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PROCEEDINGS OF THE PHYTOCHEMICAL SOCIETY

A meeting of the Society was held at the Department of Botany, University of Hull, on 12 *and* 13 *September*, 1968 *when the following papers were presented, under the general title*

Biochemical Aspects of Host Parasite Relationships in Plants

Problems of Cell Wall Hydrolysis

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BECAUSE facultative parasites spend much, if not most of their life as saprophytes, it can be assumed that they will be able to degrade all the main components of cell walls and, indeed of all other parts of plants that return to the environment after death. It is, therefore, of considerable interest to learn why there are so many diseases caused by parasites of this type in which cell wall degrading enzymes either seem to function only for a limited time or almost not at all. Even in soft rots where the action of these enzymes is so striking it is possible by altering certain conditions to produce dry, slowly spreading lesions in place of the soft, rapidly extending rot.

Little is known about what decides whether or not cell wall degrading enzymes become dominant in pathogenesis although there is some evidence that inhibitors, phenolic in nature, are important in some diseases. But in most diseases it is likely that there is a balance between the accumulation of substances that prevent growth of the pathogen and the enzymes that macerate and kill plant cells and so prevent them from producing such substances. Whether or not a progressive, rapidly spreading lesion develops is probably decided during the early growth of the pathogen in its host.

There also remain a number of difficult problems for the biochemist, mainly the identification of the substrates in cell walls that are degraded during maceration. It now seems probable that certain hydrolases and lyases that split chains of soluble pectic substances can macerate plant tissues and do so in the absence of enzymes that act upon other cell wall components. But we still do not know how these enzymes act on insoluble substrates in native cell walls to cause maceration. This is a formidable problem because of the variety of possible interactions between pectic substrates, which themselves are complex mixtures, and other polymers, salts, and other substances in cell walls. There also remains the problem of how degradation of the cell wall leads to death of the enclosed protoplast.

The Role of Pectolytic Enzymes of Sclerotinia fructigena *in Fruit Rot*

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IN FRUITS of apple and pear, the fungus *Sclerotinia fructigena* causes a characteristic firm brown rot. The fungus bursts through the fruit skin, forming conidial pustules, and the presence of these ruptures leads to tissue desiccation. Infected fruits frequently persist on the tree throughout the winter, giving rise to mummies on which fresh conidial pustules are formed during the following season.

Electron microscope studies show that, at the periphery of the lesion, there is a plasmolytic effect on the cells, which are soon killed. Loss of membrane semipermeability leads to phenolic oxidation and the typical

brown colour. Intercellular hyphal penetration is accompanied by localized wall degradation. At a later stage intracellular hyphae are present, and these can force the dead protoplasts, still intact, across the cell.[1]

In confirmation of the findings of Cole and Wood,[2] extracts of the rotted tissue contain a high level of pectin methyl esterase (which could be of host origin), some polygalacturonase and no cellulase. α-L-Arabinofuranosidase was present in extracts of rotted apple only.

In culture filtrates of the fungus grown on a pectate medium, a similar pattern of enzymes is present, together with pectin methyl-*trans*-eliminase, which has a pH optimum in the range 7 to 8, and is virtually inactive at pH 4. The absence *in vivo* of this enzyme, which is the component responsible for tissue maceration by the filtrate,[3] and of cellulase, probably accounts for the localization of wall degradation and the consequent persistence of the rotted fruits. The continuing structural integrity of the plasmalemma, even in penetrated cells, is in accord with the inability of culture filtrates to lyse isolated plant cell membranes. The biochemical mechanism by which the fungus kills the cells—probably involving an osmotic effect—remains unexplained.

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The Role of Pectolytic Enzymes in Post-harvest Decays of Citrus Fruit

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Citrus fruits are subject to post-harvest decays caused by a number of fungi of which the most important are *Penicillium digitatum*, giving green mould rotting, and *P. italicum*, the blue contact mould which cause considerable economic losses in transit. Other Penicillia cause loss in certain parts of the world while strains of black aspergilli, *Oospora* (*Geotrichum*), *Phytophthora*, *Botrytis*, *Alternaria*, *Trichoderma* and *Phomopsis* also cause citrus fruit decays. The present discussion is limited to *Penicillium digitatum*, *P. italicum* and a black *Aspergillus* (*A. fonsecaeus*).

These organisms are wound parasites and thus show no special provision for penetration of the cuticular defences of the host. Their pathogenicity thus depends on their ability to invade host citrus tissue with concomitant tissue breakdown and to survive in the presence of any toxic substances present in the host.

In initial studies *P. digitatum* was compared with *P. notatum* which does not produce citrus rot. While both organisms can grow on cut surface of albedo and the pulp vesicles *P. notatum* did not invade undamaged cells and produced no softening of surrounding tissue as found with *P. digitatum*. Pectin methylesterase and polygalacturonase can be demonstrated in culture filtrates from both organisms. When culture filtrates were allowed to act on finely divided orange rind, galacturonic acid and glucose were detected by chromatography in reaction mixtures of both organisms while arabinose was present only in the reaction mixture with *P. digitatum* culture filtrate. *P. digitatum* culture filtrate also caused maceration of orange rind discs. This suggested a possible association of an arabanase, with macerating activity in *P. digitatum* but by fractional precipitation of concentrated culture filtrate with ammonium sulphate followed by chromatography on ECTEOLA cellulose, after desalting on Sephadex, it was possible to separate the macerating factor from the arabanase activity. Macerating factor from *P. digitatum* was subsequently shown to be identical with pectin-*trans*-eliminase (P.T.E.). The fact that uronic acid monomer was not detected when partially purified P.T.E. acts on pectin is evidence that the enzyme is an endo-*trans*-eliminase causing a limited random cleavage of the polyuronide chain.

The production of P.T.E. and macerating activity by *P. italicum* and *A. fonsecaeus* has been examined under a limited range of culture conditions. The best yields from both organisms were obtained in bran cultures and aqueous extracts of the bran cultures were examined by the precipitation and chromatographic methods used for *P. digitatum*. Rather less P.T.E. is produced by *P. italicum* than by *P. digitatum* and there is evidence of macerating activity in some fractions not containing P.T.E.

With *A. fonsecaeus* little P.T.E. was produced and in the purification, fractions were found in which macerating activity appeared to be accounted for by the presence of a polygalacturonase only.

The properties of partially purified P.T.E. from *P. digitatum* and *P. italicum* are very similar in relation to pH optimum for P.T.E. and macerating activity, reaction on pectic acid, substrate affinity and heat inactivation. Chromatographic examinations of the reaction mixtures and their absorption spectra also gave similar results. Purified preparations of P.T.E. produce maceration on injection into oranges and P.T.E. activity has been demonstrated in fruit infected with both *P. digitatum* and *P. italicum*.

It is concluded that P.T.E. plays a major role in the rotting of oranges by *P. digitatum* and *P. italicum*. In *P. italicum* there is evidence of some maceration by enzymes other than P.T.E. while in *A. fonsecaeus* P.T.E. does not appear to play a major role in tissue breakdown.

Cell Wall Hydrolysis during Infection of Potatoes by Phytophthora infestans

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MOST plant pathogens produce enzymes which hydrolyse cell wall glycans, though the importance of these enzymes is open to question.[1] Previous investigations have given little evidence of the capacity of *Phytophthora infestans* to produce enzymes of this type.[2, 3]

In hydrolysates of potato tuber cell walls glucose and galactose were found to be the predominant sugars with successively smaller amounts of uronic acid, arabinose, xylose, mannose and rhamnose. Glucose was the only sugar detected in hydrolysates of the hyphal wall of *P. infestans*, suggesting that glucan is the major constituent of the wall as it is in other species of *Phytophthora*.[4]

Analysis of wall fractions from potato discs cut from tubers of a susceptible variety and inoculated with *P. infestans*, gave evidence of galactan degradation; a rapid accumulation of alkali-insoluble glucan presumably represented synthesis of fungal wall; other carbohydrate fractions increased as in the uninoculated tissue.

Culture filtrates of *P. infestans* were shown to contain an enzyme which liberates galactose from a potato pectin preparation.[5] This "galactanase" is apparently specific to β-1,4 linkages between galactose residues and acts by random cleavage. It will release galactose and galactose oligomers from tuber cell walls and has a slow macerating effect on tuber discs. Activity was also found in inoculated tissue where it may facilitate hyphal penetration of the intercellular substance.

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Respiration of Virus-Infected Leaves

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INFECTION of leaves with viruses, like many other stress conditions, leads to an increase in the "dark" respiration of the leaves. This increase, generally 10–50 per cent, depends on the plant and virus concerned, the physiological state of the plant, and also whether respiration is expressed on an area, wet weight or dry weight basis. The increases which accompany the formation of local lesions may differ qualitatively as well as quantitatively from those which occur in systemic infections.

A number of plausible explanations of these respiratory rises exist. They could, for example, result from an increased mitochondrial-type respiration produced through normal or abnormal control mechanisms, or they could be due to the development of a non-mitochondrial respiration based on some terminal oxidase other than cytochrome oxidase. The evidence available does not enable us to decide conclusively between these alternatives, although most attention is now being paid to factors which may, on infection, increase the normal respiratory processes. Recently evidence has been published[1] which suggests that when leaves of *Nicotiana glutinosa* are infected with tobacco mosaic virus, the increased respiration is due to an increase in the amount of mitochondrial material present in the leaf. However, the experimental procedure on which the evidence is based is open to a number of objections, and this has prompted a reinvestigation of the problem.[2]

Leaves of *N. glutinosa* were inoculated with TMV, and infection allowed to develop *in situ* or in discs cut from the leaves and floated on culture medium. Extracts were made from the leaves and discs when their respiration was greater than that of comparable healthy tissue; they contained similar amounts of protein

N, glycollic oxidase and mitochondrial protein N as extracts from uninfected tissue, but slightly less cytochrome oxidase and considerably more polyphenol oxidase. It was therefore considered that there is no satisfactory evidence for an increase in mitochondrial material accompanying the infection of *N. glutinosa* with TMV.

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Interactions between Venturia inaequalis *and Apple Leaf Tissue*

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AN APPLE cultivar resistant to the apple scab organism *Venturia inaequalis*, reacted within 24 hr to inoculation with conidia. The reaction took the form of increased production of fungistatic compounds, which when isolated and applied to susceptible plants conferred upon them a degree of resistance. The same compounds added to cultures of *V. inaequalis* growing in log phase caused alterations in the pigmentation of the cultures.[1]

V. inaequalis has been shown to release biologically active pigmented proteins into the extracellular liquor of stirred deep cultures.[2] Fungal protein was isolated from culture filtrates by alcohol precipitation, and fractionated by gel filtration. A subfraction thus obtained and introduced into the plant by petiole injection was able to influence solute flow in the host plant leaves by limiting the supply reaching the interveinal areas. Similar effects on host solute flow were observed in artificially inoculated plants during the development of lesions. Movement of solutes was followed by petiole injection of acid fuchsin or (^{14}C) D-glucose. Addition of a protein fraction to spore inoculum applied to a susceptible variety increased the incidence of disease several-fold compared with that on control plants receiving spore inoculum only. Protein from non-fungal sources had no effect on solute transport or incidence of disease.

The location of fungal protein in treated susceptible plants was examined. A solution of fungal protein was chemically labelled with fluorescent dye, and supplied in aqueous solution to detached apple leaves. Subsequent examination of fresh sections under the u.v. microscope showed that fluorescent protein was strongly adsorbed to the cell walls of vascular tissues. Similarly labelled non-fungal protein from a variety of sources was occasionally adsorbed, but in such cases the degree of adsorption was very weak compared with that shown by fungal protein. More detailed examination of plant material was done using the electron microscope. Fungal protein, previously treated with silver ion, was fed to detached susceptible apple leaves. Sections were taken (after fixing in formalin and embedding in epoxy resin) and shown to have electron dense areas in the cell wall lamellae, plasmodesmata, endoplasmic reticulum and other cell organelles. Silver accumulated heavily on ribosome-like particles attached to the endoplasmic reticulum. Sections taken from plant material injected in similar fashion with silver-treated bovine serum albumin did not show any accumulation of silver within the cells.

It is suggested that *V. inaequalis* has a highly developed and specialized mechanism for reorganizing the metabolism of the host plant in several respects. Solute transport appears to be partially diverted from healthy tissue to lesion areas, thereby enhancing fungal growth at the expense of the host. Further tentative evidence suggests that cell contents may be interfered with directly by fungal products although the fungus itself does not penetrate the host cells.

Acknowledgements are due to Dr. A. B. Beakbane and Miss M. M. Fuller for the electron microscopy, and to H. Hutchins and Mrs. J. Town for technical assistance.

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The Role of Toxins in Fusarium *Diseases*

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THE GENUS *Fusarium* contains many species or *formae speciales* which invade plants through their roots and develop in the vascular system. One of the results of such invasion is leaf flaccidity from which the diseases have become known as "Wilts" but other symptoms are epinasty, vascular discoloration, leaf yellowing and

necrosis and stunting of the plants. Some of these symptoms are thought to be caused by toxins secreted by the fungus and extensive studies have been made of those associated with invasion of the tomato by *F. oxysporum* f. sp. *lycopersici.*

Work at Zurich and elsewhere has shown that metabolic products from *in vitro* cultures of the tomato *Fusarium* will induce several of the symptoms of plant invasion by the fungus. Such substances include lycomarasmin, fusaric acid and several enzymes. Evidence that these substances are implicated in the natural disease syndrome has proved difficult to obtain however.

In studies by the author it was demonstrated that when aerial parts of resistant plants were grafted onto the roots of invaded susceptible plants they showed severe toxic symptoms and were much more sensitive in this respect than similarly treated aerial parts of susceptible plants. It was concluded that resistant "stems" were more sensitive than susceptible "stems" to the toxins produced during invasion of the susceptible roots.

Attempts have been made to use this difference in sensitivity to toxins as a test for the presence of these in *Fusarium* culture filtrates. Raw filtrates have been shown to affect resistant cuttings more than susceptible ones and partial purifications of such filtrates have shown that the active principle is probably not an enzyme, protein or polysaccharide, nor is it fusaric acid.

Response of Root Disease Fungi to Plant Exudates; an Example of Specificity

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Sclerotium cepivorum is a fungus which only parasitises members of the genus *Allium.* It exists in soil as sclerotia which are capable of surviving for long periods in the absence of a suitable host. Sclerotia of *S. cepivorum* only germinated in unsterile soil in the presence of species of *Allium* or of extracts made from them. Under aseptic conditions sclerotia showed a completely non-specific response to nutrients. In unsterile soil germination of sclerotia was inhibited by the mycostatic influence of the soil. Mycostasis was specifically reversed by *Allium* species although the mechanism of this reversal is at present not understood. It is not a result of antibiotics exuded by *Allium* species. Although the latter are known to contain substances (alkyl thiolsulphinates) having considerable *in vitro* antibiotic activity, no evidence has been found to suggest that they are exuded by intact *Allium* plants in sufficient quantities to affect the soil microflora.

It has now been discovered that at least part of the stimulatory effect of *Allium* species is due to volatile materials which are given off both by intact plants and by water extracts. Volatile stimulatory compounds have been collected by bubbling air through *Allium* extracts, followed by condensation in liquid air. Steam distillation of garlic extracts has also produced highly active distillates. A large number of alkyl sulphides have been identified in *Allium* spp. by other workers. Those which are available commercially are at the present time being screened for stimulatory activity. Several of these including *n*-propyl and allyl sulphides have been shown to be highly active in bringing about germination of sclerotia in soil.

Phytoalexins

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THE TERM "phytoalexin" was proposed by Müller and Börger[1] to mean a principle produced by infected plants and responsible for preventing further growth of the infecting organism. This proposal arose from theoretical considerations of the general resistance of most plants to most parasites, and of reactions which created states of "acquired immunity" to parasites after exposure to certain other organisms. The first identification of a substance as a phytoalexin was that of the chromanocoumarone pisatin.[2] Pisatin was isolated from droplets containing spores of a parasite of soft fruit incubated for several days in the seed cavities of pea pods.[3] Similar techniques led to the identification of a similar compound, phaseollin, from bean, *Phaseolus.*[4,5] Characterized antifungal compounds have been obtained from other legumes, orchid, carrot and sweet potato after challenge by non-pathogenic fungi.[6]

Little is known of the role of phytoalexins in controlling the development of specialized parasites in their usual host plants. In particular there is much question and little knowledge about the role of phytoalexins in differential resistance or susceptibility of varieties of a host species to physiological races of a pathogen.

Our research on a phytoalexin, as yet unidentified, in *Vicia faba,* shows that it is formed rapidly in response to many stimuli, including fungal infection, in fungistatic amounts. *Botrytis fabae* which slowly colonizes

bean leaves removes the phytoalexin at a much higher rate than *B. cinerea* which is also more sensitive to the phytoalexin.[7] *B. cinerea* remains limited to the initial brown infection sites. Similarly Pierre and Bateman[8] have shown that phaseollin and another unknown phytoalexin accumulate rapidly in the lesions caused by *Rhizoctonia solani* on *Phaseolus*, and prevent further growth of the parasite. Phytoalexins may have wide importance in limiting many parasitic fungi to the localized lesions typical of many diseases.

We are now investigating the sequences of events which lead to resistance or susceptibility in differential varieties of *Phaseolus* infected by races of *Colletotrichum lindemuthianum*.[7] Fungistatic amounts of phytoalexins accumulate in the early stages of infection irrespective of whether a race is able to cause a large brown lesion some days later or not. Each race of the parasite seems to be affected by the phytoalexins in a similar way. The phytoalexins may have a general role in slowing the growth of the parasites, but it is difficult to conceive that they have a special role in stopping the growth of races which are resisted. Analyses are being made of the changes in phytoalexin concentrations during the long period of 7–10 days before symptoms are apparent. Final conclusions on their role in this complex disease can then be drawn.

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Phytoalexin Production and Brown Rot in Apples

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PHYTOALEXINS were originally defined as antimicrobial products of modified host plant metabolism produced only when the host cells come into contact with a fungal parasite.[1] Some workers regard them as antifungal substances produced in plant tissues which have been subjected either to infection or other stimuli.[2]

In studies on the resistance of apple fruit to brown rot we established that phytoalexins are produced in the variety Edward VII when infected by *Sclerotinia fructigena*. These were readily detected by biological and chemical tests. Of six phenolic compounds isolated two were identified as 4-hydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid. These compounds were shown to arise from the action of the pathogen on the juice of the fruit but not on the peel or juice-free pulp. When chlorogenic and quinic acids, both of which occur in the juice of healthy fruit, were supplied separately to the brown rot pathogen growing in culture, they were both readily converted to compounds of higher antifungal activity. One of these compounds was 4-hydroxybenzoic acid.[3]

At present there is some confusion in nomenclature in regard to phytoalexins and we now propose that these substances should be subdivided into *false*, *pseudo* or *true*, depending on their mode of formation.[4] In this new concept a *false*-phytoalexin is regarded as a compound which may be detected in an infected plant; living cells of the host need not, however, be involved during its formation since a chemical precursor from the host when supplied to the pathogen in culture is converted to the same antifungal compound. A typical example of *false*-phytoalexin is 4-hydroxybenzoic acid which is produced by *S. fructigena* from chlorogenic acid in apple fruit or from chlorogenic acid added to the fungus in culture.

A *pseudo*-phytoalexin is also obtainable from an infected host plant but it may appear in the healthy host following exposure to high or low temperature, injury or treatment with certain chemicals. We suggest that several compounds which are at present regarded as phytoalexins should be reclassified as *pseudo*-phytoalexins. Compounds such as orchinol, pisatin, 3,4-dihydro-6-methoxyl-3-methyl*iso*coumarin and ipomeamarone would be included in this group.

For the production of a *true*-phytoalexin the living cells of both the parasite and its host are necessary; the formation of a *true*-phytoalexin therefore involves an interaction between two distinct metabolic systems. No *true*-phytoalexin has yet been reported.

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The Accumulation of Scopolin in Potato Tuber Tissue after Infection by Phytophthora infestans *and Its Role in Pathogenesis*

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WHEN potato tubers, infected by a number of pathogens, including fungi, bacteria and viruses, are cut open and the cut surfaces examined in u.v. light, blue fluorescent tissue can often be seen either as isolated patches surrounded by "apparently healthy" tissue or as a zone surrounding the necrotic tissue. Observations on this tissue using a fluorescent microscope show that not all the cells within the fluorescent zone fluoresce, but that the fluorescence is due to isolated patches of fluorescing cells surrounded by non-fluorescing cells. The fluorescing compounds appear to be present in the vacuole in the living tissue, but in the necrotic tissue it may become localized within the cell wall. A number of intensely fluorescing compounds have been isolated from this tissue, including scopolin, aesculin and umbelliferone. Of these, scopolin is the compound which accumulates to the greatest extent and which appears to be responsible for the major part of the fluorescence.

Experiments have been carried out to investigate the relationship between this accumulation and the resistance or susceptibility of the tuber tissue of a number of clones to infection by *Phytophthora infestans*. Evidence will be presented to show that the greatest accumulation occurs in the most susceptible clones while lesser amounts accumulate in the more resistant clones.

Experiments will also be described which have been carried out in an attempt to determine how the pathogen induces the accumulation of these compounds. Culture filtrates of *P. infestans* do not induce the accumulation of scopolin in tissue slices and neither does mechanical wounding alone. Mechanical wounding in fact induces a pattern of synthesis of phenolic and other compounds, which is qualitatively and quantitatively different from that which occurs in response to infection. However, *P. infestans* itself can synthesize the aglycones of a number of related compounds including umbelliferone, coumarin and herniarin, but not scopoletin.

The possible significance of these observations will be considered in relation to the susceptibility of tuber tissue to infection by *P. infestans* and other pathogenic organisms.

Production of Phenolic Acids by Potato Tissue Culture after Infection by Phytophthora infestans

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INGRAM and Robertson[1] and Ingram[2] have developed methods for growing suspension cultures of potato tuber cells derived from the potato varieties Majestic (rr) and Orion (R_1). When the cultures were inoculated with zoospore suspensions of *Phytophthora infestans* (race 4), Majestic cultures supported a vigorous growth of the fungus but the Orion cultures were strongly inhibitory. Several days after inoculation with this fungus the suspension liquor from Orion will cause lysis of freshly isolated zoospores of *P. infestans* whereas liquor from similarly treated Majestic cells has no effect. The toxic liquor from Orion cultures contains *p*-hydroxybenzoic, vanillic and salicylic acids which have been identified by a combination of thin-layer and gas-liquid chromatography and mass spectrometry.

The mechanism by which these three phenolic acids are biosynthesized is being investigated, and the initial results suggest that the acids may be present in cells of both varieties of potato in bound forms. The acids can be released from Orion cells but not from Majestic cells by β-glucosidase treatment; this difference in the bound forms may well be the chemical basis for the difference in the response of the two types of potato cells to attack by *P. infestans*.

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