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PHYTOALEXINS AND STRESS METABOLITES IN THE SAPWOOD OF TREES

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Key Word Index—Phytoalexins; stress metabolites; trees; sapwood; fungitoxicity; reaction zones; review.

Abstract—A wide range of organic compounds, many of them fungitoxic or fungistatic, appear in the sapwood of trees after wounding, injury or fungal attack. There is evidence that most of these compounds are formed by dying parenchyma cells and they therefore can be considered to be phytoalexins. In many cases such compounds accumulate in narrow 'reaction zones' which serve to impede further progress of pathogens.

INTRODUCTION

If, as has been claimed, the research impetus in plant pathology lags some ten to fifteen years behind that for mammals then our progress in understanding trees and their diseases must be regarded similarly when compared with our knowledge of herbaceous plants. This has not always been the case. As early as 1926 Swarbrick observed that wounding and subsequent microbial infection of sapwood resulted in the accumulation of induced secondary metabolites in a stained zone at the interface between dead infected tissue and healthy living tissue [1]. The secondary metabolites in the form of gums appeared first in the ray parenchyma cells at the expense of starch grains and later plugged the vessels and tracheids. He suggested that this accumulation of gum presented a barrier to further infection and its formation was "in some way connected with the effect of dead or dying cells upon the metabolism of the tissues in proximity to them".

Similar observations and conceptions led to the formulation in 1940 of Muller and Borger's theory of phytoalexins [2] and this has since been developed and extended particularly with regard to the soft tissue of herbaceous plants [3]. However, with the exception of some coniferous species, tree sapwood has received relatively little attention. The present paper reviews our knowledge of the production of phytoalexins and stress metabolites [4] by this tissue in response to infection, injury and damage.

Sapwood in trees is that part of the secondary xylem which contains living parenchyma cells and reserve materials [5, 6]. It is thus distinct from heartwood which forms in the inner layers of many mature trees and consists of that part of the xylem in which all the cells are dead and where the reserve materials have been converted into heartwood constituents. Preformed heartwood constituents, even where shown to be antifungal, are not considered in detail in this paper. Neither are induced metabolites of the phloem, bark, seeds, fruits and leaves of trees. Induced sapwood compounds are listed according to structure and probable biogenetic origin and pertinent aspects of their isolation, distribution, biosynthesis and toxicology are cited. Wider implications of their role in resistance to pathogens, particularly as constituents of 'reaction zones', are discussed separately.

STRUCTURES AND DISTRIBUTION

Stilbenes

The stilbenes pinosylvin (**1**) and pinosylvin monomethyl ether (**2**) occupy a central position in the history of tree phytoalexins. The important work of Jorgensen and his co-authors [7–9] demonstrated as early as 1961 that the compounds were induced non-specifically in the living cells of *Pinus resinosa* (red pine) sapwood following injury or fungal attack. Taken with their known antifungal properties [10] this established the stilbenes as 'first generation' phytoalexins although this has not always been recognized. Their excellent biosynthetic work confirmed that, in accord with expectations, phytostilbenes are derived from a phenylpropanoid precursor linked with three acetate units and that the fifteenth carbon atom is lost from the acetate moiety. The techniques used for the biosynthetic studies involved wounding excised pine branches, applying radiolabelled chemicals to the wounds, treating with solid carbon dioxide and then incubating for several days. Good incorporation rates were obtained and this may prove a most useful method for other biosynthetic studies in trees, particularly if the powerful technique of double labelling ^{13}C NMR [11] can be used to complement radiolabelling methods.

Pinosylvin and its monomethyl ether have been found to be induced in the sapwood of *Pinus* species by many fungi. These include *Fomes annosus* (*Heterobasidium annosum*) [12, 13], *Amylostereum chailletii* (the symbiotic fungus of the wood-wasp *Sirex noctilio*) [14] and the blue-stain fungus *Ceratocystis minor* [15]. Pinosylvin dimethyl ether (**3**) accumulated in *Pinus sylvestris* (Scots pine) with

the phenylpropanoid derivative eugenol methyl ether and several terpenes after infection with *Peridermium pini* [16]. Other known sapwood phytoalexins are resveratrol (**4**) [15], oxyresveratrol (**5**) and 4-prenyloxyresveratrol (**6**). The last two compounds were found in the xylem of *Morus alba* (mulberry) shoots infected with *Fusarium solani* f.sp. *mori* [17, 18].

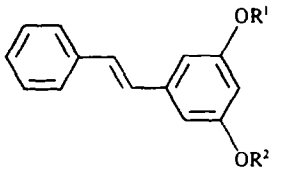
The role of phytostilbenes in decay and disease resistance has been comprehensively reviewed [19].

Lignans

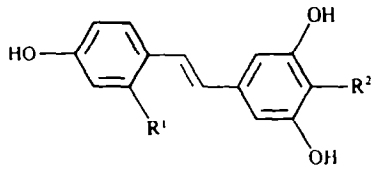
Lignans are dimers of phenylpropanoid units linked by the central carbon atoms of the side chains and they display a variety of biological activities [20]. There are several reports of lignans appearing in sapwood subsequent to fungal attack. Iso-olivil (**7**) accumulated in *Prunus jamasakura* following infection by *Coriolus versicolor* [21], and in *Picea abies* (Norway spruce) infected with *Fomes annosus* there were high levels of hydroxymatairesinol (**8**) (major component), other lignans and a 'simple' phenol, 4-methylcatechol, in a 'reaction zone' [22–25]. Hydroxymatairesinol was found to inhibit the growth of *Fomes annosus* in one investigation [22] but this was not confirmed in another [23]. Syringaresinol (**9**) and its dimethyl ether (**10**) accompanied aporphine alkaloids in injured sapwood of *Liriodendron tulipifera* (yellow poplar) [26].

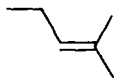
Alkaloids

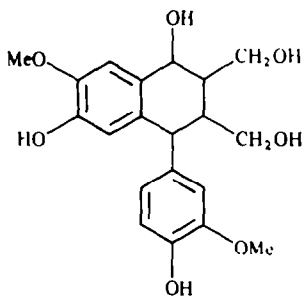
Injury, apparently caused by fire, to the tree stem of a specimen of *Liriodendron tulipifera* (yellow poplar) produced spectacular colour changes and stimulated the biosynthesis of eight phenolic and nine non-phenolic aporphine alkaloids in the discoloured sapwood [26]. The most abundant alkaloid was glaucine (**11**) which had antimicrobial activity against wood-inhabiting fungi [27].



	R^1	R^2
1	H	H
2	Me	H
3	Me	Me



	R^1	R^2
4	H	H
5	OH	H
6	OH	



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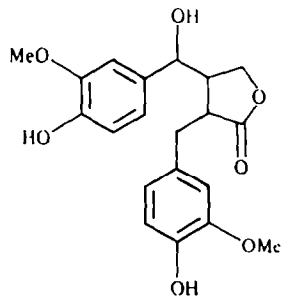

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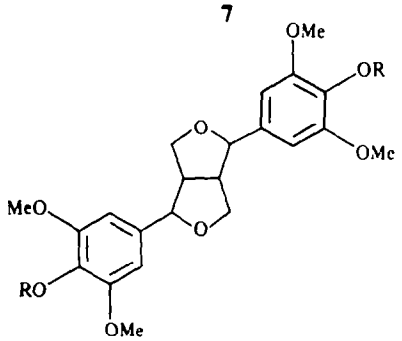
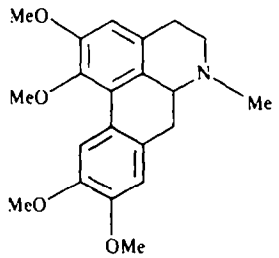


	R
9	H
10	Me



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Biaryls and derivatives

These have been discovered as sapwood phytoalexins relatively recently. Aucuparin (**12**) was isolated from the darkly pigmented interface between healthy *Malus pumila* (apple) sapwood and the stained wood colonised by the silver leaf pathogen, *Chondrostereum purpureum* [28]. It had previously been found [29] as a phytoalexin in the cortex of *Eriobotrya japonica* (loquat) shoots and its antifungal activity described [30].

Structurally related to biaryls are dibenzofurans. The antifungal α-, β- and γ-pyrufurans (14, 15 and 16 respectively) were induced in sapwood of *Pyrus communis* (perry pear) branches after inoculation with *Chondrostereum purpureum* [31, 32]. The structure of γ-pyrufuran suggests that it might have been formed from α-pyrufuran by fungal metabolism but as it was also formed on wounding it is more likely to be of host origin [32]. Another dibenzofuran cotonefuran (**17**) was isolated from a specimen of *Cotoneaster lactea* suffering from an unidentified wilt disease [33]. Because of the complexity of the substitution pattern, X-ray analysis was employed for complete structural elucidation.

The presumed biosynthetic connection between biaryls and dibenzofurans is strongly supported by the isolation of 2'-hydroxyaucuparin (**13**) and eriobofuran (**18**) from loquat shoots and leaves respectively [34]. The biaryl nucleus is uncommon in plants and its biogenesis is unknown. It may be that one ring originates from shikimate with the other arising from the acetate pathway. Alternatives are complete polyketide origin or the derivation of both rings from shikimate via oxidative coupling of hydroxybenzoic acids with decarboxyation [35]. The oxygenation patterns may afford some clues.

Flavonoids

A number of flavonoids of various structural types are known to be induced in sapwood. Thus an un-rearranged flavonoid broussonin C (**19**) was found together with the flavan broussin (**20**) in wounded xylem of *Broussonetia papyrifera* (paper mulberry) [36]. A flavanone, pinocembrin (**21**), accumulated in the damaged sapwood of *Pinus radiata* [14] and also in the 'reaction zone' of *Pinus taeda* infected with *Fomes annosus* [12]. The amount of the dihydroflavonol dihydroquercetin (taxifolin, **22**) increased in the roots of *Pseudotsuga menziessi* (Douglas fir) attacked by *Poria weirii* [37] while a 3-year-old branch of *Rhus succedanea* contained fluorescent material in a middle growth ring which appeared to arise from injury to that ring. A glycoside of the flavonol fisetin (**23**) was identified [38].

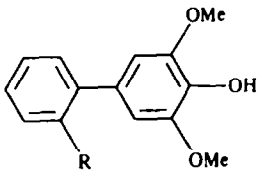
Isoflavonoids, despite their importance as phytoalexins in herbaceous members of the Leguminosae [39], have rarely been found as induced metabolites in sapwood. However, they do occur in the heartwood of leguminous trees [40] and in view of the relationship discussed in the following section (see Table 1), it may be anticipated that some will be discovered in the sapwood of such trees.

Phenylpropanoids

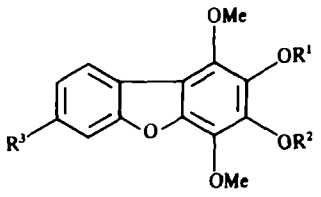
Few simple phenylpropanoids have been identified as induced stress metabolites in sapwood although their derivatives and dimers include the stilbenes, flavanoids and lignans. However, the C-9 coumarin scopoletin (**24**) was isolated from the 'pathological heartwood' produced in *Prunus domestica* (plum) by the silver leaf fungus *Stereum* (now *Chondrostereum*) *purpureum* [41]. The accumulation of eugenol methyl ether (**25**) with pinosylvin dimethyl ether and several terpenes in *Peridermium pini* infected *Pinus silvestris* (Scots pine) [16] has already been mentioned.

Terpenoids

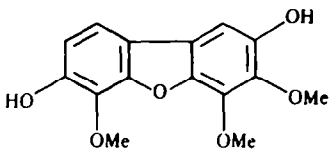
Monoterpenes. Oleoresin, which occurs in members of the Pinaceae, is a complex antimicrobial hydrophobic



	R
12	H
13	OH



	R¹	R²	R³
14	H	Me	H
15	Me	H	H
16	H	Me	OH



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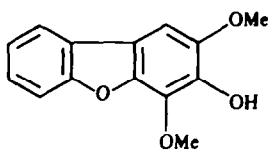

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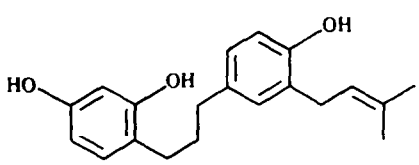

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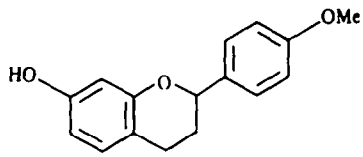

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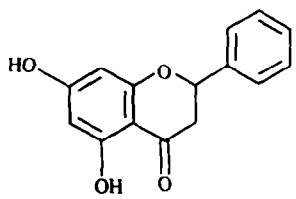

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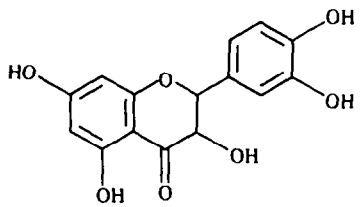

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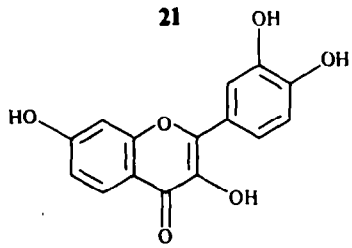

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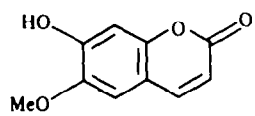

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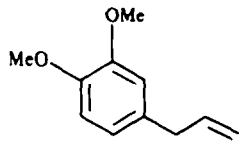

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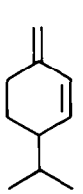
Table 1. Sapwood phytoalexins also known as heartwood constituents

Compound	Sapwood source	Heartwood source
Pinosylvin (**1**)	*Pinus resinosa* [7]	Numerous *Pinus*
and pinosylvin	*Pinus taeda* [12]	spp. [83]
mono methyl ether (**2**)	*Pinus nigra* [13]	
	Pinus radiata [14]	
Oxyresveratrol (**5**)	*Morus alba* [17]	Several *Morus* spp. [84]
Hydroxymatairesinol (**8**)	*Picea abies* [22]	*Picea abies* [22]
Syringaresinol (**9**)	*Liriodendron tulipifera* [26]	*Liriodendron tulipifera* [85]
and syringaresinol dimethyl ether (**10**)		
Glaucine (**11**)	*Liriodendron tulipifera* [26]	*Liriodendron tulipifera* [26, 85]
Aucuparin (**12**)	*Malus pumila* [28]	*Sorbus aucuparia* [86]
Mansonones E (**27**)	*Ulmus hollandica* [52]	*Mansonia altissima* [87]
and F (**28**)	and *Ulmus glabra* [57]	
7-Hydroxycalamenene (**29**)	*Ulmus glabra* [57]	Several *Ulmus* spp. [88].
	and *Tilia europea* [58]	
7-Hydroxycadalenal (**30**)	*Ulmus glabra* [57]	*Ulmus rubra* [89]

mixture of resin and fatty acids in a volatile oil composed mainly of monoterpenes [42–44]. Wounds and fungal infections of pine sapwood are accompanied by extensive resinosis, much of which is due to the flow of oleoresin from ruptured resin ducts. However, in *Pinus contorta* (lodgepole pine) sapwood attacked by the bark beetle *Dendroctonus ponderasae* and its associated microorganisms, Shrimpton has suggested [45] that the increase in monoterpenes, particularly β-phellandrene (**26**), is due to the slow death of parenchyma cells. In *Pinus elliottii* (slash pine) it appears that the process may be chemically stimulated by sublethal doses of the herbicide paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride) since 25-fold increases in light volatiles and 10-fold increases in resin acids were observed twenty-four months after injection [46]. Oleoresin production in *Pinus resinosa* was stimulated by paraquat and also by Ethrel (2-chloroethyl phosphonic acid) [47], while histochemical studies of *Azadirachta indica* sapwood confirmed that paraquat greatly enhanced metabolic activity in parenchyma cells [48]. The qualitative composition of monoterpenes in *Picea abies* altered considerably after injury [49]. The significance of these changes is unknown and the role of oleoresin in resistance to microorganisms and insects is complex and has been discussed at some length by several authors [13, 42, 43, 44, 50, 51].

Sesquiterpenes. The first sesquiterpene phytoalexins to be isolated from trees were the mansonones E (**27**) and F (**28**) from *Ulmus hollandica* branches infected with the Dutch elm pathogen, *Ceratocystis ulmi* [52]. This notorious vascular wilt ascomycete grows in the outer xylem vessels causing a dark brown intermittent streaking of the wood beneath the bark [53]. The trees wilt and eventually die, possibly as a result of the release of fungal toxins [54]. An 'aggressive strain' of *Ceratocystis ulmi*, apparently introduced into Europe from North America in the late 1960s, has been particularly damaging, causing widespread death of trees and a dramatic alteration of the landscape, particularly in Britain. There does not appear to be a correlation between the accumulation of the mansonones and resistance in that the amounts in resistant clones of *Ulmus hollandica* were not especially high while the equally susceptible *Ulmus hollandica* cl. Belgica and *Ulmus americana* had differing levels [55]. However, another report suggests [56] that more mansonones A, C, E, F and G accumulate after inoculation of *Ulmus americana* with the non-aggressive strain of *Ceratocystis ulmi* than with the aggressive strain. Mansonones E and F were quantitatively the most important.

A total of eight antifungal cadinane-type sesquiterpenes were identified in *Ulmus glabra* (Wych elm) after treatment with the aggressive strain of *Ceratocystis ulmi* [57]. These included the mansonones E and F and also 7-hydroxycalamenene (**29**). The same eight compounds were also found after inoculation with the basidiomycetes *Coriolus versicolor* and *Chondrostereum purpureum* but there were quantitative differences with these fungi in that the yellow coloured aldehyde 7-hydroxycadalenal (**30**)



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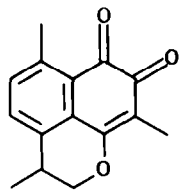

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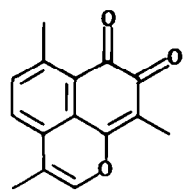

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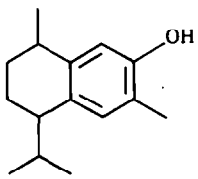

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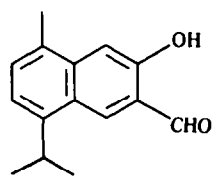

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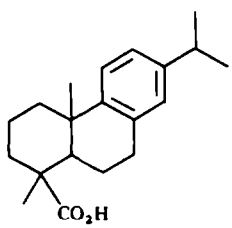

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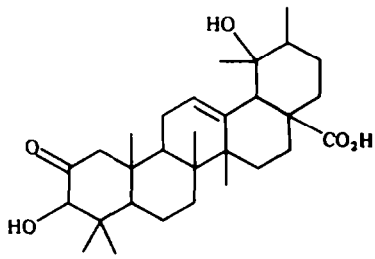

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predominated. All compounds were inhibitory towards *Cladosporium cucumerinum*.

(−)-7-Hydroxycalamenene (**29**) was also isolated in high yield from the trunk of *Tilia europea* (European lime) apparently colonized by the white-rot fungus *Ganoderma applanatum* [58]. It was found in the narrow pigmented boundary zone between the decayed inner core and the healthy sapwood.

Diterpenes. Diterpenes constitute a large portion of the resin acids of coniferous species and there are several reports of an increase on wounding. For example, the large increase in heptane-soluble compounds following mechanical wounding of *Picea glauca* (white spruce) was found to be largely due to diterpene acids of which dehydroabietic acid (**31**) predominated [51].

Triterpenes. The triterpene acid 3β,19α-dihydroxy-2-oxours-12-en-28-oic acid (**32**) was recently found to accumulate at the pigmented interface between healthy *Malus pumila* sapwood and stained wood arising from infection with *Chondrostereum purpureum* [28]. The *in vitro* fungitoxicity of the compound was low and its role in resistance remains uncertain.

Unknowns

There are many early reports of unidentified compounds increasing in wood after injury or damage. More recent examples include the increase in total polyphenols and leucoanthocyanins in cuttings of *Salix alba* (cricket bat willow) infected with the bacterium *Erwinia salicis* [59], the induction of phenolic phytoalexins in stems of *Populus tremuloides* (aspen) by *Hypoxylon mammatum* [60–63] and the accumulation of three unidentified chloroform-soluble compounds in wounded wooden discs of *Pinus resinosa* [64, 65]. Also samples of wood taken from *Acer rubrum* (red maple) and *Populus deltoides* × *P. trichocarpa* (hybrid poplar) had enhanced phenol levels in the brightly coloured marginal zone between healthy sapwood and discoloured wood resulting from drill bit wounds [66]. The phenols in the marginal zone differed from those in sapwood and discoloured wood in solubility, UV spectra and chromatographic behaviour.

ROLE IN RESISTANCE

The aforementioned compounds were found to accumulate in sapwood under a very wide range of conditions, often with minimal biological detail reported. However, the most common and important situations involved attack by fungal pathogens (Fig. 1), either after entry through wounds or by encroachment from infected heartwood. Artificial inoculation with pathogens sometimes produced similar symptoms.

The best and most widely applicable description of these fungus–sapwood interactions is due to Shain. He observed [22, 67, 68] that in infections of *Fomes annosus* in loblolly pine and Norway spruce the stained or decayed wood was separated from the sound functional sapwood by a narrow resin-soaked necrotic band which he called the 'reaction zone'. Shain suggested that reaction zones are formed in advance of pathogens as a result of the death of parenchyma cells with a concomitant increase in fungitoxic phenols (phytoalexins). The shape was typically conical with increasing penetration of the fungus towards the centre and this could be attributed to a decrease in metabolic activity of the parenchyma cells with

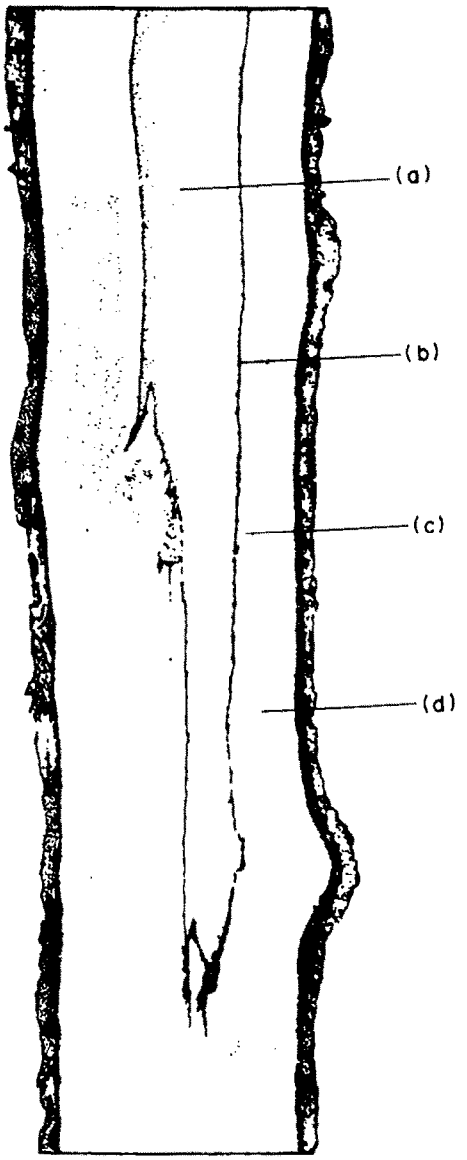

Fig. 1. Typical fungus-sapwood interaction, in this example a young branch of *Pyrus communis* (perry pear) infected with *Chondrostereum purpureum* (silver leaf pathogen) [31, 32, 69]. Nomenclature is due to Shain [22, 67, 68]. (a) Infected wood, (b) reaction zone, (c) transition zone, (d) healthy sapwood. Antifungal compounds, in this example α-, β- and γ-pyrufurans, accumulate in the pigmented reaction zone and impede further progress of the fungus.

increasing distance from the cambium. The response was seen as dynamic, keeping pace with the margin of wounded or infected tissue, with the phytoalexins impeding but not necessarily stopping the pathogen. In *Pinus nigra* (Corsican pine)–*Heterobasidium annosum* infections, Prior found a significant inverse correlation between the extent of infection and the amounts of pinosylvin and its monomethyl ether in the reaction zone [13]. A similar correlation has been observed [69] between the progress of *Chondrostereum purpureum* in *Pyrus com-*

munis (perry pear) wood and the accumulation of the pyrufurans. Reaction zones generally correspond to a number of other terms used in tree pathology including pathological heartwood, protection wood, wound-indicated discolouration [68], marginal zones [66] and pigmented boundaries or interfaces [31, 32, 58].

Shain also noted that between the necrotic reaction zone and the healthy sapwood a dry pale coloured 'transition zone' could frequently be observed. This he suggested contained living metabolically active parenchyma cells in which starch was being rapidly degraded.

It is possible to relate these observations to modern theories of phytoalexin elicitation [70] and to suggest a feasible, although hypothetical, model for the progress of a fungal pathogen through tree sapwood. Thus fungal hyphae may be envisaged to secrete diffusible enzymes, toxins or 'elicitors' which advance ahead of the pathogen and disrupt or damage the ray and axial parenchyma cells of the host. This leads in turn to the release of 'constitutive elicitors', possibly membrane or cell wall fragments [71] which stimulate *de novo* synthesis of enzymes leading to phytoalexin production. In the process the damaged cells become pigmented and eventually die. The earlier stages of this sequence of events may occur in the 'transition zone' while the accumulation of phytoalexins in dead cells takes place in the 'reaction zone'. Formation of reaction zones depends on many things including the genetics of the host tree, its age and vigour, the virulence of the pathogen, seasonal, climatic and nutritional factors and the presence of other micro-organisms where a 'succession' is involved [72, 73].

The reaction zone is presumed to be in direct contact with the fungus and will so impede its further progress. A critical factor then is the fungitoxicity of the phytoalexins and the capacity of the fungus to metabolize and detoxify them. In herbaceous plants correlations between fungal metabolism of phytoalexins and pathogenicity have been sought [74] and analogous experiments with tree pathogens are needed. Already it is known that laccase-producing fungi rapidly metabolize and inactivate fungitoxic stilbenes when grown with sub-lethal doses in culture [75].

It is important to stress that the sequence of sound sapwood–transition zone–reaction zone–infected or decayed wood is a dynamic one. However, unless the pathogen completely colonizes the tree there will be a stage at which its progress is arrested. It will then be contained within a chemical 'blockade' and, being starved of nutrient, will eventually die although this may take some considerable time. In a large tree there may be many interactions of this type at various growth stages within the xylem.

Wider aspects of the defence of trees to decay have been dealt with by Shigo and his colleagues in their hypothesis known as CODIT (Compartmentalization of Decay in Trees) [76–78]. This is an elegant model, often beautifully illustrated, which interprets defence in terms of walls. Walls 1, 2 and 3 are said to resist respectively the vertical, radial and lateral spread of pathogens while wall 4, the 'barrier zone', is formed by the vascular cambium and isolates the new wood formed after infection. In this model, phytoalexins are probably best envisaged as 'chemically strengthening' walls 2 and 3 via the axial and ray parenchyma cells after infection [78]. Wall 4, the strongest wall, has been shown in *Quercus robur* (oak) challenged with the decay fungus *Stereum gausapotum* to consist of heavily suberized cells where these are adjacent to decayed sapwood [79, 80]. While outside the scope of the present paper, suberization [81] of cell walls must be considered as a very effective physical barrier to fungal penetration, perhaps complementing the role of phytoalexins.

As mentioned in the Introduction, the numerous examples of fungitoxic heartwood constituents are not dealt with here. However, it is pertinent to note that such compounds are believed to be formed *in situ* by dying parenchyma cells in processes similar to those occurring in reaction zone formation [82]. Numerous examples are known where induced sapwood constituents occur as preformed heartwood compounds, in the same or other species (Table 1). However, important quantitative differences may occur, exemplified by the much larger pinosylvin/pinosylvin methyl ether ratio found in *Pinus taeda* reaction wood as compared with its heartwood [12].

SUMMARY

In presenting this short review of sapwood phytoalexins and stress metabolites we hope both to have summarized present knowledge and to have demonstrated challenges and opportunities. Reaction zones, easily dissected from severed branches or stems, offer considerable scope for finding new natural products in good yields and this in turn generates problems of structural elucidation, synthesis and biosynthesis. In establishing the role of the compounds in resistance to pathogens there is a great need for a combined chemical–biochemical–plant pathological approach, and modern techniques of microanalysis, microscopy, Raman microprobe, histochemistry and immunohistochemistry may usefully be employed. The relationship between the changes occurring during reaction zone induction and those involved in normal heartwood formation and in such phenomena as 'heartwood shakes' [82] is still very unclear and deserves further investigation.

There is also the question of practical exploitation. Already knowledge of chemical defense has led to new ideas for the timing and technique of pruning in order to minimize colonization by pathogens [90]. Reaction zone formation and compartmentalization processes are believed to be under strong genetic control [77] which may be reflected in varietal differences [91]. This could prove useful in breeding disease resistant varieties of trees; a knowledge of the gene products involved in resistance should aid greatly the genetic engineering of such varieties. It may also be anticipated that, as with herbaceous plants [92], chemicals and perhaps bio-control agents will be found which, while not antimicrobial *per se*, stimulate the formation of chemical defense and hence can be used for practical disease control. The use of paraquat to stimulate resinosis in pines appears to be an example whereby a natural defensive response has been chemically stimulated to produce enhanced yields of commercially valuable material, in this case rosin and turpentine [44].

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