A MECHANISM OF INDUCED DISEASE RESISTANCE IN THE BARK AND SAPWOOD OF WESTERN REDCEDAR

by

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ABSTRACT

Samples of sapwood and bark of western redcedar were collected at 3 day to 6 week intervals after injury and extracted with water, chloroform and acetone. Extracts were tested for the presence of some common heartwood compounds and *in vitro* fungitoxic properties. Extracted samples collected 6 weeks after injury were inoculated with a decay fungus, and the resulting weight losses determined. No heartwood compounds were detected in any extracts, and no extracts were fungitoxic *in vitro*. Weight losses following decay of extracted chips indicated that decay resistance was initiated in the bark and sapwood. Thus, these tissues possess a mechanism of disease resistance induced by injury. It is concluded that this resistance results from the deposition of a toxic substance that is unextractable with water, chloroform, or acetone. The alteration of sapwood, if not the bark, is analogous in certain respects to the formation of reaction zones in the sapwood of various trees, since these zones are induced by injury and are characterized by abnormal toxin formation. However, the toxins formed in other trees are normal heartwood constituents, and in this respect apparently not parallel to the toxic substance induced in western redcedar.

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INTRODUCTION

When resistant plant tissue is invaded by a pathogenic organism, it commonly responds by producing one or more toxic compounds which bring about localization or death of the invading organism. This response involves alteration of normal metabolism resulting usually in the death of the host cells involved. If the toxic compounds formed are oxidation products of compounds existing normally in the tissue, or if the level of a preexisting toxic compound increases, the result is termed a hypersensitive reaction. If the toxic compounds formed are not normally found in the plant, the result is a phytoalexin response. Both classes of response are examples of induced chemical disease resistance which provide protection against pathogens not possessing specific counter mechanisms to avoid elicitation or to degrade the toxins.

Many tree species contain extractable substances in the heartwood zone which are toxic to pathogenic fungi in vitro. Decay resistance, and hence durability of wood in use, corresponds roughly to the amounts and toxicity of the extractable toxins present. Sapwood possesses no toxic extractives, and as lumber it is much less decay resistant than most heartwood. However, even when exposed by injury, living sapwood is often resistant to decay and disease. This indicates that a mechanism which prevents disease and decay is present in resistant sapwood.

The conversion of sapwood to heartwood is somewhat analogous to the production of phytoalexins, in that fungitoxic compounds are produced upon or after the death of ray cells. The sapwood of various angiosperm trees and species in the Pinaceae forms reaction zones characterized by the synthesis of fungitoxic compounds when subjected to an irregular stimulus such as wound-

ing and subsequent fungal inoculation (Hart & Johnson, 1970; Jorgensen, 1961; Shain, 1971). In all investigated cases this altered sapwood contains toxic extractives normally found only in the heartwood. The formation of reaction zones in sapwood has been termed 'protection wood' by Jorgensen (1961), 'phytoalexin production' by Shain (1967), and is an example of induced chemical disease resistance.

This thesis attempts to determine whether disease resistance in the sapwood and bark of western redcedar can be explained by phenomena similar to those listed above for other trees. Western redcedar was selected for investigation because a great deal of work has been done on the chemistry of its heartwood extractives, certain of which have very high in vitro fungitoxicities. It has already been shown that these toxic compounds do not normally occur in the bark or sapwood (Gardner, 1962), and a technique is available for the detection of some of these extractives in minute quantities (Maclean & Gardner, 1956).

A demonstration of induced chemical resistance in the bark or sapwood of western redcedar would indicate that such reactions occur in gymnosperms outside the Pineaceae. A demonstrated absence of such a mechanism would indicate that these tissues resist disease by some other means.

LITERATURE REVIEW

A. Induced Disease Resistance in Herbaceous Plants

1. Hypersensitive Reaction

Interactions between host and pathogen are either susceptible or resistant. A susceptible interaction leads to disease, while a resistant interaction does not. Physical barriers or unfavorable conditions for pathogen survival lead to resistant interactions. In many cases unfavorable conditions are generated by the host after a pathogen becomes established. These changes result in pathogen localization. This type of response is dynamic and is a 'hypersensitive reaction'. It is extremely widespread and can be initiatedin most, if not all, higher plants (Goodman et al., 1967).

The hypersensitive reaction, according to Müller (1949), "... encompasses all morphological and histological changes that, when produced by an infectious agent, elicit the premature dying off or necrosis of the infected tissue as well as inactivation and localization of the infectious agent." Once the reaction is initiated, localized lesions develop which progressively darken. The reaction is elicited by mechanical stimuli as well as by invasion. Both types of stimuli are somewhat similar in a microscopic level, since individual cells are damaged by mechanical wounding and hyphal intrusion. In either case, the reaction is a disease resistance mechanism, since wounding results in exposure of living tissue. Exposure leads to inoculation by ubiquitous fungal spores.

Since the hypersensitive response is widespread, susceptibility to disease depends upon how quickly host metabolism alters after invasion. Alteration of normal systems is apparently irreversible, since the affected host cells ultimately die.

Rahe et al. (1969) have shown that elicitation of a hypersensitive response by inoculation with non-pathogenic fungi of Phaseolus vulgaris L. confers resistance to subsequent infections of bean anthracnose. This result indicates that infection success by this pathogen depends upon its ability to avoid or delay elicitation of the hypersensitive reaction.

Phenolic compound oxidation is implicated in the hypersensitive reaction since the activity of polyphenoloxidase correlates with intensity of reaction (Rubin et al., 1959). Also it has been shown that oxidized phenolic compounds accumulate in affected tissues of plants undergoing hypersensitive responses (Goodman et al., 1967). Oxidized phenolic compounds, such as the aglycones phloretin and hydroquinone, are more toxic in vitro than their reduced forms, the glycosides phloridzin and arbutin (Kuć, 1967). However, even when present in oxidized forms in affected cells, the concentrations of such compounds are only 1-10 percent of that required to produce an in vitro fungistatic effect (Tomiyama et al., 1967). This observation implies one of three possibilities: 1) additional factors contribute to localization; 2) the in vivo toxicities of the compounds are not the same as those in vitro; or 3) additional amounts of toxic compounds are transported from neighboring cells after a certain period of time. Tomiyama et al. (1967) has calculated that transport of 30 percent of the phenolic compounds from adjacent cells into affected cells would result in a five-fold increase in phenolics. He further calculated that transport from two cell layers bordering the affected cells would yield a twenty-fold increase, which would explain fungistatic results. Although not necessarily validating Tomiyama's theory, the following studies suggest that a transport system exists: 1) Sydow and Durbin (1962) have shown that ^{14}C from labeled phenolic compounds was transferred and accumulated at the site of infection in rust-infected leaves of susceptible wheat plants.

2) Benda (1959) has observed nuclear movement and protoplasmic streaming to infected cells.

Many phenolic compounds are involved in hypersensitive reactions. Much of the work demonstrates that amounts of preexisting phenolic substances increase after the reaction has taken place. Examples of such compounds are caffeic acid, chlorogenic acid, and the coumarins umbelliferone and scopoletin. All of these increase in concentration after injury or inoculation of sweet potato leaves (Kuć, 1964).

2. Phytoalexin Response

Phytoalexins are compounds conferring disease resistance which are synthesized de novo after injury or inoculation (sometimes only after inoculation) and are not present (within limits of detection) in healthy plants (Cruickshank, 1963). Although differences between phytoalexins and compounds involved in hypersensitive reactions are present by definition, in practice it is sometimes impossible to clearly distinguish these two categories of compounds (Tomiyama et al., 1967). In some cases, necrosis of host tissue may not occur after phytoalexin production (Cruickshank, 1963), whereas the development of necrotic lesions is an invariable symptom of a hypersensitive reaction.

The compounds pisatin from peas, trifolirhizin from red clover, and phaseollin from beans are phytoalexins. All of these compounds are isoflavones. Cruickshank recognizes three other compounds as phytoalexins: orchinol, which is apparently a stilbene and is produced in orchid leaves; isocoumarin, which is apparently derived from acetate and is produced in carrot tissue; and ipomeamarone, which is synthesized by sweet potato roots and is probably a sesquiterpene. Ipomeamarone is apparently the only terpenoid shown to have a role in disease resistance in herbaceous plants.

Contention exists over the relative contribution of induced toxic substances to disease resistance. This contention arises in part from poorly designed experimental work which does not justify the conclusion drawn. For instance, the presence of greater amounts of phenolic compounds, or abnormal compounds, in diseased plants does not necessarily demonstrate that these compounds are involved *in vivo* in disease resistance. Goodman *et al.* (1967) has suggested that this difficulty can be eliminated by testing plants with virulent and avirulent strains of the same pathogen. Host interaction with the avirulent strain initiates the factors which impart host resistance, while infection by the avirulent strain does not. A comparison of the two cases should disclose the nature of the resistance. The reciprocal situation is also useful. Isogenic lines (isolines) of a given plant differing only in a single gene for resistance are inoculated with a pathogen and infection results in one case but not in the other (Mace & Veech, 1971). In this case also, the nature of the resistance should be disclosed by a comparison of the two interactions.

B. Disease and Decay Resistance of Trees

The mechanisms of disease resistance found in herbaceous plants are also operative in trees. However, because of the growth habit of trees, tissue disintegration (decay) of wood is an important part of disease, whereas in herbaceous plants, tissue disintegration is usually a secondary factor, not caused by the primary pathogen. Thus, it has been important for forest pathologists to determine the factors which impart decay resistance to wood. Such investigations have been made for living trees where decay causes disease and for wood used as lumber where decay causes failure in service.

Due to economic factors, relative durabilities of woods have long been

recognized and taken into consideration when determining their uses. Since durability in use is approximately synonymous with decay resistance, much information has been amassed rating relative decay resistances. Scheffer and Cowling (1966) rate trees as non-resistant to highly decay resistant. However, it is imprtant to note that this scale is misleading if applied to all plant tissues. Actually non-resistant wood is considerably more resistant to decay than other types of non-woody plant tissues because of the chemical structure of wood. Wood always consists of cell walls composed of a lignin-cellulose complex. This complex is comparatively resistant to decay because few organisms possess enzymes which enable them to degrade lignin.

The composition of wood is subdivided into three artificial categories based upon chemical properties. A certain amount of material is removed from wood treated with polar solvents; additional material is removed by treatment with strong acids leaving an unaltered residue. Although the correspondence is not perfect, the following generalizations are made: The residue remaining after both treatments is lignin. The fraction removed by strong acids is cellulose. The material removed by polar solvents comprises nearly everything else found in wood and is defined as 'extractable substances' (Brown et al., 1949).

Although all woods have the same basic lignin-cellulosic structure, decay resistances of woods are variable. Thus, an additional factor(s) must be involved. Scheffer and Cowling (1966) state that "... toxic extractable substances deposited during the formation of heartwood are the principle source of decay resistance in wood." This conclusion is based upon three observations: 1) Extracts from heartwood often possess an in vitro fungal toxicity, whereas sapwood extracts from the same trees do not. 2) Decay resistance of durable heartwood can be greatly reduced or destroyed by extraction with polar solvents.

3) The in vitro toxicity of heartwood extractives of various species correlates roughly with the known durability in service of these species. These generalizations apply to both angiosperms and gymnosperms.

Specific heartwood extractive substances with recognized fungitoxic properties are nearly all aromatic. Most of these substances are phenolic compounds synthesized via the shikimic acid pathway; however, a certain number are terpenoid in nature. Some of the compounds arising via the shikimic acid pathway are dihydroquercetin or taxifolin (a flavonoid occurring in Douglas-fir), pinosylvin and pinosylvinmonomethyl ether (stilbenes occurring in pine). Many mono- and sesquiterpenes occurring throughout the Cupressaceae and certain other gymnosperm taxa have high in vitro fungitoxic properties.

Heartwood extractives are synthesized by living parenchymatous cells at the sapwood-heartwood transformation (Hillis, 1962). One school of thought is that carbohydrates already present provide the materials for this synthesis. But the amount of extant carbohydrates in some trees is not sufficient to account for the observed amounts of deposited extractives (Hillis, 1962). Therefore, additional carbohydrates may be transported to the sapwood-heartwood transition zone.

The contributions of specific heartwood extractives to decay resistance have been partially determined by in vitro toxicity tests. The conclusions drawn from such tests are probably more valid than those drawn from in vitro toxicity tests of compounds linked to induced disease resistance in living plants, since heartwood is not a true in vivo situation. Thus, these tests more nearly approximate conditions in heartwood than in living tissue.

The type of toxicity test employed has been shown to affect results in the following case: The heartwood of Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) is rated as moderately resistant to decay (Scheffer & Cowling,

1966). Kennedy (1955) concluded that taxifolin is primarily responsible for this decay resistance on the basis of two types of toxiciy tests: 1) comparison of rates of growth of decay fungi on agar to which various different fractions of heartwood extractions had been added; and 2) comparison of weight losses of inoculated heartwood blocks from which different compounds had been removed by extraction with various solvents. Kennedy observed death of his test fungi at levels varying from 0.4-0.6 percent taxifolin in agar. Rudman (1962) also tested the toxicity of taxifolin by permeating non-decay resistant wood meal with varying concentrations of pure taxifolin, inoculating this wood meal with decay fungi and then comparing weight losses. He concludes that taxifolin is only slightly toxic since 1.0 percent concentration in the meal, or that concentration which roughly equals the concentration in heartwood, produced only slightly inhibitory effects upon decay. Rudman concludes that taxifolin is not responsible for decay resistance of Douglas-fir heartwood and that Kennedy's conclusions are wrong due to a misinterpretation of his evidence. Rudman surmises that the poisoned agar test gives misleading results and that selective extraction of heartwood blocks increases water permeability, thereby enhancing decay. Although the poisoned agar test used by Kennedy gives a valid approximation of toxicity (Cruickshank & Perrin, 1964), Rudman's toxicity test may provide a more valid result in this case since his test more closely approximates actual heartwood conditions. However, the conflict between the two sets of results is unresolvable since both tests are contrived situations. It is also impossible to determine the validity of either Kennedy's or Rudman's conclusions, since one cannot distinguish the effects of water permeability from the toxicity of individual compounds. In addition, other extractives non-toxic to decay organisms <u>in vitro</u> may contribute a synergistic effect in the heartwood. Therefore, one may conclude that taxifolin is toxic

to decay organisms under some conditions and imparts some decay resistance to heartwood. Whether taxifolin is the principle source of decay resistance is an open question.

C. Induced Disease Resistance in Trees

Studies of disease resistance in trees usually have a different emphasis than similar studies of resistance in herbaceous plants. Thus, studies on non-lethal foliage diseases of trees have produced largely descriptive reports which do not apply hypersensitive and phytoalexin concepts. It is clear that large numbers of angiosperm trees produce hypersensitive reactions in their foliage, since large numbers of 'shothole' diseases are listed for these trees (Boyce, 1961). The cause of these symptoms becomes clear only when the concept of the hypersensitive reaction is applied. It is probable that parallel examples of induced disease resistance are characteristic in the foliage of some gymnosperms, but have escaped detection due to lack of investigation.

Research on foliar disease resistance has been done on a few intensely cultivated species. Chlorogenic acid has been implicated in disease resistance in certain apple and pear varieties, particularly in the fruit (Kuć, 1964). Also, the interaction between apple trees and Venturia inaequalis (Cooke) Wint. (apple scab) has been carefully investigated by Williams and Kuć (1969). It is known that resistant varieties of Malus spp. isolate the apple scab pathogen within small lesions. These lesions contain increased concentrations of the u.v. florescent compound phloretin, which is fungitoxic in vitro. Apparently the increased concentration of phloretin explains the isolation. Phloretin is a degradation product of phloridzin, the most common glycoside found in apple foliage and fruit. Whereas all apple varieties contain phloridzin, only the

resistant varieties release phloretin upon infection localizing V. inaequalis to lesions.

A number of investigations have focused attention on disease resistance in the sapwood of trees. There are two types of sapwood resistance. In the first type immune sapwood is added by growth after infection has occurred. Shigo (1966) has shown by dissections of many forest-type hardwoods that, although injuries may lead to infection and extensive decay, sapwood laid on after the time of wounding in some way resists further spread of infection. Therefore some barrier (physical and/or chemical) is produced by the immune sapwood.

The second type of sapwood resistance is a mechanism which alters extant sapwood so that the conditions for pathogenesis become unfavorable, and death or localization of the pathogen occurs. This type of resistance is a dynamic response which is induced in affected zones of sapwood. Extreme abnormal metabolic changes occur, including premature death of parenchymatous cells, and the synthesis of metabolites which are not normally present in the sapwood.

It has been shown for the genus Prunus that induced host synthesis of toxins in the sapwood leads to disease resistance. Artificial infections of P. persica (L.) Batsch (Braun & Helton, 1971) and P. domestica L. (Helton & Braun, 1971) by Cytospora cincta Fr. led to resistance to subsequent infections by the same pathogen. In the case of P. domestica the resistance does not appear to localized but is induced up to at least 120 cm from the site of the original infection. This result may indicate that sapwood metabolism is changed throughout much, if not all, of the tree.

Hart and Johnson (1970) found that injuries of the sapwood of Quercus alba L., Robinia pseudoacacia L., and Maclura pomifera (Raf.) Schn. lead to the production of abnormal zones of sapwood. These zones contain extractives

which are fungitoxic in vitro to decay organisms. However, extracts from these zones are not as toxic as control extracts from normal heartwood of the same trees. Whether this can be explained by quality or quantity of toxic extractives has not been established.

It has been shown conclusively that the sapwood of Pinus spp. responds to injury or inoculation with the production of toxins normally found only in the heartwood. Jorgensen (1961) determined that an abnormal zone of sapwood in P. resinosa Sol. (Norway pine) exists in advance of mycelial penetration by Fomes annosus (Fr.) Cooke. He found both pinosylvin and pinosylvin-monomethyl ether present in this zone, neither of which is detectable in normal sapwood. Shain (1967), in a study similar to Jorgensen's, demonstrated that pinosylvin and its monomethyl ether are produced in the sapwood of P. taeda L. (loblolly pine) in response to infection by F. annosus. Lyr (1967) showed that pinosylvins are produced by the sapwood of P. sylvestris L. (Scotch pine) in response to cambial injury.

The above three studies on three species of pine demonstrate that the abnormal synthesis of pinosylvins is a dynamic response to inoculation and injury. In each case a time interval is required before the pinosylvins appear, although trees in the field require a longer interval than living sapwood placed in controlled conditions. Jorgensen found that trees wounded artificially required 4 to 9 weeks to produce the response. Lyr found pinosylvins present after 3 weeks, but they were present in much greater quantities after 6 weeks. In controlled laboratory studies, Jorgensen found that sapwood could produce pinosylvins in as little as three days.

Differences among species in their pattern of formation of pinosylvins are present in the studies of Jorgensen and Lyr. With respect to season of year, Jorgensen found that trees respond only in the latter part of the growing

season and the dormant season, while Lyr found that trees do not respond in the dormant season. Interestingly, both authors explain these phenomena by surmising that pitch flow controls exposure of the injured sapwood to air, and thus determines the rate of desiccation and subsequent death of affected cells. Jorgensen feels that pinosylvins are not produced in the early growing season due to copious pitch flow which prevents desiccation. Reciprocally, Lyr feels that pinosylvins are not produced in the dormant season since little resin flow occurs, resulting in rapid desiccation and cell death before synthesis of pinosylvins is initiated. These conclusions do not necessarily conflict. They may suggest that extremes of desiccation of exposed sapwood lead to death rates which are either too fast or too slow; synthesis of pinosylvins will not occur in either case.

Shain (1971) has observed the formation of reaction zones in the sapwood of Picea abies Karst. (Norway spruce). These zones are formed in advance of penetration by F. annosus and are similar to those formed in pines. He detected heartwood extractives in the spruce reaction zone not normally found in the sapwood; these extractives have a demonstrated in vitro toxicity (Shain & Hillis, 1971), which leads Shain to conclude that the reaction zone in the sapwood contributes to the resistance of Norway spruce in vivo.

As for the cases of induced disease resistance in herbaceous plants and decay resistance of heartwood, a strong implication exists that specific isolated substances with known in vitro toxicities, arising from direct host-pathogen interactions, make conditions untenable for the pathogen. At the present time, no available technique, such as the infection of isolines of cultivated plants, can prove this implication. One piece of evidence suggests that conclusions based on in vitro toxicity tests of extracts of reaction zones can be misleading. Shain (1967) made extractions from portions of sapwood infected

with F. annosus which contained no pinosylvins. When tested by bioassay, these extracts proved as toxic to his test organism as extracts from uninfected reaction zones with pinosylvins present. Apparently in this case his test is not a valid indicator of what happens in vivo. However, the absence of pinosylvins in the infected zone supports the theory that these compounds contribute to disease resistance, since it is probable that pathogenesis depends upon their degradation or absence.

D. Comparison of Sapwood Reaction Zones with Heartwood

There are similarities between the formation of abnormal reaction zones in sapwood and the formation of heartwood in trees. The normal transformation of sapwood to heartwood involves alteration of metabolism of parenchymatous cells, synthesis of heartwood extractives, death of the parenchymatous cells, and release of the extractives, some of which are deposited in surrounding tracheid cells. Metabolic activity decreases along a gradient from the cambium to the heartwood transformation zone (Frey-Wyssling & Bosshard, 1959; Dietrichs, 1964; Higuchi & Fukazawa, 1966; Higuchi, et al., 1967). The amounts of carbohydrates present in the sapwood of certain tree species are represented by a similar gradient. It is known that once the heartwood zone is reached, the parenchymatous cells are dead, carbohydrates are generally no longer present, and heartwood extractives are present (Chattaway, 1952).

Shain (1967) and Jorgensen (1962) have concluded that protection wood when initiated in the sapwood of pine is produced by a sequence of events parallel to those which produce normal heartwood; altered metabolism arising from some internal stimulus leads to the synthesis of heartwood extractives in situ from local starch reserves by slowly dying parenchymatous cells.

The differences between sapwood reaction zones and normal heartwood are in site of synthesis, in circumstances of initiation, and in chemical composition. Although comparatively few compounds have been looked at in the sapwood reaction zones, differences in proportion of fungitoxic extractives have been observed in both pine and spruce. Shain (1967) observed that the proportion of pinosylvin to pinosylvinmonomethyl ether is greater in reacted sapwood than in normal heartwood. Since pinosylvin has a greater in vitro toxicity than its monomethyl ether, this possibly represents an adaptation to enhance disease resistance. Shain and Hillis (1971) observed a parallel situation for Norway spruce. Among the compounds detected and measured, one lignan with a high in vitro toxicity was found in proportionally greater quantities in reacted sapwood than in characteristic heartwood.

E. Comparison of Reacted Sapwood with Mechanisms of Induced Disease Resistance

The formation of sapwood reaction zones in trees is a response to external stimuli and imparts disease resistant qualities similar to those imparted to herbaceous plants by a phytoalexin or hypersensitive response. Reaction zone formation partially fulfills the criteria which characterize either of the latter responses. It is similar to a phytoalexin response since it consists of induced de novo synthesis of fungitoxic metabolites. It also resembles a hypersensitive response, since both involve death of affected cells and subsequent localization of the pathogen. Certain authors have established the precedent of classifying sapwood reaction zone formation as a phytoalexin response (Shain, 1967; 1971; Smith, 1970).

It is not essential to equate sapwood reaction zone formation with either a hypersensitive or phytoalexin response. The formation of reaction zones

represents a special case of induced disease resistance in living wood tissue. This formation is strikingly parallel to mechanisms of induced disease resistance in herbaceous plant tissues.

Since decay fungi are ubiquitous, and the probability is high that injuries exposing living sapwood will occur in a forest habitat over the long life of individual trees, it is likely that all trees possess some mechanism of disease resistance in the sapwood such as the formation of reaction zones. Since it is known that various pines and Norway spruce possess this mechanism, it is probable that many other gymnosperms, and especially other taxa in the Pinales, possess this capability.

To the best of this author's knowledge no other investigations have been made to determine whether other genera of gymnosperms besides Pinus and Picea form abnormal reaction zones in the sapwood. Therefore, due to the large number of gymnosperms with toxic extractives occurring in the heartwood, and the necessity of disease resistance, it is probable that the production of reaction zones in sapwood is a widespread phenomenon among many genera.

The gymnosperm taxa in which sapwood reaction zones are formed are all members of the Pinaceae. Members of the Cupressaceae belong to the same order, and many, such as western redcedar, have been shown to contain highly fungitoxic heartwood extractives. Therefore it is probable that western redcedar and perhaps other members of the Cupressaceae form sapwood reaction zones following the pattern of pine and spruce. This study is designed to investigate whether initiation of reaction zones in the sapwood and bark of western redcedar is induced by injury, and, if so, whether the fungitoxic substances produced are normally occurring heartwood compounds.

F. Summary of Investigations Made on Western Redcedar

Thuja plicata Donn. (western redcedar) is found from southern Alaska to northern California and commercially is the most important of the North American cedars. Western redcedar is used extensively for shingles, siding, poles, and fenceposts because of the large amounts of clear wood available and the high durability of the heartwood arising from its high decay resistance.

It has been recognized for many years that the high decay resistance of western redcedar wood is caused by the presence of heartwood extractives. The first recorded demonstration of this phenomenon was provided by Sowder (1929), who showed that hot water extraction of western redcedar wood flour removes durability and that the water extracts are toxic to fungi in vitro. Anderson and Sherrard (1933) isolated two isomers of an acidic compound which they separated from the steam volatile oil of western redcedar heartwood. These compounds proved ten times more toxic than creosote to F. annosus and and Lenzites trabea (Pers.) Fr. They found that amounts of 0.006 percent by weight of either compound were fungicidal to both fungi. The indentification of these compounds as β- and γ-thujaplicins and the demonstration of their fungicidal properties have been verified by various authors including Anderson and Gripenberg (1948), Erdtman and Gripenberg (1948), and Rennerfelt (1948). In addition, Rennerfelt showed that a third isomer of thujaplicin (α), also contained in western redcedar heartwood, possesses the same order of toxicity as the other two isomers. The observed high toxicities of the thujaplicins generated considerable interest in western redcedar extractives. At least partly for this reason, a large number of investigations have been directed at discovering their chemical and compositional properties.

Although western redcedar heartwood has a relatively low density, it

contains relatively large amounts of extractives. According to Gardner and Barton (1958) the heartwood may contain amounts of extractives in excess of twenty percent of the heartwood dry weight. Extraction with acetone or hot water of the butt heartwood yields a mixture of phenolic substances, arabinose, a sterol, and a complex volatile oil.

The composition of the volatile oil has proven interesting both because of the aforementioned toxicity of the components and their novel chemical nature. Contained in the oil are thujic acid, methyl thujate, various tropolones (including the thujaplicins) and a single tropone. All of these compounds contain ten carbon atoms possessing an unsaturated seven membered ring with quasiaromatic properties. Although the mode of biosynthesis of these compounds is not proven, they are probably derived from two molecules of mevalonic acid. Loomis (1967) describes the thujaplicins as unproven irregular monoterpenes. Methyl thujate is the methyl ester of thujic acid and contributes much of the odor associated with the heartwood. The tropolones comprise a family of compounds first recognized by Dewar (1945) as derivatives of 2-hydroxy-2,4,6-cycloheptatriene-1-one. Five of the simplest naturally occurring tropolones are present in western redcedar heartwood. The tree thujaplicins, α-, β-, and γ-, are isomers of isopropyl tropolone with the aryl group attached at the 3, 4, and 5 positions respectively. β-thujaplicinol is similar to β-thujaplicin in structure differing by the addition of a hydroxyl group at the seven position. β-dolabrin is also similar to β-thujaplicin differing by its reduction state so that the three carbon aryl group is isopropenyl. The one tropone present in the heartwood is nezukone (4-isopropyl-2,4,6-cycloheptatriene-1-one). This compound was first discovered in the heartwood of T. standishii Carr. by Hirose et al. (1966); its presence was later confirmed in western redcedar (Hirose & Nakatsuka, 1967).

Table I lists the composition of the volatile oil of western redcedar heartwood.

The tropolones have several interesting chemical properties due to the presence of adjacent carbonyl and hydroxyl groups at the 1 and 2 positions of the ring. This association makes them substantially more acidic than normal phenols and also causes them to be steam volatile. In addition, they form chelate complexes with heavy metals. These complexes absorb visible light, enabling detection and quantification of extremely low concentrations of metal-thujaplicin complex by densitometric techniques (MacLean & Gardner, 1956).

Besides the toxicity tests of the three thujaplicins, additional tests have been performed on other redcedar extractives showing that many of these are also toxic. Roff and Whittaker (1959) tested the toxicity of β-thujaplicinol on various brown and white rot types of decay fungi. They found that β-thujaplicinol is approximately as toxic as the thujaplicins to the brown rot fungi tested, but much less toxic to the white rot fungi tested. The same study showed slight differences in toxicity of γ-thujaplicin to the two types of decay fungi.

Roff and Atkinson (1954) made a toxicity test on the phenolic fraction of water extracts of western redcedar heartwood. They describe this fraction as 'thujaplicin free', which probably means tropolone free. They found that concentrations of this fraction greater than 1.0 percent in malt agar are fungistatic, but concentrations as high as 8.0 percent are not fungicidal. They conclude that although the toxicity of the phenolic fraction is not high, it still plays an important role in providing decay resistance since this fraction represents 4 to 5 percent of the oven-dry weight of the heartwood.

Since nezukone occurs in relatively large quantities in western redcedar heartwood (Jiang, 1968), it may also contribute to decay resistance.

TABLE I

Composition of the Volatile

Oil of Western Redcedar

Component	Reference I	Reference II
Methyl thujate (other neutrals)	0.17 %*	-
Thujic acid	0.08	-
Tropolones	0.56	
β-thujaplicin	0.30	0.03 %
α-thujaplicin	0.01	-
γ-thujaplicin	0.20	0.30
β-dolabrin	0.0003	-
β-thujaplicinol	0.07	0.15
Nezoukone	-	0.22

*%--percent by weight of oven-dry heartwood

After: I. Gardner & Barton (1958a)
II. Jiang (1968)

The extent of this contribution, if any, is unknown. However, it is possible that nezukone may have contributed to extract toxicity, or at least have been present, in some of the studies already mentioned.

Raa and Goksøyr (1965;1966) have tried to determine the mechanism of toxicity of β-thujaplicin to Saccharomyces cerevisiae Meyen ex Hansen. Although they found that β-thujaplicin injibits formation of acetyl coenzyme A, they were unable to delineate a mechanism of toxicity. Raa and Goksøyr did show that the stable soluble copper chelate complex of thujaplicin, formed from an excess of cupric sulfate added to a solution of β-thujaplicin, is approximately two orders of magnitude more toxic to baker's yeast in liquid culture than plain thujaplicin. They showed that ferric complexes do not produce this amplified effect. Although copper is a well known fungicide, the enhancement of toxicity of thujaplicin by formation of the copper chelate is suprising. Before the importance of this discovery can be evaluated, further tests are necessary to determine the toxic effects on decay fungi within cedar heartwood.

Whereas decay resistance of western redcedar heartwood depends upon the amount of normal extractives present, various studies have shown that these extractives vary in amounts between trees and within trees. Gardner (1962; MacLean & Gardner, 1956a) found thujaplicins in amounts increasing radially outward from the pith until they cease to appear at the sapwood-heartwood transition zone. In addition these compounds occur in decreasing amounts from butt to apex. Jiang (1968) has verified this pattern for thujaplicins and has shown that it holds for the distribution of nezukone and various lignans. In no cases have thujaplicins been shown to be present in the sapwood or bark.

Although the above pattern of distribution of heartwood extractives is physiologically normal in western redcedar, microbial invasion of the heartwood

also affects the observed pattern of distribution. In some trees with butt-rot present, clearly defined concentric color zones exist which represent zones of microbial succession (van der Kamp, 1971). These zones move radially outward over time, apparently dependent upon the speed at which a pioneer organism is capable of degrading thujaplicins. This degradation changes the environment such that other organisms can invade, resulting ultimately in wood decay. It is possible that the absence of thujaplicins in the pith of undecayed trees allows the pioneer organism to get a foothold at this position. Such a succession is not known to occur in the opposite direction -- decay starting at the periphery of a tree and progressing in toward the pith.

MATERIALS AND METHODS

In the summer of 1970 an attempt was made to develop a thin layer chromatography (TLC) system, using silica-gel as the adsorbent, which would resolve extracts of western redcedar heartwood into components.

In late summer and early fall of 1970, portions of living bark were exposed, inoculated, collected after a time interval, and extracted with hot water. These extracts were tested for the presence of heartwood extractives. In the summer of 1971, portions of living bark and sapwood of western redcedar were exposed, collected at various time intervals, and extracted with various solvents. These extracts were tested for the presence of thujaplicins and *in vitro* fungitoxicity by several bioassay techniques. The extracted bark and sapwood collected after the longest interval was tested for any unextracted toxic factors by a weight-loss test.

Table II is a complete list of all the samples of bark and sapwood collected from August, 1970 to August, 1971. It summarizes the type of treatment each sample underwent, the date of the beginning of laboratory processing, the numbers and ages of trees from which each sample was taken, and the treatment time interval for each sample.

Figure 1 is a generalized flow chart of the methods utilized in field treatment and laboratory processing of samples. No individual sample was processed with all the techniques listed, although all samples in each collection block were treated in precisely the same manner.

Samples of living bark which had been exposed and inoculated were collected from trees 2 and 3 in August and October, 1970 respectively (Collection blocks 1 and 2). These trees were located southeast of Thunderbird Stadium, U.B.C. Endowment Lands, Vancouver, B.C. Two squares, 10 cm on

TABLE II

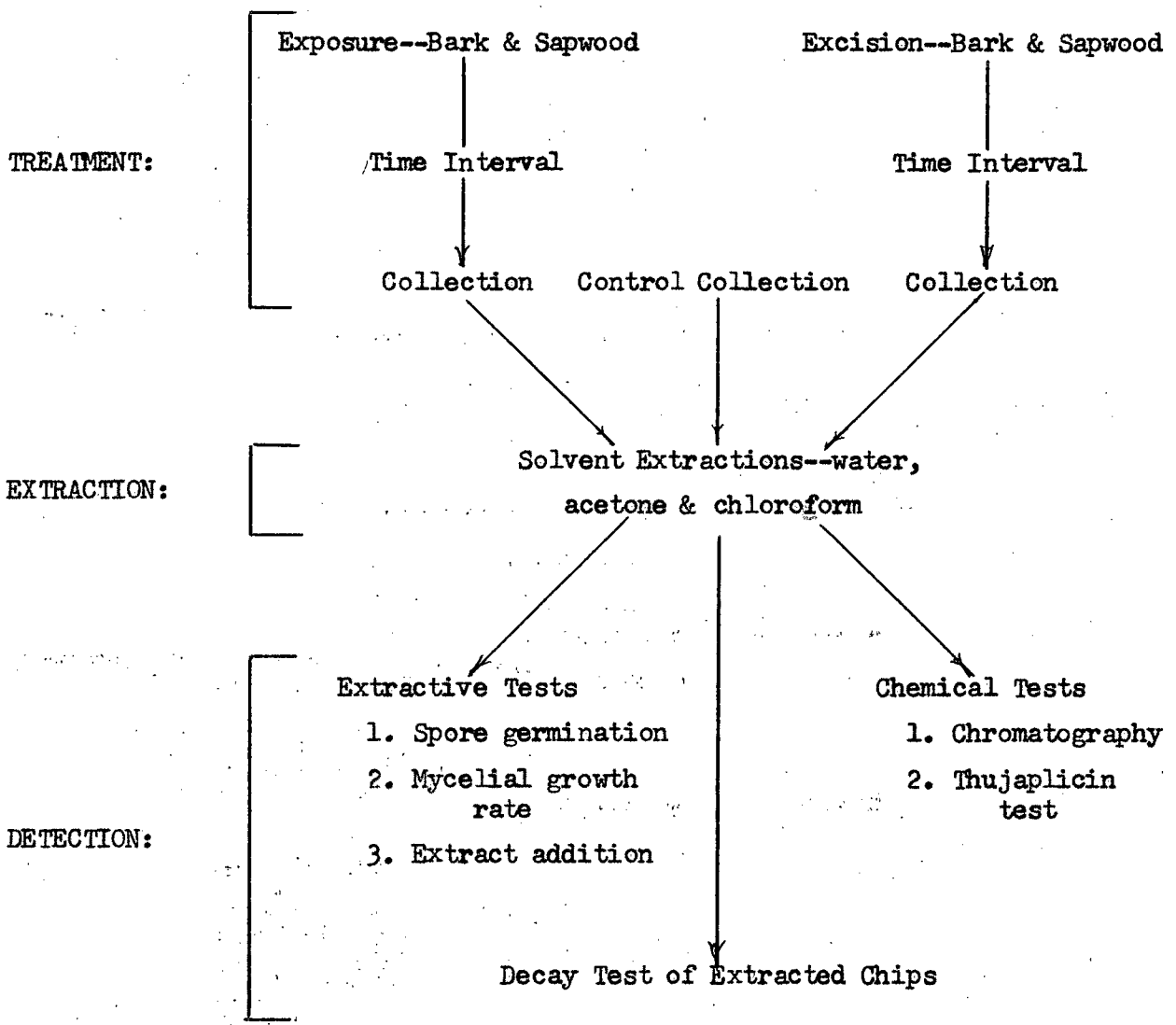
Summary of Sapwood and Bark Collections

Collection Block	Sample Number	Tree Number	Approx. Age	Treatment Description	Treatment Interval	Date Begin Process*
1	101	2	50 yrs.	Control bark	0	Aug. 24
	102	2		Inoculated bark	3 days	Aug. 27
2	203	3	50 yrs.	Control bark	0	Oct. 16
	204	3		Inoculated bark	3 days	Oct. 19
3	301	5	250 yrs.	Excised bark	1 wk.	June 22
	302	5		Excised sapwood	1 wk.	"
	303	4	250 yrs.	Exposed bark	1 wk.	"
	304	4		Control bark	0	"
	305	5		Exposed sapwood	1 wk.	"
	306	5		Control sapwood	0	"
4	407	5		Excised sapwood	3 wk.	July 5
5	508	7	250 yrs.	Exposed bark	4 wk.	July 20
	509	7		Bark under 508	4 wk.	"
	510	7		Control bark	0	"
	511	6	250 yrs.	Excised bark	4 wk.	"
	512	5		Exposed sapwood	4 wk.	"
6	613	4		Control bark	0	Aug. 4
	614	4		Control sapwood	0	"
	615	4		Exposed bark	6 wk.	"
	616	6		Exposed sapwood	6 wk.	"

* -- Collection blocks 1 & 2 processed in 1970; collection blocks 3-6 processed in 1971.

FIGURE 1

Methods Used to Detect Formation of Reaction Zones



a side, were laid out on each tree. The dead bark was removed from these squares exposing two 'windows' of living bark. One of these windows was painted with a dense spore suspension of Trichoderma sp., while the outer layer of the other was cut away with a stainless steel knife to serve as a control. The control strips, 2-4 mm thick, were chopped into chips about 6-10 mm square and extracted with about 10 times their living weight of 90-95°C water for 4 hours. Three days later the inoculated bark was collected and extracted in the same manner as the control bark.

Each hot water extract was simply distilled; 4 aliquots (tree 2--40 ml; tree 3--50 ml) were collected of extract distillations. These samples were extracted with chloroform (tree 2 samples with 20 ml chloroform; tree 3 samples first with 20 ml and consolidated with a subsequent 10 ml). Distillation residues were also extracted, first with 30 ml hexane and consolidated with a subsequent 20 ml hexane. In each case the aqueous phase was discarded, and the organic phases were evaporated, leaving crude oily residues. Each of these residues was dissolved in 1 ml chloroform and tested for the presence of known heartwood extractives by three techniques: 1) the analytical method for thujaplicins described by MacLean and Gardner (1956); 2) TLC chromatograms using silica-gel as an adsorbent developed with 10:1 mixture of chloroform to t-butanol (v/v); and 3) paper chromatograms prepared and developed after the method of Zavarin and Anderson (1956).

In the summer of 1971 investigation designed to detect induced formation of reaction zones was continued in an expanded form. Sapwood was included in addition to bark, and time intervals after treatment were extended. Instead of inoculation with Trichoderma sp., living bark and sapwood exposed by treatment were left exposed to natural sources of inoculum. Various solvents were used for extraction in place of, or in addition to, hot water. And samples

of living bark and sapwood were excised from trees and placed for various intervals in an incubating oven at room temperature, in the dark, at nearly 100 percent humidity.

The depth of reaction zone formation, if it exists in western redcedar, is not known. Thus, all samples, including collection bolcks 1 and 2, were removed in strips as thin as practicable, since this procedure assures that any abnormal metabolites will be minimally diluted with unreacted portions of tissue.

Various bioassays, as well as chemical tests used to detect tropolones, were used to indicate any formation of reaction zones in the samples comprising collection blocks 3-6. All the bioassay tests were in vitro. F. annosus (U.B.C. Forest Pathology Culture #25) was chosen as the test organism since this fungus is widely used in parallel studies and is adaptable to the following four types of bioassay used in this investigation: 1) Mycelial growth rate--a plug of actively growing mycelium, taken with a #4 cork borer, is deposited on a standard medium of 2 percent malt extract and 2 percent agar to which a specified amount of test substance dissolved in ethanol has been added, and the growth rate is observed. 2) Extract additions--the same as 1 except that the test substance is added in the form of a crudely extracted thick oil to a spot on the medium, and it is noted whether the mycelial front will grow over the extract. 3) Spore germination--spore producing colonies of test fungus are inverted over plates containing standard medium to which test substances have been added, and the effects on spore germination are observed. 4) Decay test of extracted chips--extracted chips are incubated on actively growing cultures of decay fungus for a time interval, and weight losses are calculated.

Samples comprising collection blocks 3-6 were taken from four trees (4-7)

located north of Blaney Lake, U.B.C. Research Forest, Maple Ridge, B.C. Treatments were of three types: 1) Exposure by stripping away the dead bark or all of the bark followed by a time interval. 2) Maintainence of sapwood or bark tissue excised from the tree and kept in the dark at about 25°C at nearly 100 percent hunidity for a time interval. 3) Collection of controls in the same manner used for the other samples without a time interval.

Samples in collection blocks 3-6 were rectangles 17.5 by 35.0 cm. The outer 2-4 mm of exposed bark were cut away with a knife. The sapwood samples were collected thicker than the bark samples (3-6mm) since they were removed with a chisel neccessitating a coarser cut.. Excised bark samples maintained under artificial conditions were collected thicker than other bark samples to facilitate removal of samples in one piece. In this case all of the living bark was removed resulting in cambial exposure. Before extraction all samples were cut into small chips 10-25 mm on a side.

Samples in collection block 3 consisted of bark and sapwood exposed for 1 week, excised bark and sapwood which had been incubated for 1 week, and bark and sapwood controls. The following procedure was followed for each of the 6 samples: Each was extracted with about 3 times its weight of 90-95°C water for 4 hours; 2.0 g agar and 2.0 g malt extract were added to a 100 ml aliquot of each extract before cooling, while the excess was frozen. Each aliquot was poured into 5 sterile petrie plates so each contained 20 ml of medium. After cooling, 2 of the 5 plates were tested with the spore germination test, and the remaining three plates were tested with the mycelial growth rate test.

Collection block 4 consisted of one sample of excised sapwood kept under artificial conditions for 3 weeks. It was extracted in a Soxhlet extraction apparatus for 4 hours with about 10 times its weight of chloroform.

A 10 ml aliquot of the chloroform solution was tested for the presence of thujaplicins by the method of MacLean and Gardner (1956). The remainder of the chloroform was flash-evaporated at about 30°C to dryness; the residue, dissolved in 1 ml ethanol, was added to 15 ml of standard growth medium. Three control plates without test substance containing the same percentage ethanol were also made. These four plates were tested by the mycelial growth rate test.

Samples comprising collection block 5 consisted of bark and sapwood exposed for 4 weeks, excised bark maintained for 4 weeks, and control bark. Each of these samples was air-dried for 3 days and then extracted with 10 times its air-dried weight of chloroform for 2 days. 10 ml aliquots of chloroform extracts from each sample were tested for the presence of thujaplicins. The remainder of these extracts were filtered, flash-evaporated at about 30°C, and the residue wights determined. About 50 mg of each residues were tested by extract addition tests. The bulk of each residue, dissolved in 1 ml ethanol, was added to 30 ml standard growth media and tested by mycelial growth rate and spore germination tests. Control plates without test substance containing equal amounts of ethanol were also tested.

Samples comprising collection block 6 consisted of bark and sapwood exposed for 6 weeks and control bark and sapwood. Each sample was air-dried for 6 days and then extracted for 2 days with 10 volumes (w/v) of acetone. After filtration each sample was flash-evaporated at about 30°C to dryness leaving oily residues. After weighing these residues were dissolved in 1 ml ethanol, added to 30 ml standard medium, and tested by mycelial growth rate and spore germination tests. In this case it was not possible to dissolve all the residue with ethanol, so portions of undissolved residue were tested by extract addition tests. The acetone extracted chips, after drying in a

fume hood for 3 days, were further extracted with 10 volumes (w/v) of 90-95°C water for 4 hours. These extracts were flash-evaporated at about 60°C to about 1 ml, added to 30 ml of standard medium, and tested by mycelial growth rate and spore germination tests. Since previous experience had shown that it was undesirable to evaporate the water extracts to dryness, residue weights were calculated indirectly by comparison of air-dried chip weights before and after the water extraction.

After water extraction, the samples of collection block 6 were air-dried and then oven-dried for 3 days at about 95-100°C. Seven groups of 10 chips, randomly selected from each sample, were weighed and deposited on cultures of F. annosus growing on standard medium. Four groups from each sample were placed on 11 week old cultures of F. annosus, while the remaining three groups were placed on 3 week old cultures. Each of these plates was incubated at room temperature in the dark at nearly 100 percent humidity for 8 weeks. After removal the chips were oven-dried for 3 days and their weights determined. The percentage weight losses for each of the 28 groups of chips were calculated, and various t-tests were performed on this data.

RESULTS

When living bark of western redcedar is exposed to air by stripping of the dead outer bark, or by excision from the tree, it rapidly darkens in color from a pale cream to a deep brown. This color change takes place within a few minutes after exposure. After this rapid change, the color continues to darken at a much slower rate for a day or so. Sapwood exposed to air also darkens although much more slowly and with a less noticeable color change--from cream to yellow. This change takes place over several days. The appearance of collections taken at 4 weeks did not differ visibly from collections taken after 3 days or 1 week. However, collections of bark and sapwood exposed for 6 weeks exhibited certain differences from samples collected after shorter exposure intervals. Both the bark and sapwood had exuded resins which made the samples sticky, a characteristic not noted for earlier collections. In addition, the presence of blue staining was macroscopically noticeable on the sapwood surface. Microscopic examination showed that hyphae were present, indicating that the staining was of fungal origin. This blue-staining fungus was not observed on any bark samples.

Table III lists sample weights (Column III), extracted residue weights (Column IV), and material extracted expressed as percentages of sample weights (Column V) and as percentages of the test medium (Column VI). A comparison of the values in Column V indicates notable differences only between percentages of material extracted with acetone from control samples and samples exposed for 6 weeks. A comparison between the values in Column V and VI indicates that the concentrations at which the extracted residues were tested, were of the same order as the concentrations naturally present in air-dried samples.

Attempts to resolve heartwood extracts by TLC were unsuccessful.

TABLE III

Sample Weights, Extract Weights and Percentages of Extractives

I. Sample #	II. Treatment Descrip.	III. Sample wt.	IV. Extracted Residue Weight			V. Extracted Residue as % sample wt.			VI. Extracted Residue as % test medium		
			chlfm.	acetone	H_2O	chlfm.	acetone	H_2O	chlfm.	acetone	H_2O
101	Control bark	20.0 g			ND*			ND			
102	Inoc. bark	15.6			ND			ND			
203	Control bark	54.5			ND			ND			
204	Inoc. bark	24.0			ND			ND			
301	Excised bark	120.4			ND			ND			ND
302	Excised sapwd.	191.8			ND			ND			ND
303	Exposed bark	56.4			ND			ND			ND
304	Control bark	46.4			ND			ND			ND
305	Exposed sapwd.	67.2			ND			ND			ND
306	Control sapwd.	109.3			ND			ND			ND
407	Excised sapwd.	63.0	0.47g			0.75%			3.0%		
508	Exposed bark	25.03	0.09			0.36			1.2		
509	Bk. under 508	39.69	0.13			0.33			1.1		
510	Control bark	50.73	0.17			0.34			1.1		
511	Excised bark	39.34	0.11			0.28			0.9		
512	Exposed sapwd.	52.83	0.11			0.19			0.6		
613	Control bark	56.50		0.536g	3.24g		0.95%	5.74%		0.56%	10.4%
614	Control sapwd.	45.64		0.134	2.75		0.29	6.03		0.32	8.8
615	Exposed bark	31.46		1.896	1.75		6.04	5.57		2.23	5.6
616	Exposed sapwd.	40.92		0.212	0.30		0.52	0.73		0.31	0.96

*ND--Not Determined

Preliminary attempts indicated that several heartwood compounds were separated on silica-gel developed with a solvent of chloroform:t-butanol (10:1; v/v). However, this system did not separate tropolones, all of which streaked. For this reason, the system was abandoned.

All tests made to detect the presence of thujaplicins in bark and sapwood samples, after the method of MacLean and Gardner (1956), produced negative results. This test easily detects 100µg of tropolone present in a 10 ml solution of n-hexane. At this range of sensitivity, assuming thujaplicins were efficiently extracted, this test would have detected the presence of thujaplicins in samples at 0.01 percent by weight.

Bioassays of extracted test substances were of three types--mycelial growth rate, spore germination, and extract additions. In all cases no differences in effects were observed between treated or control sample extracts, either for sapwood or bark. No toxic effects were observed in any instance, and water-extracted compounds from both the sapwood and bark enhanced mycelial growth rate over that on unamended medium.

Table IV lists the results of the controlled decay test performed on sapwood and bark samples exposed for 6 weeks and their controls, all previously extracted with acetone and hot water. These results are expressed as actual weight losses and as percentages of weight lost from each group. Table V lists the relevant statistical parameters describing the percentages of weights lost from these samples. The calculated means, variances, and number of observations are listed for each of the blocks of the four samples incubated on 11 week old cultures of F. annosus, for samples incubated on 3 week old cultures, and for the combined data. Table V also lists calculated t values which compare the mean weight losses of control bark and sapwood with exposed bark and sapwood for each of the three blocks of observations.

TABLE IV

Weight Losses after Decay of 6 Week Samples

CALCULATION		SAMPLE--- #613 Control bark	#615 6-week bark	#614 Control spwd.	#616 6-wk. spwd.	F. annosus CULTURE AGE
OVEN DRY WEIGHT	Trial 1	1.5829 g	0.6657 g	1.0562 g	1.1160 g	11 weeks
	2	1.5949	0.8415	0.8842	1.0162	
	3	1.4696	0.7469	0.8717	1.1342	
	4	1.6289	0.6533	1.0045	0.9360	
	5	1.7012	0.7553	1.0875	0.9207	3 weeks
	6	1.3227	0.7347	0.8692	1.1875	
	7	1.4644	0.6604	1.0280	0.9579	
WEIGHT LOSS	Trial 1	0.2552 g	0.0624 g	0.0570 g	0.0179 g	11 weeks
	2	0.2189	0.0765	0.0436	0.0094	
	3	0.2118	0.0715	0.0371	0.0204	
	4	0.2607	0.0671	0.0435	0.0266	
	5	0.2759	0.0773	0.0324	0.0545	3 weeks
	6	0.1926	0.0737	0.0478	0.0678	
	7	0.2272	0.0618	0.0464	0.0406	
% WEIGHT LOSS	Trial 1	16.12 %	9.37 %	5.39 %	1.60 %	11 weeks
	2	13.72	9.09	4.93	0.92	
	3	14.41	9.57	4.25	1.79	
	4	16.00	10.27	4.33	2.84	
	5	16.21	10.23	2.97	5.91	3 weeks
	6	14.56	10.03	5.49	5.70	
	7	15.51	9.35	4.51	4.23	

TABLE V

Comparison of Weight Losses after Decay

CULTURE BLOCK	STATISTIC	SAMPLE---- #613 Control bark	#615 6-week bark	#614 Control spwd.	#616 6-wk. spwd.
11 week	n	4	4	4	4
	$\bar{x}$	15.06 %	9.58 %	4.72 %	1.78 %
	s^2	1.41	0.25	0.29	0.63
	t (6 df)	8.51**		6.13**	
3 week	n	3	3	3	3
	$\bar{x}$	15.42 %	9.87 %	4.32 %	5.28 %
	s^2	0.69	0.21	1.62	0.84
	t (4 df)	11.71**		1.33	
All-- 11 & 3 wk. combined	n	7	7	7	7
	$\bar{x}$	15.21 %	9.70 %	4.55 %	3.28 %
	s^2	0.97	0.22	0.73	4.08
	t (12 df)	13.37**		1.54	

**- Significant at 0.01 probability level.

Tablulated Values of t

df	t(0.01)	t(0.05)
4	4.604	2.776
6	3.707	2.447
12	3.055	2.179

These values indicate that there is less than a one percent chance that the observed differences arose due to chance in four of the six comparisons.

DISCUSSION

This investigation tested whether the synthesis of fungitoxic compounds is initiated upon injury and inoculation of sapwood or bark of western redcedar and, if so, whether these toxic compounds are heartwood extractives normally not present in the sapwood or bark. The experimental results indicate four conclusions which provide the basis for an evaluation of this hypothesis. This evaluation in turn provides certain implications concerning a mechanism of disease resistance in western redcedar.

I. Physiological changes were observed in exposed samples which are analogous in some respects to those accompanying reaction zone formation in the sapwood of pine. The physical appearances of the 6 week samples of bark and sapwood indicate the initiation of changes. Along with darkening of color, a pronounced increase in resin exudation was noted over the exposed surfaces. This observation may explain the results of Table III which shows that greater amounts of acetone extractable substances were recovered for samples exposed for 6 weeks than for controls.

The presence of resins on exposed surfaces is unusual, since western redcedar does not contain resin canals nor is it capable of forming traumatic resin canals (Brown et al., 1949). The absence of traumatic resin canals in exposed sapwood samples collected in this investigation was verified by microscopic examination.

Since a network of resin canals is present in pine (Brown et al., 1949), resinosis accompanying reaction zone formation can occur within hours. Although the increased amounts of resins detected on samples in this study arise after a much greater interval and from a different transport system, a basic similarity in macroscopic changes accompanying injury exists between pine

and cedar.

The presence of the blue-stain fungus on the surface of the sapwood seems to be inconsistent with the formation of a reaction zone. However, it is possible that this organism became established before initiation of the reaction. It is also possible that the increased resin flow and other changes associated with reaction zone formation in the sapwood were stimulated by the presence of the blue-stain fungus, although this is unlikely since parallel changes occurred in the bark where no blue stain fungus was present. A third possibility regarding the significance of the presence of the blue stain fungus is discussed under II.

II. Toxic compounds which are unextractable by either hot water or acetone are formed within 6 weeks both in the living bark and sapwood exposed on a tree as a result of injury. The results of Table V indicate that bark exposed for 6 weeks is significantly more resistant to decay by F. annosus regardless of culture age, while sapwood exposed for 6 weeks is significantly more resistant to decay by 11 week old cultures of F. annosus than the control but not resistant to decay by 3 week old cultures of F. annosus. Since both controls and exposed samples were extracted first with acetone and then with 90-95°C water, it is probable that a large percentage of the normal extractable substances were removed by this treatment. Therefore percent weight losses probably represent a close approximation of percentage decay of lignin and cellulose and do not represent metabolism of carbohydrates in any of the samples. Since it is extremely unlikely that increases in decay resistance arose due to host-mediated structural alterations of tissues formed before wounding, a toxic factor(s) was formed and deposited in the bark and sapwood which caused the observed differences in decay resistance between exposed samples and controls. Any physical changes of samples that might arise from the extraction

procedure, such as permeability changes, might quite possibly alter susceptibility to decay, but could not account for the observed differences in decay resistance between samples and controls (See Tables IV & V).

Bark samples exposed for 6 weeks resisted decay regardless of colony age of the decay fungus, while sapwood samples exposed for 6 weeks resisted decay by 11 week old colonies, but were less able to resist decay by 3 week old colonies. Thus, colony age of F. annosus is a significant factor in determining weight losses in the sapwood samples, but not in the bark samples. A possible explanation for this unexpected result is that the concentration of toxic factor in the sapwood samples was at the threshold level needed to produce inhibition in vitro, so that the younger, more vigorous colonies of F. annosus were able to overcome the toxic effects more quickly than the older, less vigorous colonies of the same pathogen. Apparently the toxin concentration in the bark samples was greater than in the sapwood samples, since colony age does not significantly affect decay in the bark. Since reaction zone formation may be limited to only a few cell layers below the exposed surface, samples were cut away in thin strips to minimize dilution with unaffected portions of sapwood and bark. Since it was possible to collect thinner layers of bark than sapwood due to the cutting characteristics of each, this dilution factor may be directly responsible for the lesser toxicity exhibited by the sapwood sample. An alternate explanation of the lesser toxicity is that the toxic factor was present in vivo at a lower concentration in the sapwood than in the bark. The presence of blue-stain fungus on the sapwood but not bark samples exposed for 6 weeks tends to support this alternate explanation if growth of the fungus was not inhibited after initiation of the reaction zone in the sapwood.

III. No toxic compounds extractable by hot water, chloroform, or acetone

were formed after any of the experimental treatments. This conclusion is based on the demonstrated complete lack of toxic effects exhibited by all extracts for all three types of bioassay on amended standard medium. This conclusion follows from these results provided the following four conditions are valid: 1) that the extraction procedure used would remove significant amounts of normal extractable substances. This condition is nearly a definition and needs no further comment. 2) That F. annosus is a good test organism and would have responded to any toxic effects had they been present. This condition is probably justified since F. annosus is not a common pathogen of western redcedar, even though the distributions of the two species overlap. Therefore, redcedar may possess some means of resistance to this widespread tree pathogen. In addition the differences in weight losses within collection block 6 after decay by F. annosus indicate that F. annosus was sensitive to the toxic factor present and would probably have responded to any extractable toxic effects, if they had been present. 3) That a high proportion of the exposed samples of bark and sapwood underwent physiological changes associated with formation of reaction zones. This condition is probably justified since the sampling technique was designed to minimize dilution of samples with unreacted tissues. The weight loss results indicate that this condition was met for the bark and the sapwood also, although perhaps to a lesser degree in the sapwood for the reasons discussed under II. 4) That the amount of extractable materials added to the standard medium represented a large enough percentage such that if any toxic factor was extracted, it would have been present in the media at concentrations roughly the same as those at which it occurs in the reacted tissues. Providing that the former three conditions are true, this condition is justified for any materials readily soluble in the extracting solvents, since the

values listed for collection blocks 4-6 in Table III indicate that the percentages by weight of extracted materials present in the test media were either greater or of the same order of magnitude as the corresponding percentages in all of the samples from these blocks.

IV. None of the normal extractable substances commonly recognized as contributors to heartwood decay resistance are formed in response to exposure in either the bark or the sapwood. In the case of tropolones, this conclusion is very definite because the analytical technique of MacLean and Gardner (1956) is highly sensitive, and the extraction procedures used would have removed a large percentage of any tropolones present that had been deposited in samples parallel to the manner of deposition in heartwood. If any heartwood compounds were formed and extracted from samples, these compounds were not fungitoxic in vitro.

Providing that the above four conclusions are justified, the following overall conclusion may be drawn: there is a mechanism of disease resistance in western redcedar bark and sapwood which is initiated in response to exposure resulting from injury. This mechanism involves the formation of reaction zones in the bark and sapwood containing a toxic substance which is neither a normal heartwood compound nor extractable by polar solvents. To the best of this author's knowledge this observation has not been made before for western redcedar, and no exactly parallel observations have been made for any other tree.

The presence in reaction zones of toxins in sufficient concentrations to produce fungal inhibition, automatically confers a certain amount of disease resistance to the host. However, the formation of toxins as demonstrated in this investigation does not provide the basis for a quantitative assessment of the contribution of this component to the overall disease resistance of

western redcedar. The weight losses observed after decay of samples from collection block 6 indicate the presence of a factor which reduced decay. Nevertheless, any inference drawn from these results can only be applied tentatively to natural host-pathogen interactions, since the results are derived from a contrived situation.

Although only further work will reveal the chemical nature of the toxic factor and the reason it is unextractable, experience in pulp and paper research has shown that some extractives are difficult to extract from pulp. Hillis et al. (1966) reports that a stilbene (3,3'-dimethoxy-4,4'-dihydroxystilbene), causing reddening of pine pulp, is strongly adsorbed so that its removal requires special extraction procedures. Since other stilbenes are well-known fungitoxins, this case is particularly interesting in light of phenomena observed for western redcedar. It is possible that a parallel exists between these two situations.

Determination of the significance of this mechanism of induced disease resistance in the sapwood and bark is dependent upon further knowledge of a variety of factors. Is this the only mechanism of resistance or are there additional systems superimposed--either chemical or physical? How important are host and environmental conditions in determining the occurrence or amplitude of this reaction? What factors or interactions occur on a microscopic and biochemical scale which control initiation? What is the nature of the toxic factor? Only carefully planned and executed experimentation can answer these questions.

Although unreported to this time, it is probable that phenomena similar to those observed in western redcedar occur in other members of the Cupressaceae and possibly in other gymnosperms as well.

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