ppm thujaplicin and isolate 26-4 considerable tolerance. Gradual increase of ratio to control was observed for isolate 6-2 and variable stimulation occurred with 11-2. Generally, Thuja isolates were stimulated or increased their tolerance to five ppm thujaplicin during the three generations, whereas non-Thuja isolate tolerance decreased.

Field Examinations

Laboratory tests and field inoculations have shown that non-Thuja isolates of Poria weirii are incapable of decaying heartwood (12) or infecting living roots of Thuja plicata (44). However, T. plicata roots that had been in contact for an extended period with infected Pseudotsuga menziesii roots had Poria weirii mycelium on the bark surface and the wood of 2 of 123 roots had been invaded. Mycelial growth in the wood was very limited (47).

If P. weirii inhabiting Pseudotsuga menziesii roots is able to infect roots of Thuja plicata, are its cultural and physiological characteristics typical of a Thuja or non-Thuja isolate? To clarify this point, root systems of four living T. plicata trees growing adjacent to infected Pseudotsuga menziesii were excavated. Three trees were located in a large infection center near Cowichan Lake, B. C. and the fourth was near Enderby, B. C. Figure 8 shows one Cowichan excavation; points of contact between P. menziesii and Thuja plicata roots (numbers) and the inoculum sources and stump of the attacked T. plicata are marked.

Twelve of twenty-six contacts between roots of the two species resulted in infection of the contacting T. plicata root. Average extent of decay or stain was 12 cm and one root had been killed. In most instances the fungus had penetrated only slightly into the wood (Fig. 9 contacts 6 and 8).

Fig. 8. Root excavation of an infected Thuja plicata and the adjacent Pseudotsuga menziesii trees that were the Poria weirii inoculum source

Zone lines marked the extent of decay or stain in one-third of the infections (Fig. 9 contact 4). Wounding of T. plicata roots was evident at 90 per cent of contacts. Wounding per se or in combination with Poria weirii mycelium had apparently induced heartwood formation at many contacts.

The P. weirii inoculum sources for infections on one Thuja plicata were two dead and one living Pseudotsuga menziesii (Fig. 8), and for the other three, one dead P. menziesii. The extent to which

P. menziesii trees had been colonized by Poria weirii varied, and the percentage of contacts resulting in infection was proportional to the degree of inoculum source colonization.

Fig. 9. Thuja plicata roots at three contacts

Wood chips taken from Pseudotsuga menziesii and Thuja plicata roots at the contacts were cultured on malt extract agar. Cultural characteristics and tolerance to 7.5 ppm thujaplicin of four pairs of isolates were compared with those of Thuja isolate 11-2 and non-Thuja isolate 26-4. The incubation period for the thujaplicin test was 19 days.

On malt agar, the mat colour and growth rates of isolates comprising a pair were identical. Mycelium of isolate pairs was a darker brown than either 11-2 or 26-4. The numbers, hosts, locations, collection dates and ratios to control of isolate pairs tested are given in Table X.

Table X. Collection data and ratios to control of isolate pairs tested for tolerance to 7.5 ppm thujaplicin

Isolate No.	Host	Location and Date of Collection		Ratio to Control
Pw 11-2	<u>Thuja plicata</u>	Enderby	1962	0.854
Pw 26-4	<u>Pseudotsuga menziesii</u>	Cowichan	1962	0.649
Pwm 15-1	<u>T. plicata</u>	Enderby	1967	no growth
Pwm 15-2	<u>P. menziesii</u>	Enderby	1967	0.317
Pwm 16-1	<u>T. plicata</u>	Cowichan	1968	0.582
Pwm 16-2	<u>P. menziesii</u>	Cowichan	1968	0.798
Pwm 18-1	<u>T. plicata</u>	Cowichan	1968	0.322
Pwm 18-2	<u>P. menziesii</u>	Cowichan	1968	0.362
Pwm 19-1	<u>T. plicata</u>	Cowichan	1968	0.545
Pwm 19-2	<u>P. menziesii</u>	Cowichan	1968	0.622

The ratios to control of 11-2 and 26-4 were nearly identical to those determined previously (Table IX). Growth of isolate 15-1 on control medium was poor, however, in an earlier experiment its ratio to control was about 0.30 at 7.5 ppm thujaplicin. The ratios of isolates from Thuja plicata were consistently lower than those from Pseudotsuga menziesii.

Discussion

Non-Thuja isolates were unable to decay Thuja plicata heartwood blocks (13), or to tolerate T. plicata heartwood extractives diffused into agar (13). Hot-water extraction of T. plicata heartwood increased its susceptibility to decay by a number of wood-destroying fungi (43). These results suggest that the inhibitory compounds are hot-water soluble heartwood extractives.

The experiments that measured isolate growth along wood micro-sections established that the compounds inhibitory to non-Thuja isolates are found only in T. plicata heartwood and confirmed that these substances are hot-water soluble. Poorer growth of the non-Thuja isolate on extracted wood micro-sections may be attributed to inhibitory hot-water insoluble extractives that have little or no effect on Thuja isolates.

A hot-water extract equivalent to that used in this study would contain steam volatile (thujaplicins, thujic acid and methyl thujate) and non-volatile components (water-soluble phenols, protein and carbohydrate). Hexane extraction of the hot-water extract would remove the steam volatiles, principally thujaplicins.

Slight differences between isolate groups in response to thujaplicin-free hot-water extract were noted (Fig. 5) and these are probably due to the presence of the water-soluble phenols. Carbohydrate and protein in the hot-water and thujaplicin-free extracts cause the growth stimulation and lessen the inhibitory effects of the thujaplicins in the former. Pectic substances present in the extracts were shown to be a much better carbon source than glucose (Appendix).

The ability of carbohydrates and protein to mask growth inhibition by thujaplicins is less marked with non-Thuja isolates. Water-soluble phenols, known to be slightly inhibitory to Basidiomycetes (36) may, like the thujaplicins, have a greater effect on non-Thuja than on Thuja isolates. Mycelial growth rate of Thuja isolates on gallic or tannic acid medium was higher than that of non-Thuja isolates (13); this suggests that the former have greater polyphenol oxidase activity and hence greater apparent tolerance of water-soluble phenols.

There were highly significant differences between isolate groups in tolerance to 7.5 ppm thujaplicin. Thuja isolates showed greater stimulation to higher concentrations than non-Thuja isolates. The concentration toxic to Thuja isolates was 1.3 to 1.8 times that toxic to non-Thuja isolates. Therefore, with respect to thujaplicin tolerance, a Poria weirii isolate belongs to one of two distinct populations.

Explanations of the stimulation phenomenon were proposed by Lyr (25) and Schultze-Dewitz (39,40), but are beyond the scope of this discussion.

Static or toxic fungicide concentrations are affected by the substrate type used in their determination. On malt agar and wood substrates, γ -thujaplicin concentrations toxic to Lentinus lepideus were 20 ppm and 1 to 1.2 per cent, respectively (Table II). Similarly, values determined using malt agar and liquid media could not be compared.

B- and γ -thujaplicin inhibited glycolysis (34), oxidative phosphorylation (26) and succinic dehydrogenase (26,34) in certain

fungi or isolated liver mitochondria. The greater tolerance of Thuja isolates to thujaplicins suggests that they have the ability to adapt the blocked enzyme system(s) or to detoxify thujaplicins. During serial generations, decreasing non-Thuja tolerance to five ppm thujaplicin contrasted with increasing Thuja tolerance of the same concentration indicates that the former group lacks these enzymes. Furthermore, exposure to non-Thuja isolates to Thuja plicata heartwood for several years under field conditions did not increase their thujaplicin tolerance; in fact, they apparently became more intolerant.

When inoculum potential was large enough, non-Thuja isolates were able to overcome T. plicata heartwood extractive toxicity. Occasionally areas of cambium near contacts were killed; killing of roots seldom occurred. Wound-induced heartwood appears to limit the extent of successful infections. Zone lines produced by Poria weirii were shown to retard replacement of the fungus by antagonistic soil organisms (32). In addition, they are apparently laid down by non-Thuja isolates to separate an infected area from uncolonized Thuja plicata heartwood. Survival of the fungus may thereby be facilitated.

A strain or physiologic race has been defined as one of a group of forms, like in morphology, but unlike in certain cultural, physiological, biochemical, pathological or other characteristics (1). Differences under the above characteristics between the Thuja and non-Thuja isolate groups warrant their designation as strains. Studies on compatibility between the groups and apparent differences in enzyme complements are required before variety or species status

is considered for the Thuja and non-Thuja isolate groups.

Poria weirii causes a butt decay in Thuja plicata, whereas it frequently kills or leads to death of other hosts. Wood volume lost through killing is more significant and as a result research on possible controls has concentrated on hosts other than T. plicata. Within an infection center, Poria weirii spreads from tree to tree across root contacts. Any measure that breaks or prevents root contacts between susceptible species would reduce spread of the disease. Trenching around infection centers, wide spacing of susceptible trees and planting of non-susceptible species are possible controls. Trenching effectively prevented increase of infection center size, however, it was too expensive to apply generally (46). Without constant attention, widely spaced stands tend to fill in through natural regeneration. Planting infection centers with non-susceptible species, alone or mixed with susceptible ones, would probably be the most effective and least expensive control.

Laboratory and field studies showed that non-Thuja isolates are unable to increase their thujaplicin tolerance to the level required to attack Thuja plicata. When inoculum potential was great enough to overcome thujaplicin toxicity, the resulting infections on T. plicata roots were usually localized. This evidence indicates that T. plicata trees, when planted around and within an infection center caused by a non-Thuja isolate, would limit its size by reducing the number of root contacts between susceptible species.

Conclusions

1. Thuja plicata extractives inhibitory to non-Thuja isolates of Poria weirii were shown to be hot-water soluble heartwood components.
2. Thujaplicins were more inhibitory to Poria weirii isolates than water soluble phenols.
3. Tolerance of Poria weirii Thuja isolates to thujaplicin was significantly higher than that of non-Thuja isolates.
4. Non-Thuja isolates were unable to increase thujaplicin tolerance to the level required to attack Thuja plicata.
5. The results have significance as means for controlling Poria weirii infection centers caused by non-Thuja isolates.

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Appendix

Nutrition of Poria weirii

The literature on Poria weirii contains no reference to nutritional requirements except to state that the fungus grows well on malt agar. To determine vitamin requirements, if any, and efficiency of specific compounds as carbon or nitrogen sources, a brief nutritional study was carried out. Thuja isolate 11-2 and non-Thuja isolate 27-3 were used throughout.

Vitamin Requirement

The effect on mycelial growth of omitting one of seven vitamins in rotation was determined. Treatments containing and omitting all vitamins were included. Vitamins tested and per liter amounts added were as follows: pyridoxine, 100 ug; p-aminobenzoic acid, 100 ug; Ca-d-;pantothenate, 100 ug; inositol, 5 ug; biotin, 5 ug; thiamine, 100 ug and niacinamide, 100 ug. Vitamin solutions were made up in 20 per cent ethanol. The basal medium contained 20.0 g glucose, 0.75 g MgSO_4 , 1.75 g KH_2PO_4 , and 1.75 g $(\text{NH}_4)_2\text{SO}_4$ per liter. All glassware used in this experiment was acid washed and rinsed several times in distilled water. Twenty-five ml of a solution containing minerals and nitrogen source was placed in 250 ml erlenmeyer flasks. These flasks and others of carbon source were autoclaved separately; vitamins were added after the carbon source

had cooled. Twenty-five ml of carbon source plus vitamins were added to each flask. Flasks were inoculated with four mm diameter plugs from the second serial transfer on water agar and incubated at 20 to 22 C for 28 days. The four replicates in each treatment were combined and mycelium was harvested by filtering on tared 5.5 cm Whatman No. 1 filter papers. Filter papers were dried at 80 C for eight hours and weighed.

Fig. 10. Response of Poria weirii isolates to media deficient in various vitamins

The results shown in Figure 10 indicate a definite thiamine requirement.

Carbon Source

The efficiency of glucose, sucrose, xylose, cellobiose, pectin, starch and glycerol as carbon sources was compared. The basal medium contained 0.75 g MgSO_4 , 1.75 g KH_2PO_4 , 2.0 g L-asparagine and 100 ug thiamine per liter. Carbon sources were added at a rate of 10.0 g per liter. Methods used were identical to those described for the vitamin experiment. The incubation period was 28 days.

Fig. 11. Efficiency of several carbon sources relative to glucose

Results, expressed as ratios to mycelium production on glucose, are shown in Figure 11. Glycerol and sucrose were less efficient carbon sources than glucose; xylose and cellobiose were somewhat more efficient and starch and pectin were much superior.

Nitrogen Source

At a nitrogen concentration of 0.04 per cent, ammonium sulphate, ammonium tartrate, sodium nitrate and L-asparagine were compared. Glucose, at 10.0 g per liter served as carbon source. Methods were as described.

Fig. 12. Growth of *Poria weirii* isolates on four nitrogen sources relative to nitrogen-free control

Results expressed as ratios to a nitrogen-free control are graphed in Figure 12. *Poria weirii*, like many wood-destroying Basidiomycetes, is unable to utilize nitrate nitrogen. L-asparagine was superior to ammonium sulfate and ammonium tartrate. A much shorter incubation period was responsible for the smaller mycelium production of 11-2 relative to 27-3.

At the beginning and end of the preceeding nutrition experiments, medium pH was measured. Initially, pH of the various media used was between four and five. In a few treatments, pH increased slightly, however, decreases of one-half point were usual. The magnitude of pH change was directly related to mycelium production.