PHYTOALEXINS AND THE RESISTANCE AND SUSCEPTIBILITY OF RED CLOVER TO *SCLEROTINIA* AND *BOTRYTIS* SPP.

By

Cyrus Algernon Macfoy

B.Sc., M.Sc., D.I.C.

A thesis presented in part fulfilment of the requirements for the degree of Doctor of Philosophy of the University of London

Department of Botany and Plant Technology

Imperial College of Science and Technology

London S W 7

NOVEMBER 1978

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ABSTRACT

The pathogenicity of *Sclerotinia trifoliorum*, a clover pathogen, and *Botrytis cinerea*, an unspecialised pathogen, to red clover (*Trifoliorum pratense* L.) was compared with their sensitivity to and ability to degrade the clover phytoalexins, maackiain and medicarpin. The study was also extended to other *Sclerotinia* and *Botrytis* species.

Up to six days after inoculating clover leaves, *S. fructicola* mycelium formed no lesions apart from minute necrotic flecks. *S. fructigena* and *B. allii* formed flecking lesions whereas *B. cinerea* and *B. fabae* formed limited lesions and *S. trifoliorum*, spreading lesions.

In 24 h, *S. trifoliorum* and *B. cinerea* significantly metabolized both phytoalexins *in vitro* to less inhibitory hydroxylated derivatives. *S. trifoliorum* degraded at a faster rate than *B. cinerea*. *B. fabae* also significantly degraded these phytoalexins to unknown products, but *B. allii* and the other *Sclerotinia* species did so little or not at all. A cell-free preparation capable of degrading maackiain was obtained from *Sclerotinia trifoliorum* mycelium, and partially characterised. An initial increase in phytoalexin concentration in leaves after inoculation with *S. trifoliorum* was followed by a significant decrease to low levels as tissues became completely necrotic and colonised by the fungus. *B. cinerea* mycelium-inoculated tissue accumulated high phytoalexin concentrations in spite of some metabolism. No phytoalexins were present in diffusates from *S. trifoliorum*, *B. cinerea* or *B. fabae* inoculated tissue. This was probably due to degradation. In lesions caused by both *S. trifoliorum* and *B. cinerea* the same phytoalexin degradation products were detected as those from *in vitro* studies. Higher phytoalexin concentration accumulated and little or no hydroxy degradation products were present in leaves inoculated with *B. cinerea* spores in 5% w/v glucose solution. Following inoculation with *S. fructigena*, *S. fructicola* and *B. allii*, high phytoalexin concentrations accumulated in tissue and in diffusates and no hydroxy degradation products were detected. These three latter fungi were more sensitive to the phytoalexins with respect to mycelial growth than the three other fungi. The respiration of *S. fructicola* was also more sensitive to maackiain than that of *S. trifoliorum* and *B. cinerea*.

Using crude extracts of cultures, partially purified culture filtrates and extracts of mycelial wall containing elicitors, the capacity of *S. trifoliorum* and *B. cinerea* to induce phytoalexin biosynthesis was compared.

Production of toxin by *S. trifoliorum* was confirmed and partially characterized.

The specificity of *S. trifoliorum* and the non-pathogenicity of the other fungi are discussed in terms of the possible biochemical mechanisms involved.

CONTENTS

INTRODUCTION

The role of phytoalexins in disease resistance has received much attention in recent years, but to date their precise function has not yet been proven unequivocally.

Using the red clover - Sclerotinia trifoliorum interaction as a convenient model, Debnam and Smith (1976) demonstrated that the known clover phytoalexins, maackiain and medicarpin may be involved in the resistance of clover to Botrytis cinerea, and that their degradation may contribute to the pathogenicity of S. trifoliorum to this plant.

The object of the present investigation was to compare these two fungi more rigorously for their relative ability to induce and to degrade clover phytoalexins in vitro and in vivo. Also, their differential sensitivity to these phytoalexins needed examination. Other Sclerotinia and Botrytis species were included in the study to determine if these phytoalexins are involved in the specificity of parasitism. A cell free system from S. trifoliorum capable of degrading maackiain was also investigated.

LITERATURE REVIEW

Susceptibility of plants to disease is the exception rather than the rule. Plants in their natural communities either tolerate parasites or are resistant to microbial invasion. Resistance is therefore the rule. Inherent mechanisms must exist in plants which are responsible for the failure of microorganisms to infect and for the specificity of pathogens to their particular host plant(s). The basis of this resistance to disease has been of special interests to biochemists and plant pathologists and understanding of natural resistance may well lead to the development of more satisfactory methods of controlling disease. Despite this interest only recently has an active biochemical and molecular orientation been pursued. In this respect, a biochemically-based disease resistance mechanism involving 'antibiotics' called phytoalexins has been implicated as having a role in the determination of host-parasite interactions.

Since Müller and Börger (1940) reported a phenomenon in plants similar to induced immunity in animals and the eventual development of the 'phytoalexin theory', work on phytoalexins has started. Phytoalexins can be defined as antimicrobial compounds produced *de novo* by many plants as a result of physical, chemical or biological stimuli. Several reviews have appeared in the literature (Cruickshank, 1963; Cruickshank *et al.*,1971; Deverall, 1972; Ingham, 1973; Kuc, 1972 and 1976b; Stoessl *et al.*, 1976). In healthy plants, little or no phytoalexins are present, but on infection high concentrations accumulate.

In 1960 Cruickshank and Perrin isolated and identified the first phytoalexin, called pisatin, from peas (*Pisum sativum*). Since then a large number of phytoalexins have been extracted and purified from a wide range of plants.(Table 1).

There are several reports which indicate that these compounds are involved in the protection of plants from fungal colonisation, and research still continues in this area of plant pathology.

Table 1. Phytoalexins produced in some species of the leguminosae

Plant species	Phytoalexin	Reference
Medicago sativa L. (alfalfa)	medicaprin	Smith *et al.*, 1971
	sativan	Ingham and Millar, 1973
Lotus corniculatus L. (birdsfoot trefoil)	vestitol	Bonde *et al.*, 1973
	sativan	" " " "
Vicia faba L. (broad bean)	wyerol	Fawcett *et al.*, 1968
	wyerone	" " " "
	wyerone acid	Letcher *et al.*, 1970
	wyerone epoxide	Hargreaves *et al.*, 1976a
	medicarpin	" " "., 1976b
Cicer arietinum L. (chickpea)	medicarpin	Keen, 1975
	maackiain	" "
Vigna sinesis L. (cowpea)	phaseollin	Bailey, 1973
	phaseollidin	" "
	kievitone	" "
Phaseolus vulgaris L. (French beans)	phaseollin	Perrin, 1964
	phaseollidin	Perrin, *et al.*, 1972
	Phaseollin isoflavan	Burden, *et al.*, 1972
	kievitone	Smith, *et al.*, 1973
	2'-methoxyphaseollin	Van Etten and Smith, 1975
Pisum sativum L. (pea)	pisatin	Perrin and Bottomley, 1962
	maackiain	Stoessl, 1972
	hydroxytrimethoxy)	Pueppke and Van Etten, 1975
	hydroxydimethoxy)pterocarpan	" " " "
	trimethoxy)	" " " "
Trifolium pratense L. (red clover)	maackiain	Higgins and Smith, 1972
	medicarpin	" " "
	pisatin	Debnam and Smith, 1976
	homopisatin	" " "
Trigonella sp.	maackiain	Ingham and Harborne, 1976
	medicarpin	" " "
	vestitol	" " "
	sativan	" " "

One of the most widely studied plant families in this respect, is the leguminosae, where with a few exceptions the phytoalexins are isoflavonoid with a pterocarpan backbone (Fig. 1). This is not surprising since the type of phytoalexin produced as a result of fungal induction is almost completely family specific (Harborne, 1977). Apart from pterocarpans other compounds including isoflavones (Ingham, 1976c) are also known phytoalexins in the leguminosae (Fig. 2).

The very simple view of one phytoalexin per plant (Letcher et al., 1970 Perrin and Bottomley, 1962; Perrin, 1964; Smith et al., 1971) and the explanation of resistance by the accumulation of just this one inhibitor (Deverall, 1972; Higgins, 1972; Mansfield and Deverall, 1974), is now superseded by the discovery of several phytoalexins in many plants (Table 1).

This so called multicomponent phytoalexin response would therefore result in a more complex antifungal environment than was formerly envisaged.

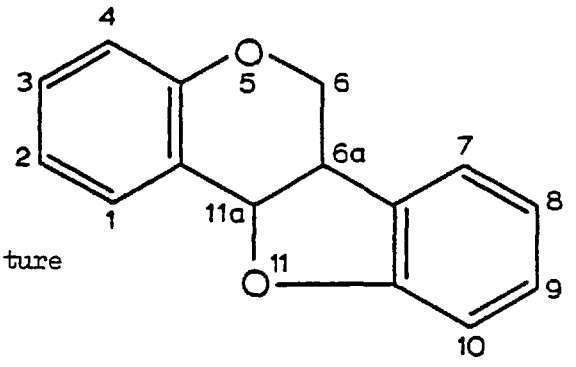
Clover and its antifungal compounds

Clover is a leguminous forage crop, which has been used in agriculture for many different purposes. This crop has been shown to contain several antifungal compounds. Virtanen, Hietala and Wahlroos (1957) found that the press juice of clover before flowering had a strong antifungal effect on Fusarium nivale and S. trifoliorum. In agar, both fungi were inhibited by 0.02% and 0.04% solution respectively of the antifungal extract. The compound responsible was later found to be the isoflavone biochanin A (Virtanen and Hietala, 1957; Bredenberg, 1961).

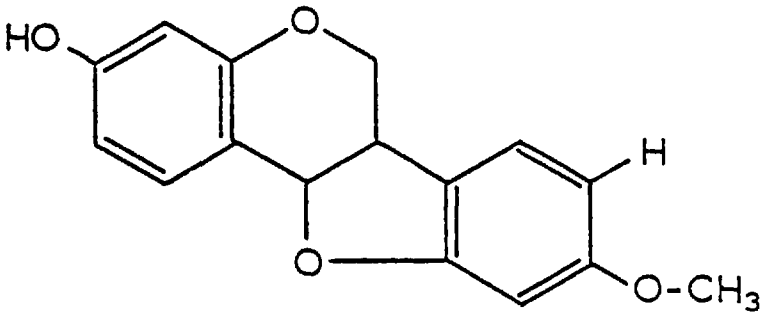
An anti-Sclerotinia factor from red clover had previously been found by Virtanen and Hietala (1958) to be the isoflavone, formononetin. Both isoflavones occur in several other plants (Harborne et al., 1975). Usually they are present as the glycosides in healthy cells (Frances, Millington and Bailey, 1967; Schultz, 1967) but on infection glycosidase enzyme probably

Figure 1. Pterocarpan phytoalexins

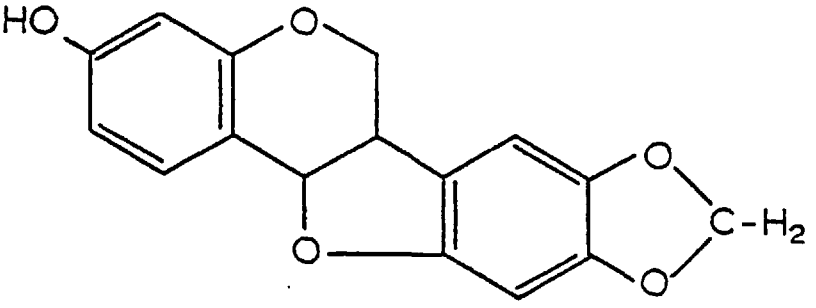
Skeletal structure



Medicarpin



Maackiain



Phaseollin

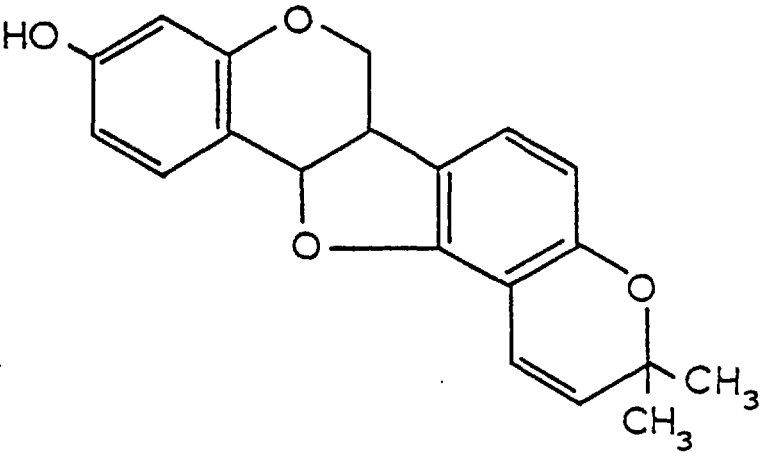
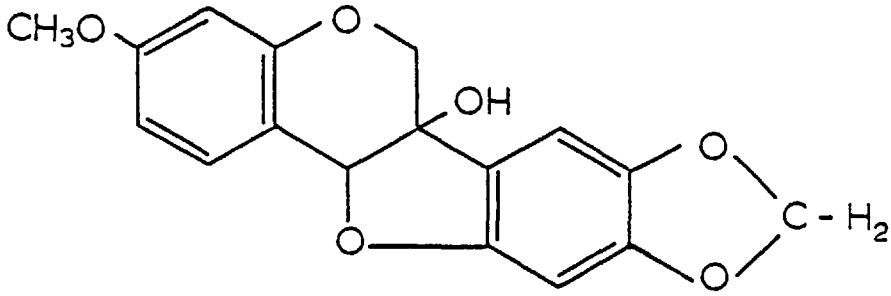


Figure 1. contd.....

Pisatin



Glyceollin

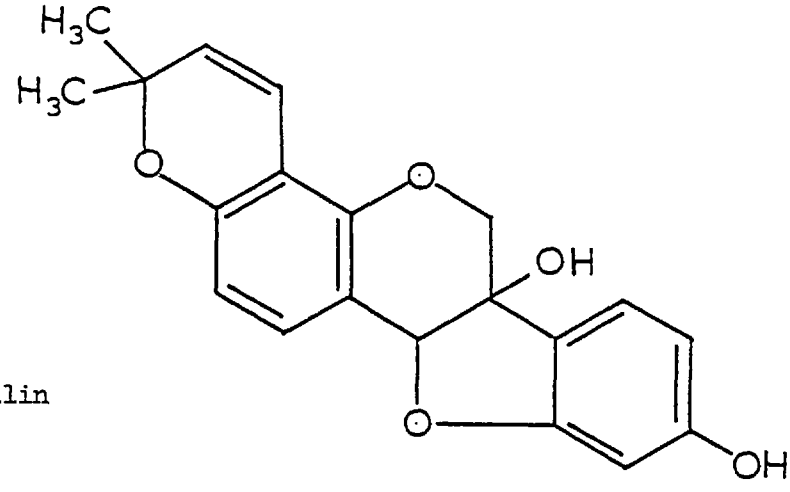
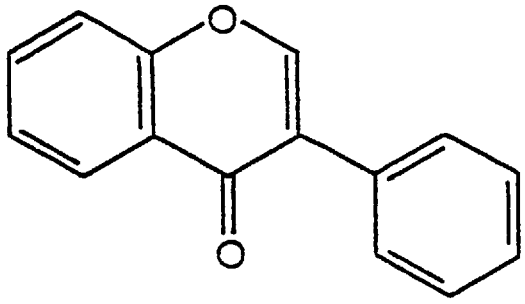
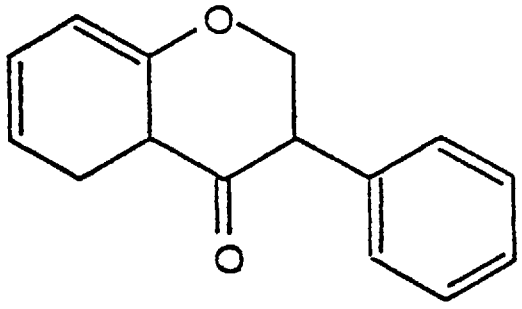


Figure 2. Skeletal structure of other types of phytoalexins in the leguminosae

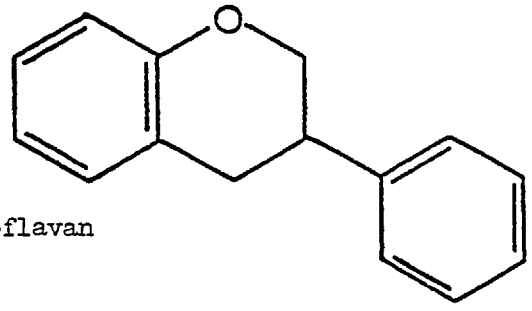
Isoflavone
e.g. cajanin



Isoflavanone
e.g. kievitone



Isoflavan
e.g. Phaseollin isoflavan



Hydroxystilbene
e.g. resveratrol

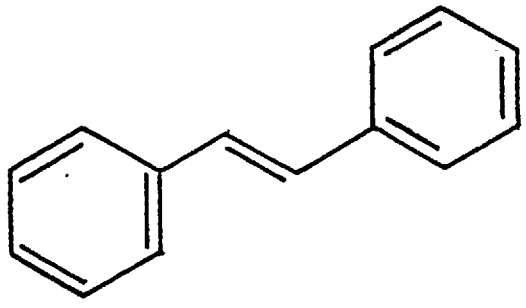
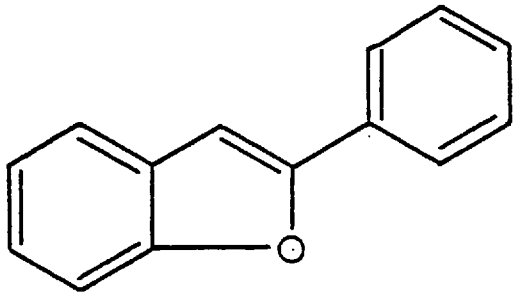
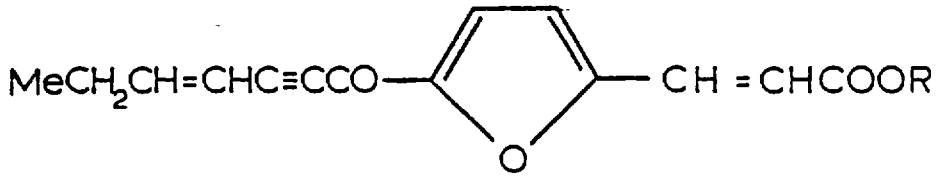


Figure 2. contd.....



Benzofuran

e.g. vignafuran



Furaneacetylene

e.g. wyerone

converts the glycosides to the aglycones (Olah and Sherwood, 1973). Despite the above reports that biochanin A and formononetin may act as anti-*Sclerotinia* factors, neither compound nor the newly identified genistein or daidzein appeared to be of major significance in the resistance of red clover to *B. cinerea* (Debnam and Smith, 1976) or in the resistance of chickpeas to *H. carbonum* (Ingham, 1976b).

Trifolirhizin which was found to be an antifungal glycoside in clover roots (Bredenberg and Hietala, 1961a, b) is also isoflavonoid, but of the pterocarpan type (Harper, Kemp and Underwood, 1965). It is absent in healthy shoots and is hydrolysed to the aglycone, when homogenised roots are left for 2 - 3 days in water.

Cruickshank (1965) first reported a phytoalexin in red clover and suggested that it was the aglycone of trifolirhizin, 3-hydroxy-8,9-methylene-dioxypterocarpan, also called maackiain (Suginome, 1962) demethylpterocarpin and inermin (Cocker, Dahl, Carmel, Dempsey and McMurry, 1962). Following the identification of the pterocarpan phytoalexin, medicarpin, from lucerne (Smith, McInnes, Higgins and Millar, 1971), this compound together with maackiain (Higgins, 1971; Higgins and Smith, 1972; Debnam and Smith, 1976; Ingham, 1976a) was also observed in red clover leaves inoculated with fungi. The structures of these phytoalexins are shown in Fig. 1. Two characteristics of isoflavonoid phytoalexin production are evident with maackiain and medicarpin i.e. a given phytoalexin is often produced by several plant species and secondly a plant species may produce more than one isoflavonoid phytoalexin.

Pterocarpan biosynthesis

Very little study has been made on the biosynthetic pathways which result in phytoalexin production. Knowledge of these are needed to support previous evidence that phytoalexins are synthesized from remote precursors and not from conversion or hydrolytic release from close precursors. Also,

to assess the importance of specific enzymic steps in the pathway, which may be important in manipulating the natural disease resistance mechanisms.

Three sequential phases in the biosynthesis of pterocarpans can be observed.

1. Early reaction pathways shared with other phenolic secondary metabolites;

2. Reactions common to flavonoid and isoflavonoid biosynthesis;

3. Reaction steps unique to isoflavonoids.

(1) above is well understood but not (2) and (3).

It is known that several pterocarpans are biosynthesized from phenylalanine, since this amino acid is the only good phenylpropanoid precursor of flavonoids in the Leguminosae (Towers, 1974). ^{14}C-phenylalanine was incorporated into pisatin (Hadwiger, 1966), phaseollin (Hess, Hadwiger and Schwochau, 1971) hydroxyphaseollin, i.e. glyceollin (Keen et al., 1972) and into medicarpin and maackiain (Dewick, 1975). The enzyme phenylalanine ammonia lyase (PAL) which oxidatively deaminates phenylalanine to trans cinnamic acid has been shown to increase before cells become necrotic (Rathmell, 1973) or concurrent with increased phenol synthesis (Camm and Towers, 1973; Dixon and Fuller, 1976; Schwochau and Hadwiger, 1968). Extensive research has been done on this enzyme (Camm and Towers, 1973; Creasy and Zucker, 1974) but, although increased PAL is associated with phytoalexin induction, its significance is still unresolved (Cruickshank et al., 1971; Dixon and Fuller, 1976; Hadwiger et al., 1970; Rathmell, 1973).

In some plants isoflavonoids are not specifically induced, but are part of a general change in phenolic metabolism (Bickoff et al., 1967; Olah and Sherwood, 1971, 1973; Smith, 1970). In other plants, isoflavonoid biosynthesis is accelerated, especially to pterocarpans while other phenols remain little changed compared with healthy tissues (Paxton et al., 1974;

Keen et al., 1972a,b;Rathmell, 1973; Rathmell and Bendall, 1971). So it is apparent that the mechanism underlying enzyme activation is still not fully understood. This awaits a complete knowledge of the isoflavonoid biosynthetic pathway.

An increase in protein and certain RNA fractions followed pisatin production in pea pods (Hadwiger, et al., 1973 and references therein) and from further work by this same group, it was suggested that the diverse groups of phytoalexin inducers affect multiple segments of nuclear DNA and that the changes in DNA conformation which occur in the globular regions improve the accessibility or "melting in" of polymerase to regions previously inaccessible (Hadwiger et al., 1974).

Dewick (1975) using $CuCl_2$-treated clover seedlings showed that maackiain and medicarpin could be biosynthesized from labelled phenylalanine. Hijwegen (1973) had previously reported that trifolirhizin in red clover roots was synthesized at the expense of isoflavones. Although exogenous formononetin was a good precursor of maackiain and medicarpin in red clover roots, the main formononetin pool in roots was not involved in pterocarpan biosynthesis (Dewick, 1975). That this may be so was suggested by Debnam and Smith (1976) who inferred that the existing isoflavones make only a minor contribution to the pterocarpan biosynthesis in B. cinerea lesions on red clover leaves. This relationship was not clear in S. trifoliorum lesions.

Other compounds have been tested for their ability to synthesize maackiain and medicarpin and these include $2^1,4^1,4$ trihydroxychalcone. This compound was efficient as a precursor of both phytoalexins and formononetin, whereas $2^1,4^1$ dihydroxy-4-methoxychalcone and the dihydroxyisoflavone, daidzein were very poor precursors (Dewick, 1975). This latter compound was however found by Hess et al. (1971) to be an efficient precursor of phaseollin in Phaseolus vulgaris. Isoliquiritigenin was an efficient precursor of glyceollin (Keen et al., 1972b). More recently Dewick (1977) reported that $7,2^1$-dihydroxy-4^1-methoxy-isoflavone and isoflavanone were efficient precursors of medicarpin in $CuCl_2$-treated red clover seedlings. Neither these two compounds

nor medicarpin itself were precursors of the other clover phytoalexin maackiain. Also in this study, 3-hydroxy-9-methoxypterocarp-6a-ene was found to be a poor precursor of both phytoalexins. From the results a biosynthetic pathway to medicarpin via 2^1-hydroxylation of formononetin, and subsequent reduction to the isoflavanone was proposed. Dewick suggested that the conversion of the isoflavanone to the pterocarpan, medicarpin, may involve the corresponding isoflavonol and a carbonium ion intermediate. The branch point to maackiain is probably at the formononetin stage, since neither the isoflavone nor isoflavanone were good precursors of maackiain. Not regarded as significantly utilized for maackiain or medicarpin production was the 3-hydroxy-9-methoxypterocarp-6a-ene. Therefore a hypothesis for pterocarpan biosynthesis involving pterocarp-6a-ene intermediate as originally proposed (Fig. 3) could not be made. Fig. 4 illustrates a possible pathway to medicarpin and maackiain biosynthesis. That the pathway of isoflavonoids may resemble a metabolic grid rather than a sequential precursor to end point was suggested by Van Etten and Pueppke (1976), and more recently by Biggs and Lane (1978) who have also identified important intermediates in the maackiain biosynthetic pathway.

Elicitors of phytoalexin biosynthesis

Apart from the numerous abiotic agents capable of inducing phytoalexins, several active inducers of fungal origin have been identified. Monilicolin A, a polypeptide from the mycelium of Monilinia fructicola (Cruickshank and Perrin, 1968) was not phytotoxic but induced phaseollin in French beans. No pisatin was however induced in peas. Keen et al. (1972a) have coined the word 'elicitor' for inducers of biological origin. But though the mechanism of initiation of phytoalexin biosynthesis is still unknown, Albersheim's group (Anderson, 1978; Ayers et al., 1976) and Keen's (Keen et al., 1972a; Keen, 1975b) have isolated fractions with elicitor activity from culture fluids and/or cell wall of fungi. These elicitors were capable of inducing necrosis and phytoalexin accumulation. Other reports in this area of plant pathology are from Daniels and Hadwiger (1976),

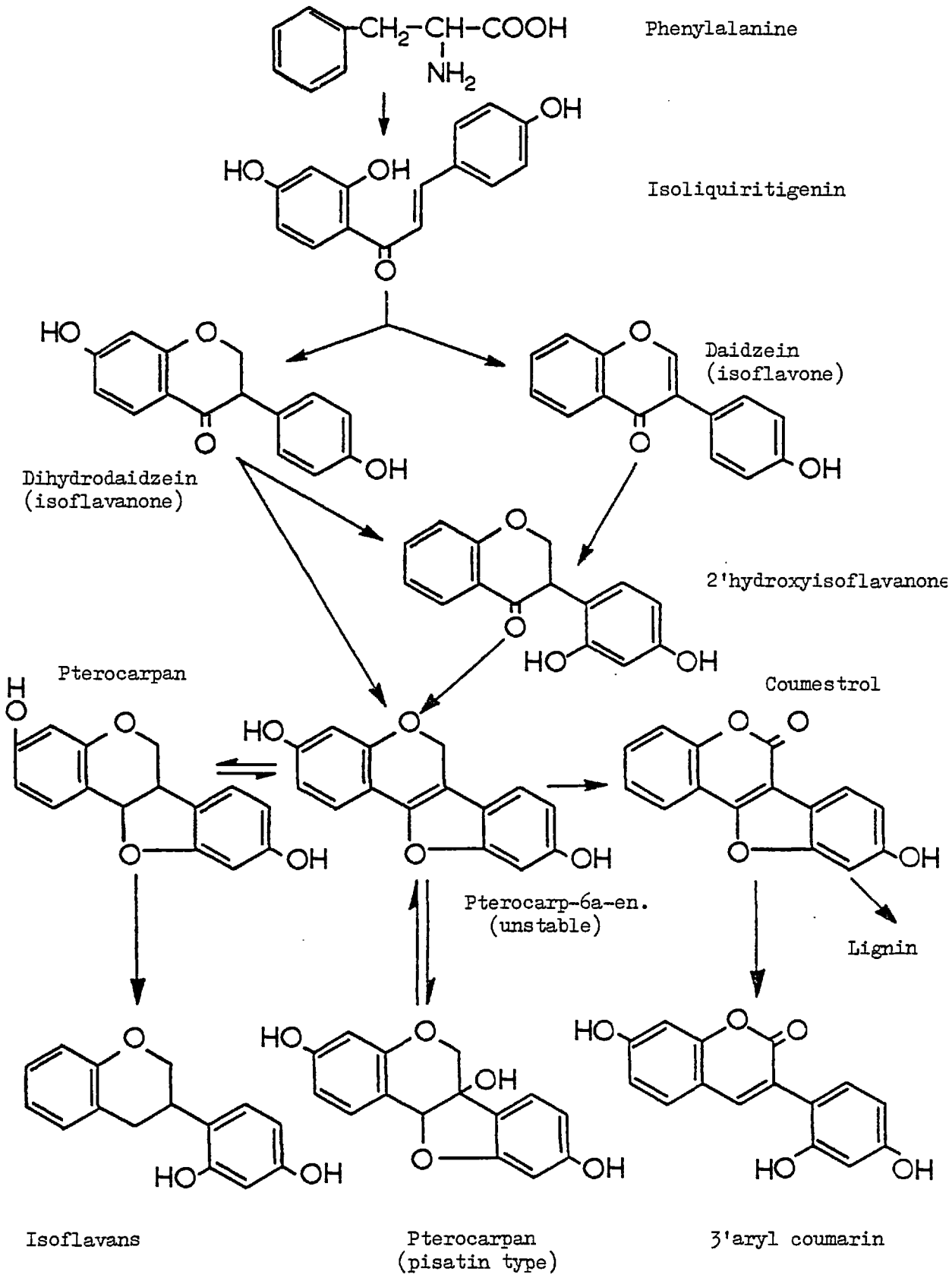
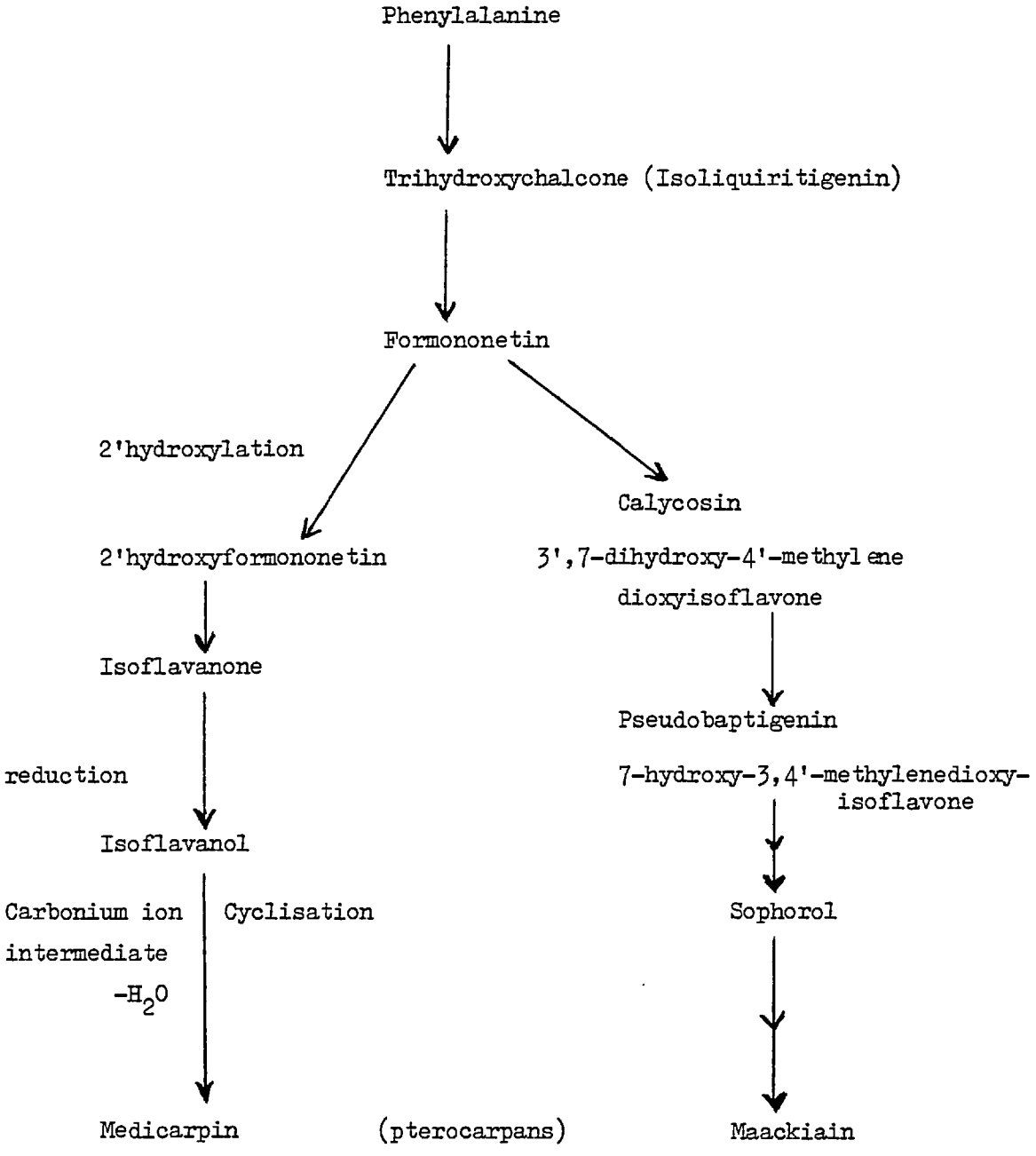


Figure 3. Proposed general pathway of the biosynthesis of pterocarpanoids (after Hijwegen, 1973)

Figure 4. Possible biosynthetic pathway to medicarpin and maackiain adapted from Dewick (1977) and Biggs & Lane (1978)



Duczek and Higgins (1976a), Higgins and Millar (1969a), Lister and Kuc (1977) and Stekoll et al. (1978).

Anderson and Albersheim (1975) postulated that the defence reaction may be due to the interaction between carbohydrate-containing molecules on the surface of the fungus and protein receptors on the plasmalemma of the host. Recently Dixon and Fuller (1977) isolated and partially characterised an elicitor from Botrytis cinerea culture filtrates. This elicitor was capable of inducing phaseollin biosynthesis in cell-suspension cultures of Phaseolus vulgaris. Spore germination fluid of pathogens and non-pathogens had previously been found to elicit medicarpin biosynthesis in alfalfa (Higgins and Millar, 1969a; Sakuma and Millar, 1972). In a later study (Duczek and Higgins, 1976a), culture fluid from spores (germinated in a weak solution of maackiain and medicarpin) was used. Germination fluid from Stemphylium botryosum induced 4 - 12 times more phytoalexin than germination fluid from controls (spores germinated in 2% ethanol). This was not so for Stemphylium sarcinaeforme or Helminthosporum carbonum, since germination fluid from treatment and control induced about the same amount of phytoalexins.

The pathogen and disease cycle

One of the most serious diseases of red clover is clover rot caused by the fungus Sclerotinia trifoliorum Erikss. Sclerotinia species are members of the Helotiales of the class ascomycete.

Apart from attacking red clover (T. pratense L.), S. trifoliorum will sometimes attack crimson clover (T. incarnatum L.), alsike clover (T. hybridum), lucerne (Medicago sativa), white clover (T. repens), and bird'sfoot trefoil (Lotus corniculatus) (Loveless, 1951b; Barr and Callen, 1963; Western, 1971). Field beans are more commonly attacked by a different variety S. trifoliorum var. fabae Keay. (Keay, 1939; Loveless, 1951a).

Different races of S. trifoliorum showing different aggressiveness to clover have been reported (Frandsen, 1946; Björling, 1951). Also, Held (1955) demonstrated that the only difference between a virulent strain and

a degenerate non-virulent strain derived from the former, was the production of a wilt-inducing toxin by the virulent strain.

Clover rot usually becomes apparent in Autumn as brown ascospore lesions (Dijkstra, 1966) which then spread in favourable conditions and cover the whole leaf. Leaves then wilt and the disease spreads to the stem and crown through the petiole. Black sclerotia are then produced which can give rise to either mycelia or apothecia. Both mycelia (Frandsen, 1946) and ascospores(Justham and Ogilve,1950; Loveless, 1951b) can infect clover.

Histopathology

Purdy (1958) used leaf tissue to investigate the formation of appressoria by germinating ascospores of S. sclerotiorum (he regarded S. trifoliorum as a form of S. sclerotiorum). Appressorial formation was dependent on nutrients and the stimulus of mechanical contact. Mycelia from germinating sclerotia were also capable of initiating infection in leaf tissue but only in the presence of a nutrient source of non-living organic matter. Other reports have also established the importance of a food source in the infection process (McLean, 1950; Purdy and Bardin, 1953; Natti, 1971). S. trifoliorum produces distinct, organised infection cushions (Prior and Owen, 1964), similar to those reported for Thanatephorus cucumeris (Flentje, Dodman and Kerr, 1963). Penetration of clover varieties and alfalfa by S. trifoliorum differed. On Kenland red and crimson clover varieties, infection was by direct penetration as a result of mechanical pressure from the infection cushion rupturing the cuticle and breaking the epidermal cell, or enzymatic action of the appressorium, which destroys the cuticle and the epidermis, growth of the hyphae through stomata,or any combination of these methods. Once inside the host plant mycelial growth was primarily intercellular, but sometimes intracellular growth was also observed. Growth was restricted to the parenchymatous tissues but spread occasionally to the tracheids and vessels of the xylem. The thicker hyphal

strands formed branches of smaller hyphae ramifying throughout infected tissue, frequently in a longitudinal pattern. The smaller hyphae frequently invaded the more resistant tissues. Penetration of Louisiana S-1 clover was only through stomata but growth in the tissue of this variety and in Cal Ladino clover was similar to Kenland and crimson varieties. Enzymatic disintegration of tissues was however only observed in advanced stages.

S. sclerotiorum infection of bean was found to be similar when infected oat kernels were used as inoculum (Lumsden and Dow, 1973), including the indiscriminate inter and intracellular penetration by hyphae.

The histology of the infection process was studied on clover and alfalfa leaves inoculated with three necrotrophic fungi (Duczek and Higgins, 1976a). As reported by Pierre and Millar (1965), Stemphylium botryosum was pathogenic to alfalfa, and S. sarcinaeforme was pathogenic to red clover, alfalfa and white clover. Both fungi penetrate into varieties of these plants. However, S. botryosum entered mainly through the stomata whereas S. sarcinaeforme penetrated mostly between apidermal cells. Once in the plant, the two fungi produce thick bulbous primary hyphae within the tissue of both suscept and non-suscept plants. A major limitation to pathogenicity was the inability to develop beyond the primary hyphal stage in non-suscept plant, where growth was inhibited by 24 h. In suscept plants secondary hyphae develop from the primary ones and cause intercellular ramifications in all directions. On alfalfa leaves which are highly resistant to Helminthosporum carbonum conidia germinated and produced appressoria and penetration was direct. However, by 120 h, growth was restricted to a few cells (Higgins and Millar, 1968a). Although conidia of this fungus germinated similarly on red clover, no internal hyphae were observed (Duczek and Higgins, 1976a). A recent ultrastructural comparison of S. botryosum and S. sarcinaeforme on clover was made by Lazarovits and Higgins (1977) but from their results they concluded that the different effects of both fungi on leaf cells failed to explain the cessation of growth by S. botryosum.

Antifungal activity of phytoalexins in leguminosae

Sensitivity to phytoalexins has been studied by the antifungal activity of these inhibitors to fungi *in vitro*. The bioassays used were : effect of phytalexin on spore germination and germ tube length, on accumulation of fungal mycelium in liquid culture and on growth of mycelium on solid media. Since research workers hardly ever standardize the methods among themselves it can sometimes be difficult to draw general conclusions.

Phytoalexins have been reported to be active between 1 - 100 µg/ml (Bailey and Burden, 1973; Cruickshank, 1962; Cruickshank and Perrin, 1971; Van Etten, 1976). This being the range they are usually tested.

In 1962 Cruickshank observed that only five out of fifty fungal isolates were insensitive to pisatin. The five insensitive ones were pathogens of pea. Similar results have been obtained for phaseollin (Cruickshank and Perrin, 1971) and for medicarpin (Higgins, 1972). Further research has revealed additional exceptions for pisatin (Pueppke and Van Etten, 1974; Van Etten, 1973a) phaseollin (Bailey and Burden, 1973; Heuval and Glazener, 1975; Van Etten, 1973a) and kievitone (Smith *et al.*, 1975). A notable example is *Aphanomyces euteiches* which is pathogenic on peas, but is sensitive to pisatin *in vitro* (Pueppke and Van Etten, 1976).

Working with the alfalfa phytoalexin, medicarpin, Higgins (1972) obtained results which show that four alfalfa pathogens were only slightly inhibited by medicarpin(0 - 12% by 75 µg/ml) while *H. turcicum* (*carbonum*), a non-pathogen was greatly inhibited (50% inhibition by 25 - 50 µg/ml). This high sensitivity of the non-pathogen was confirmed by Ingham (1976a)and Duczek and Higgins (1976 a). Both groups demonstrated that this fungus was sensitive to maackiain as well. *B. cinerea*, however, was less sensitive to both phytoalexins (Debnam and Smith, 1976; Ingham, 1976 a). Debnam and Smith (1976) also reported that the clover pathogen *Sclerotinia trifoliorum* was less sensitive to maackiain and medicarpin than *B. cinerea*. The broad bean pathogen, *Botrytis fabae* has also been shown to be less sensitive to broad bean phytoalexins than *B. cinerea* is (Hargreaves, Mansfield and Rossall, (1977).

Degradation of phytoalexins by fungi in vitro

Even when phytoalexins are present, fungi can still cause disease. This may be due to degradation by the fungus. Host tissue may also degrade phytoalexins and the degradation products from either source may be less inhibitory. If these do occur, assessment of the role of phytoalexins in disease resistance becomes more complicated.

Although Müller (1958) suggested that fungi may degrade phytoalexins as a form of adaptation to these inhibitors, Uehara (1964) was the first to show that the isoflavonoid pea phytoalexin, pisatin, could be degraded in vitro by pea pathogens. This was confirmed by Wit Elshove (1968, 1969) who also showed that non-pathogens of pea could not bring about this degradation. In this study there was a 60% recovery of pisatin with Botrytis fabae and 87% with Sclerotinia fructigena. Fusarium oxysporum f. pisi and F. solani f. pisi did not degrade pisatin if the concentration of glucose or sucrose was high. However, if the concentration of glucose was less than 2750 µg/ml complete degradation occurred to CO_2. This suggests catabolite repression. With Ascochyta pisi there was no catabolite repression(Wit Elshove and Fuchs, 1971).The degradation product from this latter interaction was 6a-hydroxymaackiain (Land, Wiersma-Van Duin and Fuchs, 1975a). Stemphylium botryosum and Fusarium solani f. sp. pisi also degraded pisatin by O-demethylation at carbon 3 to 6a-hydroxymaackiain (Van Etten et al., 1975) (Fig. 5).

Fig. 5. Degradation of pisatin by fungi

This first metabolic step may be regarded as a detoxification process, since 100 µg/ml hydroxymaackiain had no effect on all races of Ascochyta pisi (virulent and avirulent), A. fabae or M. fructicola, whereas 100 µg/ml pisatin completely inhibited spore germination of A. fabae and M. fructicola.

Another isoflavonoid phytoalexin, phaseollin, from French beans is metabolised by three different methods. Colletotrichum lindemuthianum degraded phaseollin (Bailey, 1974) first by 6a-hydroxylation and then by a second hydroxylation at position 7 (Burden et al., 1974; Heuvel and Vollaard, 1976). Both 6a-hydroxyphaseollin and 6a,7-dehydroxyphaseollin were antifungal in Cladosporium TLC Bioassay (Burden et al., 1974). The metabolite, 6a, 7-dehydroxyphaseollin was as inhibitory as phaseollin to Colletotrichum lindemuthianum race δ11, but was only slightly inhibitory to races α1, α2 and γ1 (Heuvel and Vollaard, 1976). Other fungi can also degrade phaseollin.

Heuvel and Glazener (1975) showed that five out of seven pathogens and three out of five non-pathogens degraded this phytoalexin. Further work by Heuvel (1976) using six isolates of Botrytis cinerea showed that the three pathogenic isolates detoxified phaseollin to 6a-hydroxyphaseollin, while the non-pathogenic isolates were not able or less able to do so. Metabolism by Stemphylium botryosum resulted in phaseolliniso-flavan (Higgins et al., 1974), while Cladosporium herbarum (Heuvel and Glazener, 1975) and Fusarium solani (Heuvel et al., 1974) metabolized phaseollin to 1a-hydroxyphaseollone. F. solani was able to degrade an inhibitory concentration provided it was first exposed to a non-inhibitory dosage (Heuvel and Van Etten, 1973). Recent work by Bailey et al. (1977) demonstrated that Septoria nodorum detoxified phaseollin to 12, 13-dihydrodihydroxy phaseollin cis and trans isomers. Other non-pathogens also degraded phaseollin, thus further showing that detoxification mechanisms are not confined to pathogenic fungi. The isoflavanone kievitone was reported by Kuhn et al., (1977) to be detoxified by F. solani f. sp. phaseoli, to kievitone hydrate. The furano-acetylenic wyerone acid was also detoxified by Botrytis fabae and B. cinerea, but the former apparently degraded at a

faster rate (Mansfield and Widdowson, 1973).

Experiments with the alfalfa phytoalexin, medicarpin, have also shown that the ability of fungi to degrade is common. Stemphylium loti, a weak alfalfa pathogen and Colletotrichum phomoides, a non-pathogen of this plant, both degrade medicarpin. The former to a compound still inhibitory to both fungi and the latter slowly to a product similar to medicarpin and inhibitory to C. phomoides. Helminthosporum carbonum was however, unable to degrade medicarpin (Higgins and Millar, 1969a, 1970). It has also been reported that Stemphylium botryosum, pathogenic to alfalfa, can degrade medicarpin but at a faster rate than it degrades pisatin and phaseollin (Higgins and Millar, 1968b, 1969a, b, Heath and Higgins, 1973). As for phaseollin, the product formed was also an isoflavan - vestitol. Another isoflavan, dihydroxymaackiain was formed when this fungus degraded maackiain (Duczek and Higgins, 1973; Higgins, 1975; Steiner and Millar, 1974). The isoflavans are usually as inhibitory as the parent phytoalexins. Higgins (1975) demonstrated that the maackiain degradation system can be induced with maackiain, medicarpin or phaseollin and that maackiain could also induce the conversion of the two latter phytoalexins. In 1976 Duczek and Higgins confirmed the degradation of maackiain and medicarpin by S. botryosum and reported their conversion by the clover pathogen S. sarcinaeforme to unidentified products, and their non-degradation by H. carbonum, a non-pathogen of clover. Ingham (1976a) also demonstrated degradation of maackiain and/or medicarpin with Colletotrichum lindemuthianum, Colletotrichum coffeanum and Botrytis cinerea in leaf diffusates. The first conversion product was the 6a-hydroxylated derivative and the second the 6a,7-dihydroxylated derivative. B. cinerea was unable to bring about the second reaction on leaf diffusates and in vitro. Debnam and Smith (1976) and Bilton et al. (1976) have also shown that Botrytis cinerea can degrade both clover phytoalexins. Sclerotinia trifoliorum a clover pathogen degraded maackiain and medicarpin in vitro to the 6a-hydroxylated derivatives.

Degradation of phytoalexins by fungi is therefore a widespread phenomenon.

Degradation of phytoalexins in plants

Cruickshank (1963) considered phytoalexins as end products of a host-parasite interaction, but there is now evidence which suggests that isoflavonoids can be metabolized by plant tissue, i.e. there is a turnover of phytoalexins (Barz, 1975; Rahe and Arnold, 1975). Recently Dewick (1975) and Dewick and Martin (1976) produced results which suggest that the clover phytoalexins, medicarpin and maackiain were subjected to turnover in clover plants. Phaseollin was also degraded by cell suspension cultures of Phaseolus vulgaris (Glazener and Van Etten, 1978; Skipp et al., 1977) to phaseollin isoflavan (Hargreaves and Selby, 1978). Phaseolus aureus also degraded phaseollin (Glazener and Van Etten, 1978). Metabolism of capsidiol (Stoessl et al., 1977) and rishitin (Murai et al., 1977) in plant tissue has also been reported.

Degradation of phytoalexins in vitro by fungi does not necessarily imply degradation in vivo, since the co-existence of the pathogen and phytoalexins in plant tissue does not necessarily mean the two interact. However, the detection of the in vitro degradation products in vivo may suggest the interaction of fungus and phytoalexin in situ (Duczek and Higgins, 1976a; Heath and Higgins, 1973; Land et al., 1975a; Van Etten and Smith (1975). If this metabolite could also be of plant origin (Higgins et al., 1974) the situation becomes more complicated. Phaseollin isoflavan, e.g. is produced as a phytoalexin (Bailey and Burden, 1973) and also as a metabolite of phaseollin degradation (Higgins, 1975; Higgins et al., 1974). Van Etten and Smith (1975) were able to detect 1a-hydroxy-phaseollone, a metabolite from the in vitro degradation of phaseollin, in

F. solani f. sp. phaseoli - infected tissue. More recently Van den Heuvel and Grootveld (1978) reported that French beans inoculated with isolates of Botrytis cinerea accumulated the in vitro phaseollin metabolite, 6a-hydroxy-phaseollin, as well as the other bean phytoalexins. Other fungal metabolites are often difficult or impossible to detect in tissue (Cruickshank et al., 1974; Higgins, 1975; Duczek and Higgins, 1976a)but some degradation products have been recovered from diffusates (Ingham, 1976a).

Enzymology of degradation of Flavonoids

To date no fungal enzymes capable of degrading phytoalexins have been isolated. Nevertheless cell-free preparations and purified enzymes which degrade other flavonoids have been obtained from fungi, bacteria and plants (Westlake et al., 1961a; Schultz and Wood, 1972; Hosel and Barz, 1972). There is a turnover of flavonoids in plants and these compounds are also known to be degraded by bacteria and fungi as sources for energy and carbon. To obtain carbon and energy for growth and differentiation, the flavonoid substrates must be transformed in such a way that aliphatic ring fission products are obtained. These aliphatic intermediates are eventually funnelled into the high energy release reactions of the primary metabolism (Gibson, 1968; Dagley, 1971).

Harborne and Sherratt (1957) described the activity of a fungal "anthocyanase" enzyme in the culture filtrate of Aspergillus species. This rapidly decolourised anthocyanins and produced anthocyanidins and sugars. Aspergillus species grown on rutin were also reported to produce extracellular enzyme systems which degrade rutin (Westlake et al., 1959, 1961a, 1961b). This flavonol glucoside was degraded to yield phloroglucinol carboxylic acid, protocatechuic acid, rutinose and carbon monoxide, and all the enzymes have been isolated and characterised. The dihydrochalcone, phloridzin has been shown to be degraded by a variety of fungi. Moreover a cell-free preparation

from macerated mycelium of A. niger catalysed the hydrolysis of phloridzin to phloroglucinol and phloretic acid (Jayasanker et al., 1969). This same group of researchers (Minamikawa et al., 1970) have extracted an inducible enzyme from acetone powders of A. niger mycelium grown in the presence of phloridzin. The enzyme was capable of hydrolysing phloretin to phloroglucinol and phloretic acid and have been partially purified and characterized.

Although phytoalexin metabolism is a common phenomenon, none of the enzymes responsible have to date been isolated. However, Christenson and Hadwiger (1973) observed that culture filtrates of Fusarium solani f. sp. phaseoli could transform radioactively labelled pisatin. Higgins (1972) claimed that culture filtrates of Leptosphaerulina briosiana altered medicarpin. In both cases no products were identified. Since no prior exposure of the organisms to the phytoalexins was necessary the activity in the culture filtrates must be constitutive. S. botryosum (Higgins, 1975), F. solani (Heuvel and Van Etten, 1973) and B. cinerea (Ingham, 1976a)required an induction prior to phytoalexin degradation. This suggests an inducible degradation system. Moreover, Higgins (1975) found that the protein synthesis inhibitor cycloheximide, delays or inhibits the degradation of maackiain by S. botryosum. However, an attempt to obtain a cell-free preparation from the culture filtrate of this fungus was unsuccessful. Heuvel and Van Etten (1973) were also unsuccessful in their attempts to obtain cell-free preparations from F. solani f. sp. phaseoli which can degrade phaseollin to 1a-hydroxyphaseollone.

The role of phytoalexins in disease resistance

The role of phytoalexins as part of the defence mechanism of plants is well documented (Deverall, 1976; 1977; Kuc, 1976a; Smith, 1978; Van Etten and Pueppke, 1976). Many studies have shown that in a resistant interaction high levels of phytoalexins accumulate rapidly whereas in a susceptible interaction the accumulation is slower (Bailey, 1974; Bailey

and Deverall, 1971; Khan and Milton, 1978; Keen, 1971; Kuc et al., 1976; Rahe, 1973). Lower levels of phytoalexins were also recovered from susceptible interactions than from resistant interaction (Debnam and Smith, 1976; Garcia-Arenal et al., 1978; Hargreaves et al., 1977; Yoshikawa et al., 1978a) These differences in phytoalexin levels may be related to the rate of induction of biosynthesis, rate of degradation by the fungus and the host plant, or to suppression of biosynthesis by the fungus. Sensitivity of the fungi to phytoalexins is also important since the restriction of fungal growth is often temporally correlated with the accumulation of high phytoalexin concentrations in some interactions (Bailey, 1974; Bailey and Deverall, 1971; Hargreaves et al., 1977; Keen and Kennedy, 1974; Pueppke and Van Etten, 1974; Smith et al., 1975; Yoshikawa et al., 1978a) These latter researchers provided evidence which strongly suggested that glyceollin accumulation accounted for cessation of fungal growth in resistant soybean hypocotyls inoculated with Phytophthora megasperma var. sojae. Further evidence in support of phytoalexins as part of plant's defence mechanisms came from cross-protection studies. Preinoculation or treatment which depress the rate of phytoalexin biosynthesis often convert a resistant reaction into a susceptible reaction (Cruickshank and Perrin, 1967; Keen, 1971). Also, preinoculation treatment which stimulate phytoalexin biosynthesis sometimes render a plant less susceptible (Bridge and Klarman, 1973).

Some workers have however shown that phytoalexins may not play a significant role in lesion limitation in some host-parasite interactions. (Duczek and Higgins, 1976a; Higgins, 1972; Teasdale et al., 1974). A notable system which provides direct evidence against the phytoalexin theory is the Aphanomyces euteiches - Pisum sativum (Peas) interaction where high phytoalexin concentrations accumulate but the pathogen is not restricted, even though it is sensitive to pisatin in vitro (Pueppke and

Van Etten, 1974, 1976). This appears incompatible with the phytoalexin theory and must await further research.

In general evidence for the detailed rates of accumulation and metabolism of phytoalexins *in vivo* is lacking. The exact localisation of these inhibitors in zones of tissue in relation to the fungus could be critical when distinguishing resistance and susceptibility. Also of importance could be the state of the fungus, e.g. whether it is just a single germ tube from a spore or a complex mycelium from an agar plug should make some difference to the host-parasite balance.

MATERIALS AND METHODS

1. Biological materials

(a) Higher plants

Seeds of a late flowering red clover (Trifolium pratense L.) cultivar Merkur, a susceptible diploid variety, were supplied by Charles Sharpe and Co., Ltd. (Sleaford).

Plants were grown at the University of London Botanical Supply Unit, Egham, Surrey. They were grown in a mixture of John Innes No. 2 compost and coarse sand in black plastic pots in greenhouses with additional lighting until eight weeks old. Thereafter they were kept in the Imperial College roof glasshouse at South Kensington, where a 16 h photoperiod was maintained. Night temperatures were about 18 - 20°C, and day temperatures the same or ambient (if higher) on normal days, but rising to 30°C or more on very sunny days. Illuminance was about 5 - 6000 lumen M^{-2}.

Clover plants were occasionally infected with powdery mildew (Erysiphe polygoni DC.), but this was controlled by picking off infected leaves. For experiments only leaves with no visible signs of mildew infection were used.

(b) Fungi

All cultures were from the Imperial College culture collection.

Sclerotinia fructigena Pers. (No. 16), Sclerotinia fructicola Wint. (No. 83) and Sclerotinia trifoliorum Eriksson (No. 56) were maintained on slants of V-8 juice agar. The latter fungus was also maintained as sclerotia.

Botrytis cinerea Pers. (No. 2), Botrytis fabae Sard.(No. 19) and Botrytis allii Munn. (No. 38) were stored on slants of Medium X (Purkayastha and Deverall, 1965 a).

Cladosporium cucumerinum Ell. and Arth. (No. 80) obtained from Dr.J.A.Bailey at Wye College was maintained in SCA medium.

All stock cultures were kept under sterile liquid paraffin at 4°C.

2. Culture media

(a) Medium X

Mycological peptone	2 g
Casein hydrolysate (acid)	3 g
Glucose	10 g
KH_2PO_4	1.5 g
$NaNO_3$	6 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
Yeast nucleic acid	0.5 g
Agar	20 g
Distilled water	1 litre

The above was heated until all solid had dissolved and then dispensed in 30 - 40 ml aliquots in 250 ml Erlenmeyer flasks, before autoclaving at 1 kg/cm^2 for 15 min.

(b) Acid V-8 juice agar

V-8 juice	200 ml
Distilled water	800 ml
Agar	20 g

Preparation was as for Medium X.

(c) Sucrose casamino acids - SCA medium

Casamino acids (vitamin free)	4.6 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g

Glucose		0.5 g
Sucrose		15.0 g
Trace element solution		10 ml
Glass distilled water	to	1 litre

When solid SCA medium was required 20 g Oxoid agar was added per litre of medium.

The solution of trace elements was prepared as follows :

Trace element	Salt	Amount in mg/litre	ppm trace element in final soln.
Iron	$FeSO_4 \cdot 7H_2O$	249	0.5
Zinc	$ZnSO_4 \cdot 7H_2O$	44	0.1
Molybdenum	$Na_2MoO_4 \cdot 2H_2O$	51	0.2
Copper	$CuSO_4 \cdot 7H_2O$	40	0.1
Manganese	$MnSO_4 \cdot 7H_2O$	41	0.1

Distilled water to 1 litre

SCA medium was dispensed as 100 ml aliquots in 250 ml Erlenmeyer flasks.

(d) Potato dextrose agar - PDA medium

PDA (Oxoid CM 139)	39 g
Distilled water	1 litre

1 litre contains:

Potato extract (Oxoid L101)	4 g
Dextrose	20 g
Agar	15 g

pH 5.6 (approx.)

3. Culture methods

(a) Production of conidia

250 ml "Pyrex" Erlenmeyer flasks containing V-8 juice agar (for Sclerotinia species) or Medium X (for Botrytis species) were inoculated with 1 ml of a thick suspension of conidia in sterile distilled water. Flasks were then kept at 20°C in an incubator containing a "Philips Black light", fluorescent tube with an emission spectrum range of 410 - 310 nm (λ max. 360 nm). Cultures of Botrytis species sporulated heavily under these conditions, whereas those of S. fructigena and S. fructicola were moderate.

(b) Preparation of conidial suspensions

Eight-to ten-day old cultures of fungi were used to prepare suspensions. About 15 ml of sterile distilled water was added to the flask containing the sporulating culture, and the flask shaken vigorously. The spore suspension was then filtered through four layers of muslin to remove larger mycelial fragments, and the suspension washed two times with sterile distilled water, centrifuging at 850 g for 2 min each time. The spore concentration was estimated by haemocytometer counts and the suspension diluted to the required concentration. When spores were to be suspended in a medium other than water, a spore suspension was centrifuged and the conidia resuspended in the appropriate medium. All procedures were accomplished under sterile conditions.

(c) Production of mycelium on solid medium

S. trifoliorum was cultured on potato dextrose agar in petri dish as follows : Sclerotia were first sterilized in 10% sodium hypochlorite solution for 5 min; rinsed in sterile distilled water and cut aseptically into small pieces. Each piece was seeded into the centre of PDA petri dishes

and incubated at 20 - 25°C. Mycelia took 4 - 8 days to grow out from the sclerotia. For production of large numbers of sclerotia, 20 g bran (purchased from a pet shop) was put in a 1 litre flask and 150 ml distilled water added and autoclaved for 15 min at 1 kg/cm^{-2}. On cooling the bran was seeded with four S. trifoliorum agar plugs, and stored in a cupboard for five weeks. The bran and sclerotia were then removed from the flask and dried overnight on the laboratory bench with the aid of a fan. After this the bran was separated from the sclerotia with a sieve.

For Sclerotinia fructigena, S. fructicola, B. fabae and B. allii an agar plug from sporulating cultures was seeded on to the centre of PDA petri dishes and incubated at 25°C. Mycelia took 1 - 2 days to grow out from the plugs.

(d) Preparation of mycelial inoculum

For S. trifoliorum, three 6 mm diameter mycelial discs were cut with a cork borer from the periphery of the advancing culture growing on PDA petri dishes. Agar plugs were aseptically transferred to 100 ml SCA liquid medium and incubated for 6 days at 25°C. Similar procedures were utilised for the other fungi except that one agar plug was used for S. fructicola and B. fabae and two plugs for S. fructigena and B. allii. These procedures resulted in approximately 40 mg dry wt. mycelium of each fungus per flask, after 6 days incubation.

B. cinerea could not be handled in this way since mycelial agar plugs of this isolate failed to grow in liquid medium. Mycelial growth in liquid medium was therefore achieved by dispensing 0.5 ml of a 1 x 10^6 spores/ml conidial suspension in 100 ml SCA and incubating for 6 days at 25°C.

After 6 days incubation, the mycelial mats of each fungus were homogenised in SCA medium with a Sorvall Omnimixer at setting 6 for 20 s.

This was the homogenized mycelial inoculum used for various experiments.

4. Inoculation technique

Whole leaves were detached from the plant by excising with a sharp scalpel. The cut petioles were immediately immersed in a beaker of water. Leaves were then gently rubbed acropetally through the thumb and fore finger to disperse the wax bloom, care being taken not to damage the epidermal cells. Leaves were placed on expanded metal grids so that the petioles were immersed in 75 ppm benzimidazole solution in transparent polystyrene sandwich boxes (17.2 cm x 11.2 cm x 3.2 cm). Usually six to eight 20 µl droplets of homogenised mycelium or conidial suspension prepared as above were placed on the upper surface of each leaflet by means of an "Agla" micrometer syringe fitted with a hypodermic needle. Sandwich boxes were tightly shut and incubated in a cooled illuminated incubator held at 20°C.

5. Chemicals

All chemicals routinely used were of Analar grade and were obtained from British Drug Houses (BDH) Ltd., Koch Light Ltd., Oxoid Ltd., Hopkins and Williams Ltd. Ethanol and methanol from James Burrough Ltd., Phenyl methyl sulphonyl fluoride from Sigma Chemical Company, Ethylene diamine-tetra-acetic acid and Mercaptoethanol from BDH.

6. Preparation of Elicitor

(a) Fungal cell wall

Large quantities of fungal mycelium were produced by inoculating 500 ml SCA medium contained in 1 litre Erlenmeyer flasks, with 10 ml of approximately 10^8 spores/ml *B. cinerea* or 40 ml homogenised *S. trifoliorum* mycelium. Flasks were then incubated in a shaker for 8 days at 25°C at a speed of 100 r.p.m.

(i) Isolation of mycelial wall from fungi

The method of Anderson & Albersheim(1975)was used. 8 day old mycelia were vacuum-filtered through Whatman No.1 filter paper,washed two times with distilled water and weighed - this was the wet wt. Mycelia were then homogenised in water (5 ml H_2O/g wet wt. mycelium). The homogenate was filtered as above and the residue homogenised three times with water, filtering as above each time. Homogenisation was then carried out once with chloroform : methanol (1 : 1) and finally with acetone. The final residue was air-dried overnight and was referred to as mycelial wall.

(ii) Extraction of "Elicitor" from mycelial wall

Dried mycelial wall was weighed and suspended in distilled water (1 g wall/100 ml H_2O). This suspension was autoclaved at 121°C for 20 min and then filtered through Whatman No. 1 filter paper (without vacuum) and the filtrate dialysed in 2 litres of water overnight in a cold room. The dialysate was then concentrated to a known carbohydrate content and this was used as the cell wall elicitor. Treatment of leaflets was as for mycelial inoculation.

(b) Culture filtrates

(i) Preparation of crude culture filtrate

Fungal mycelium was grown in SCA liquid medium for 6 days as mentioned above for production of mycelial inoculum. After this time, the cultures were harvested and the mycelium removed by centrifugation at 3000 r.p.m. for 15 min. The supernatant above the mycelial mats was decanted and either used immediately as culture filtrate or stored at -20°C until required. Before use, culture filtrates were sterilized by microfiltration through 0.2 μm pore size Millipore membrane filters

and leaf treatment as for mycelial homogenate.

(ii) Preparation of ethanol-soluble and ethanol-insoluble fractions

A modified method of Dixon and Fuller (1977) was used. Four hundred ml of absolute alcohol was added slowly while stirring all the time to 100 ml of culture filtrate. After standing for 45 min, the precipitate which formed was centrifuged at 25,000 g for 30 min. The supernatant was evaporated to dryness in vacuo at 45°C and the residue taken up in 100 ml distilled water. This fraction was the 80% ethanol-soluble fraction of the culture filtrate. The precipitate (ethanol-insoluble pellet) was washed twice with 80% ethanol and taken up in 100 ml distilled water. Before use, fractions were membrane sterilized as for crude culture filtrate and leaf treatment as for mycelial inoculation.

7. Preparation of material for light microscopy

Leaf discs were cut with a 6 mm diameter cork borer, fixed in chloroform : ethanol : acetic acid (30 : 60 : 10) for 24 h and cleared in 1% phenol in xylene for 24 h. Discs were then stained with cotton blue (0.1% in lactophenol) or trypan blue (0.2% in lactophenol) for 24 h.

Excess stain was washed off with lactophenol and the leaf discs mounted in lactophenol.

35 mm PANF black and white film and an Exacta VX 1000 camera mounted on a Reichert Zetopan microscope were used for photography.

8. Extraction of phytoalexins from plants

(a) Leaf tissue

Inoculum droplets collectively referred to as diffusates were removed with a syringe. The underlying tissue was excised with a 5 mm diameter cork

borer. Rings of tissue surrounding this were excised with larger diameter borers or with a scalpel so that an area of ostensibly live tissue 2 mm in diameter was obtained. The collected tissue was then weighed and either combined with the diffusates or extracted separately as follows.

Samples were homogenised in ethanol with a Sorvall Omnimixer (at least 20 ml ethanol/g wet wt.), at setting 6 for 30 s. The homogenate and several washings from the mixer were filtered by vacuum filtration, and the residue washed three times with 5 ml aliquots of ethanol. The combined ethanol filtrates were evaporated to dryness in vacuo at 45°C and the residue dissolved in 70% ethanol. This ethanolic phase was partitioned twice with equal volumes of petroleum ether (40° - 60°C) to remove pigments and lipids. The 70% ethanol phase was evaporated to dryness and the residue taken up in the minimum volume of ethyl acetate for thin layer chromatography (t.l.c.)

(b) Diffusates

Known volumes of inoculum droplets were extracted three times with equal volumes of ethyl acetate in test tubes (150 mm x 15 mm). Thorough mixing of the aqueous and ethyl acetate phases was achieved by the use of a Griffin flask shaker. The combined organic phases were then reduced to dryness *in vacuo* at 45°C prior to chromatography.

9. Methods for obtaining phytoalexins for *in vitro* studies

(a) Maackiain

As red clover roots contain large quantities of the maackiain glycoside, trifolirhizin, the phytoalexin maackiain was obtained from this source.

Roots from 2 - 3 months old plants were thoroughly washed with water. Soil-free roots were then cut into small bits with scissors and homogenised in distilled water with acid-washed sand and a pestle and mortar. The pulp was left at room temperature in plastic sandwich boxes for at least 6 h,

during which time the endogenous β-glucosidase enzyme brings about hydrolysis of trifolirhizin to the aglycone, maackiain.

The homogenate was sieved through 4 layers of muslin and washed several times with ethanol. The combined washings were then filtered through Whatman No. 1 filter paper by vacuum filtration and the filtrate dried down in vacuo to about 25% of the original volume. This aqueous phase was then partitioned three times with equal volumes of diethyl ether. The combined ether phases were evaporated down in vacuo and the residue taken up in a small volume of ethyl acetate for TLC.

Pure 6a-hydroxymaackiain was produced by incubating homogenised clover roots with comminuted S. trifoliorum mycelium for at least 48 h. Extraction was with ethyl acetate and chromatography as for maackiain.

(b) Medicarpin

This is the phytoalexin from Jack beans (Keen, 1972, 1975a).

Jack bean seeds were allowed to imbibe in water for 24 h. Soaked seeds were then thinly sliced into 1 - 3 mm thick pieces with a razor and placed in sandwich boxes moist with 0.01M KH_2SO_4 pH 7.5, by placing pieces directly with the solution and incubated for 3 - 5 days at 25°C. No micro-organisms were added to the germinating seeds but they were challenged with their natural microflora, which increased greatly by multiplication during incubation. The seeds became yellow-brown during incubation. After incubation, seeds were comminuted in 95% ethanol with a Sorvall Omnimixer at setting 6 for 30 s. The resulting slurry was vacuum filtered through Whatman No. 1 filter paper and the residue washed twice with 50 ml aliquots of the solvent. The ethanol was removed in vacuo at 45°C on a rotary evaporator and the aqueous phase partitioned three times with equal volumes of ethyl acetate. The combined ethyl acetate phases were dehydrated with anhydrous sodium sulphate and dried down at 45°C on a rotary evaporator. The residue was taken up in a minimum volume of ethyl acetate for thin layer chromatography.

10. Chromatographic techniques

Ascending thin-layer chromatography (TLC) was used as a preparative technique throughout this research. TLC plates used were plastic-backed precoated silica gel with luminescence 254 nm and layer thickness of 0.20 mm. Extracts in ethyl acetate were applied as a streak 2.5 cm from the bottom edge using a drawn-out pasteur pipette. A Murphy Richards hair dryer blowing cold air was used to evaporate the solvent during application. TLC plates were then developed in unequilibrated Shandon S/P Chromotanks containing 100 ml solvents, until the solvent front was about 1.5 cm from the top. The following solvent systems were used :

Chloroform : methanol (98 : 2, v/v) and for further chromatographic purification if necessary

petroleum ether : diethyl ether (50 : 50, v/v) and/or

n-pentane : diethyl ether : acetic acid (75 : 25 : 1, v/v)

11. Location of phytoalexins

(a) UV absorption

After ascending chromatography, developed chromatograms were dried and examined under 254 nm or 366 nm UV light. Maackiain and medicarpin and their degradation products appear as a dark area at the same R_f as authentic standards. These values corresponded to the published R_f values. The bands were outlined with a pencil and eluted with ethanol after careful removal of the silica gel from the plates with a microspatula. Elution was in sintered glass funnels lined with filter paper to prevent blockage by the silica gel.

(b) UV spectroscopy

Eluates in ethanol were scanned on a Spectrophotometer between 350 nm

and 240 nm and the spectra compared with those of authentic compounds.

(c) Locating reagents

Phytoalexins were located on chromatograms by spraying TLC plates with the following reagents :

(i) Diazotised sulphanilic acid

A. 9 g sulphanilic acid, 40 ml conc. HCl and 900 ml H_2O.

B. 5% $NaNO_2$.

C. 10% anhydrous Na_2CO_3.

Equal volumes of A and B were mixed and left at 10°C for 5 minutes. 2 volumes of C were then added and the solution used immediately. This reagent shows phenols and aromatic amines that can couple.

(ii) 4-nitroanailine diazotised (PNA)

5 ml 0.5% 4-nitroaniline solution in 2 N HCl were mixed under cooling with 0.5 ml 5% aqueous $NaNO_2$ solution and 15 ml 20% aqueous sodium acetate solution was added. Phenolic compounds form products of various colours with this reagent.

(iii) 2,6-dibromoquinonechloroimide (Gibb's reagent) stains phenols.

Spray solution : Freshly prepared 0.4% methanolic solution of 2,6-dibromoquinonechloroimide. After spraying, spray again with 10% aqueous sodium carbonate solution.

(iv) Antimony pentachloride : 2 parts to 8 parts of chloroform. Shows phenols and steroids. If plate is heated at 120°C, colour changes may occur.

12. Quantification of phytoalexins

Spectra of the phytoalexins in 95% ethanol in 1 cm diameter cuvettes were obtained by scanning on a spectrophotometer between 350 nm and 240 nm. Concentrations were calculated by using their molar extinction coefficient ($\mathcal{E}$). This is a proportionality constant, and it is the optical density for a 1 M solution in a cell with a path length of 1 cm.

From Beer's Law

$$\text{O.D.} = \mathcal{E} \times C \times \text{path length}$$

C = concentration in mole/litre

O.D.= optical density

$$\text{Concentration in } \mu g/ml = \frac{\text{O.D. x MW x } 10^3}{\mathcal{E}}$$

MW = molecular weight of compound

Published extinction coefficients are :-

1. Maackiain $\log \mathcal{E} = 3.63$ (at 287 nm)

   .'. O.D. of 1.0 = 66 µg/ml (Bredenberg and Hietala, 1961 a,b)

2. Medicarpin $\log \mathcal{E} = 3.90$ (at 287 nm)

   .'. O.D. of 1.0 = 34 µg/ml (Smith et al., 1971)

3. 6a-hydroxymaackiain $\log \mathcal{E} = 3.64$ (at 286.5 nm)

   .'. O.D. of 1.0 = 69 µg/ml

   or $\log \mathcal{E} = 3.87$ at 309 nm (Van Etten et al., 1975)

4. 6a-hydroxymedicarpin - based on $\log \mathcal{E}$ for medicarpin.

Maackiain and medicarpin run together on t.l.c. and could not be easily separated by any simple procedure. Therefore, in clover extracts, they were quantitated from the O.D. of the mixtures measured at 287 and 310 nm. The separate contribution of each compound to the mixed spectrum was calculated from the extinction coefficient of the pure compounds at these wavelengths by use of simultaneous equations.

$0.0294\ x + 0.0150\ y$ = O.D. at 287 nm

$0.0052\ x + 0.0238\ y$ = O.D. at 310 nm

x = medicarpin concentration

y = maackiain concentration

Figures are based on the O.D. of a 1 μg/ml solution at the appropriate wavelength.

Similar procedures were used for calculating the concentrations of 6a-hydroxymaackiain and 6a-hydroxymedicarpin, from their mixed spectra.

$0.0294\ x + 0.0145\ y$ = O.D. at 287 nm

$0.0052\ x + 0.0177\ y$ = O.D. at 310 nm

x = hydroxymedicarpin concentration

y = hydroxymaackiain concentration

Figures are also based on the O.D. of a 1 μg/ml solution at the appropriate wavelength.

13. Measurement of oxygen uptake

Oxygen uptake by respiring fungi was determined in a Rank oxygen electrode (Rank Bros. Ltd.) with a 5 ml cell and a voltage of 7 mV. Changes in electrode current were recorded on a Servoscribe potentiometric recorder (R.E. 511.20 Philips). Two electrodes were maintained at a constant temperature of 20°C by a water jacket connected in series and maintained by a Julabo "Paratherm II" heater (Science Western Ltd.). The electrodes were

calibrated with air-saturated distilled water which contained 0.29 µmole O_2/ ml at 20°C and zeroed with a few granules of sodium dithionite. Aliquots (5 ml) of homogenised mycelium (prepared as for mycelial growth test) were placed into the electrode cells and continuously agitated with a rotating magnetic flea. After stabilisation, 50 µl DMSO or phytoalexin in 50 µl DMSO was added to each cell and oxygen uptake measured over 15 - 20 min periods at hourly intervals.

14. Metabolism of phytoalexins by fungi in vitro

Purified phytoalexin dissolved in 50 µl dimethyl sulphoxide (DMSO) was added to 50 ml Erlenmeyer flasks containing 5 ml homogenised mycelium, which had been left for 24 h to recover from homogenisation. The final DMSO concentration of 1% was not inhibitory to fungal growth and was necessary to keep the phytoalexins in solution. Sterile conditions were maintained throughout these experiments, and the cultures incubated at 25°C without shaking.

At time zero minutes and at various time intervals thereafter phytoalexin concentration and/or fungal growth in duplicate or triplicate flasks were monitored. The contents of each flask was centrifuged at 1800 g and the supernatant pasteur-pipetted into 50 ml boiling tubes. Fungal pellets were washed with 5 ml distilled water and the combined supernatants partitioned twice with equal volumes of carbon tetrachloride (CCl_4) and then twice again with ethyl acetate (Et OAc). Thorough mixing of aqueous and organic phases was achieved with a Griffin flask shaker. The CCl_4 and Et OAc extracts were dried down in vacuo at 45°C and the residue taken up in 3 or 4 ml ethanol for spectrophotometry.

The fractions were scanned from 350 nm to 240 nm, with ethanol in the reference cuvette (optical path length = 1 cm). The extinction at 287 nm minus the background at 350 nm was taken as a measure of the relative quantity of phytoalexin present. In some experiments, supernatants from fungal

cultures were extracted three times with Et OAc and then subjected to TLC in chloroform : methanol (98 : 2, v/v).

15. Bioassay techniques

(a) Cladosporium on TLC plates

Cladosporium cucumerinum has been used as an indicator for antifungal compounds (Bailey and Burden, 1973) because it grows quickly, it is dark in colour and is reputed to be particularly sensitive to antifungal compounds. This fungus was therefore used to locate antifungal compounds directly on TLC plates.

A dense spore suspension of Cladosporium was prepared by putting 10 -15 ml sterile SCA medium into 5 - 7 day old cultures, gently scraping the culture surface and shaking the flask. The resulting suspension was filtered through two layers of muslin and then sprayed on to developed TLC plates which had been dried to remove traces of solvents. Spraying was accomplished with a Shandon spray gun. The plates were allowed to dry and then given a second spray with the suspension, ensuring homogeneity throughout the plate, and then dried off in a fume cupboard until the plates were just moist, but with no free surface medium. Damp plates were then incubated about 3.5 cm from the bottom, of moist plastic boxes at 25°C for 3 - 4 days. After this period, the fungus would have grown over the whole plate except in those areas where inhibitory compounds were present which remained white.

(b) Spore germination test

The method was based on that of Purkayastha and Deverall(1965b). Soda glass slides were cleaned by rubbing in hot water and detergent (Teepol) and repeatedly rinsed in tap water, and then in distilled water. They were soaked overnight in 5% acetic acid and then overnight in distilled water. They were then soaked for 4 h in acetone and dried in an oven at 100°C for 6 h. This method gave slides which had no deleterious effect on spore

germination nor spread of the test drops.

Twenty microlitre (µl) drops of the solution to be bioassayed were pipetted onto separate slides. Usually three separate treatments were allocated to each slide and there were three replicates per treatment. One µl of conidial suspension (5×10^5 conidia/ml) was added to the centre of each drop using an 'Agla' micrometer syringe so that there were 500 spores/drop. Only drops shaped as shallow, approximately circular domes were used, other drops were discarded, thereby standardising the position of spores relative to the edge and surface of the drop. Slides were supported on test tubes, over moist tissue paper, in sandwich boxes (171 mm x 112 mm x 50 mm) and incubated at 20°C for 21 h in the dark.

At the end of this period, conidia were killed and stained by adding a drop of cotton blue in lactophenol to each drop. Percentage germination was measured by examining 100 spores/drop, germination being considered as the production of a germ-tube at least the length of the spore diameter. Germ-tube lengths were measured by use of a calibrated micrometer eyepiece. The length of thirty germ-tubes per drop was measured. All results are expressed as replicate means.

(c) Mycelial growth test

5 ml samples of homogenised fungal mycelium (approx. 2 mg dry wt.) in SCA medium were pipetted into 50 ml Erlenmeyer flasks and allowed to recover from homogenisation for 24 h. Phytoalexin was added in 50 µl DMSO giving a final DMSO concentration of 1%. Control flasks received only DMSO, and sterile conditions were maintained throughout. Flasks were incubated at room temperature without shaking for 24 h. After this time, the fungal mycelium was then centrifuged at 1800 g and the dry wt. determined after heating in an oven at 80°C for 24 h.

16. Electrolyte measurements

(a) Flame photometry

A flame photometric assay for K^+, Ca^{2+}, and Na^+ ions was used. Recovered homogenised mycelium as used for mycelial growth test was used and the leakage and/or uptake of the ions monitored with an Eel flame photometer (Evans Electroselenium Ltd.). The photometer was calibrated using potassium, calcium or sodium filters as appropriate, with stock solutions containing 3 ppm K^+, 40 ppm Ca^{2+}, and 2 ppm Na^+. Zeroing of this instrument was with distilled water.

(b) Conductivity measurements

Total electrolyte loss or uptake from mycelium prepared as for mycelial growth test, was measured using a Chandras conductivity meter, and noting the change in conductivity against a water blank.

17. Carbohydrate determination

The anthrone method was used. 4 ml anthrone reagent (0.2% anthrone in conc. H_2SO_4) was added to 1 ml of test solution with rapid mixing. Tubes were covered with aluminium foil to prevent water loss by evaporation and then placed in a boiling water bath for 10 min. After cooling the extinction at 620 nm was read against a reagent blank, and the amount of glucose calculated from a standard curve using a 100 µg/ml glucose solution each time.

18. Protein estimation

(a) Folin-Lowry method

(i) Materials

1. 2% Na_2CO_3 in 0.1N NaOH
2. 0.5% $CuSO_4$ in 1% Na, K tartrate (prepared fresh by mixing

stock solutions)

3. 'Alkaline solution' (prepared on day of use by mixing 50 ml of (1) and 1 ml of (2)
4. Folin-Ciocalteu reagent (Commercial reagent diluted 1 : 1 with distilled water on day of use)
5. Protein standard (0.2 mg/ml albumin)

(ii) Method

5 ml (3) was added to 1 ml test solution thoroughly mixed and allowed to stand at room temperature for 15 min.0.5 ml of (4) was then rapidly added with immediate mixing. After 30 min the extinction at 750 nm was read against a reagent blank, and the protein concentration estimated from a standard curve using (5) above as standard each time.

(b) UV absorption (Layne, 1957, after Warburg and Christian, 1941)

The optical density of the sample was read in cuvettes with a 1 cm light path at 260 nm and 280 nm. The ratio $OD\ \frac{280}{260} = F$ was calculated and substituted in the following equation :

$$\text{Protein concentration (mg ml}^{-1}) = F \times \frac{1}{d} \times \text{OD at 280 nm}$$

where d = length (in cm) of light path in cuvette

19. Ultrafiltration

This was carried out in a stirred ultrafiltration cell (Amicon Ltd. model 52) fitted with a Diaflo ultrafiltration membrane size PM 10. The pore size of this membrane has a retentivity $>$ 10,000 MW. The contents of the cell was kept at $\underline{c}$ 2°C and the carrier gas was N_2 maintained at a pressure of 4-kg. cm^{-2}.

20. Degradation of phytoalexin by 'cell free' fungal extract

(a) Induction

Large quantities of mycelium was produced as in preparation of elicitor. 5 g washed mycelium was resuspended in 40 ml 0.1 M phosphate buffer pH 7.2 and the phytoalexin degrading system induced with a concentration of 10 µg/ml maackiain for 6 h.

(b) Preparation of 'cell free' extract

Induced mycelium was harvested by centrifugation (1800 g x 10 min) and washed three times with cold buffer and finally taken up in 20 ml buffer. To this the following were added :-

1 mM EDTA, 10 mM Mercaptoethanol and phenyl methyl sulphonyl fluoride (4.8 mg in 0.3 ml isopropyl alcohol).

Approximately 5 ml of the resulting suspension were then placed into each of the two cooled flat bottomed glass mickled containers (60 mm x 40 mm). An equal volume of glass Ballotini beads (No. 12) was added to each cup which were then closed with rubber stoppers, and screwed into the disintegrator arms and shaken at maximum amplitude for 15 min in a cold room. The homogenate was then centrifuged (1800 g x 10 min) to separate unbroken cells and ballotini beads from the supernatant, which was further centrifuged at 10,000 g for 30 min. The supernatant was decanted and was the 'cell free extract'.

21. Measurement of pH

Hydrogen ion concentrations (pH) were measured with a pH meter (Pye unicam model 290) or narrow range indicator papers (BDH) for small volumes.

22. Production of toxin

Flasks inoculated with mycelium as in preparation of mycelial inoculum were incubated at 25°C for 20 days. After this period, mycelium was removed by centrifugation at 1800 g for 15 min and the supernatant treated as in crude culture filtrate preparation and referred to as crude toxin.

23. Chemical test for oxalic acid

The test used involved the formation of aniline blue (Hodkinson, 1977). Acetic acid (20 ml) was added to 10 ml of 20-day old culture filtrate or 10 ml SCA medium. This acetic acid solution was then treated with calcium chloride and the suspension centrifuged at 1,800 g for 10 min. The supernatant was decanted and the precipitate freed from water by washing with 5 ml absolute alcohol followed by 5 ml diethyl ether. After drying in a stream of air 0.1 g diphenylamine was added to the precipitate followed by 1 ml syrupy phosphoric acid. The tube was then heated over a free flame and the melt allowed to cool down before taken up in 3 ml alcohol. A blue colour indicates the presence of oxalic acid. Crystalline sample was assayed by melting directly with diphenylamine.

EXPERIMENTAL

1. Detection and characterisation of phytoalexins from inoculated red clover leaves

The investigation of antifungal compounds produced by red clover leaves was based mainly on the induced pterocarpans. This section deals with the development of a suitable procedure for extracting pterocarpans. The detection and characterisation of these induced antifungal compounds is also described.

Several methods have been used for phytoalexin extraction in clover leaf tissue. Debnam and Smith (1976) removed pigments and lipids from the ethanolic extract with petroleum ether, hydrolysed glycosides to the aglycones with hydrochloric acid then extracted phytoalexins with diethyl ether. Duczek and Higgins (1976a)on the other hand, subjected the entire ethanol soluble extract to thin layer chromatography (TLC). However, when this latter method was attempted, pigments obscured the phytoalexin bands, and since hydrolysis with HCl did not increase the phytoalexin content, a combined method of the two was therefore developed.

In this method, pigments were first removed from the ethanol extracts with petroleum ether. The ethanol extract was then subjected to TLC in a chloroform : methanol (98 : 2, v/v) solvent system. This method resulted in fairly clean separation of compounds which can then be eluted and quantitated by ultraviolet spectrophotometry.

Phytoalexins from diffusates have been extracted with (CCl_4) carbon tetrachloride (Duczek and Higgins, 1976a)but this method differentially extracts phytoalexins but not their hydroxylated derivatives (these being more polar). The method of Ingham (1976a)was therefore adapted. In this method inoculation droplets (diffusates) were collected and extracted three times with ethyl acetate. This extracts both phytoalexins and their hydroxylated derivatives, which can then be subjected to TLC on chloroform : methanol as for leaf tissue.

For the detection of induced antifungal compounds 3-day old S. trifoliorum-infected clover leaves were extracted and chromatographed as above. After development, TLC plates were sprayed with a thick spore suspension of Cladosporium cucumerinum in SCA medium and the plates incubated for 2 - 4 days. After this time antifungal compounds appeared as whitish zones on a dark background. The most prominent induced antifungal band had an R_f of 0.65. Less pronounced bands were at R_f 0.16, 0.07 and 0.80. Other inhibitory bands were identified as the known preformed inhibitors of clover leaves (Plate 1).

In a second experiment, developed TLC plates were viewed under UV light (254/366 nm) and bands corresponding to the induced antifungal compounds appeared as dark absorbing bands at 254 nm. The area of silica gel corresponding to the main band was eluted with ethanol and the UV absorption spectrum recorded. The eluate of this band (R_f 0.65) gave a peak at 287 nm, a shoulder at 282 nm and another peak at 310 nm which was less pronounced than that at 287 nm (Fig. 6). These characteristics were similar to the medicarpin/maackiain mixture reported by Debnam and Smith (1976), so the band was further characterised by R_f values in other solvent systems, colour reactions and spectral changes in acid and alkali media (Table 2).

These results are consistent with the phenolic nature of these compounds and with data described in the literature, that the pterocarpans maackiain and medicarpin are the main phytoalexins in red clover leaves. The faintly absorbing band at R_f 0.16 was also eluted and scanned in a UV spectrophotometer. Sometimes the spectrum was obscured by partial overlap with the isoflavone, genistein. In this case, the compound was further purified by rechromatography in diethyl ether : petroleum ether (50 : 50, v/v) x 3. The now purified compound had a typical pterocarpan spectrum and resembled that of maackiain/medicarpin mixture, with a main peak at 287 nm, a shoulder at 282 nm and another peak at 310 nm (Fig. 7). The characteristics of this band are

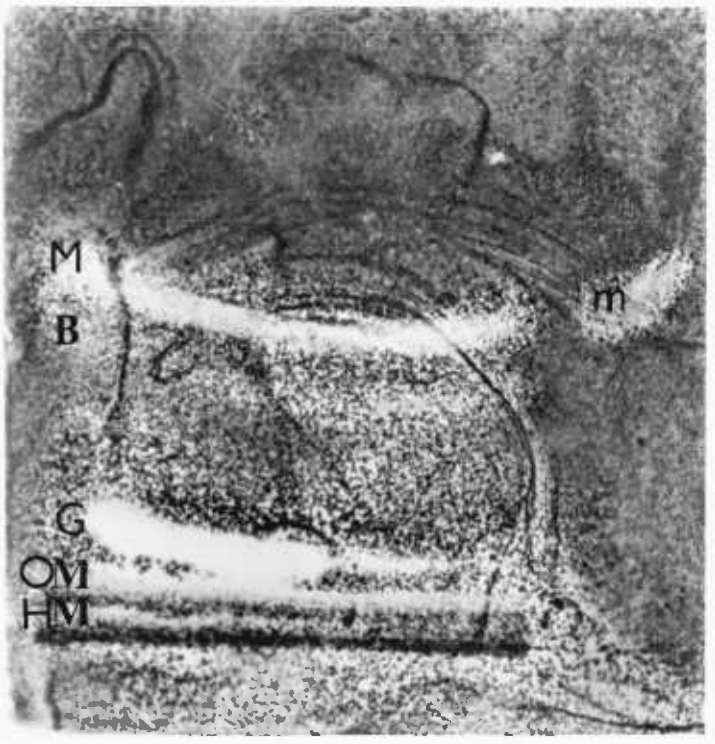


Plate 1. Cladosporium bioassay of S. trifoliorum-infected leaf tissue.

M = Maackiain/Medicarpin

m = Authentic maackiain from clover roots

B = Biochanin A

G = Genistein

OM= 6a-hydroxymaackiain/6a-hydroxymedicarpin

HM- 6a,7 dihydroxymaackiain/6a,7 dihydroxymedicarpin

Band at R_f 0.8 corresponding to pisatin/homopisatin not present on this plate.

Figure 6. UV absorption spectrum of a mixture of maackiain and medicarpin from extracts of S. trifoliorum inoculated tissue.

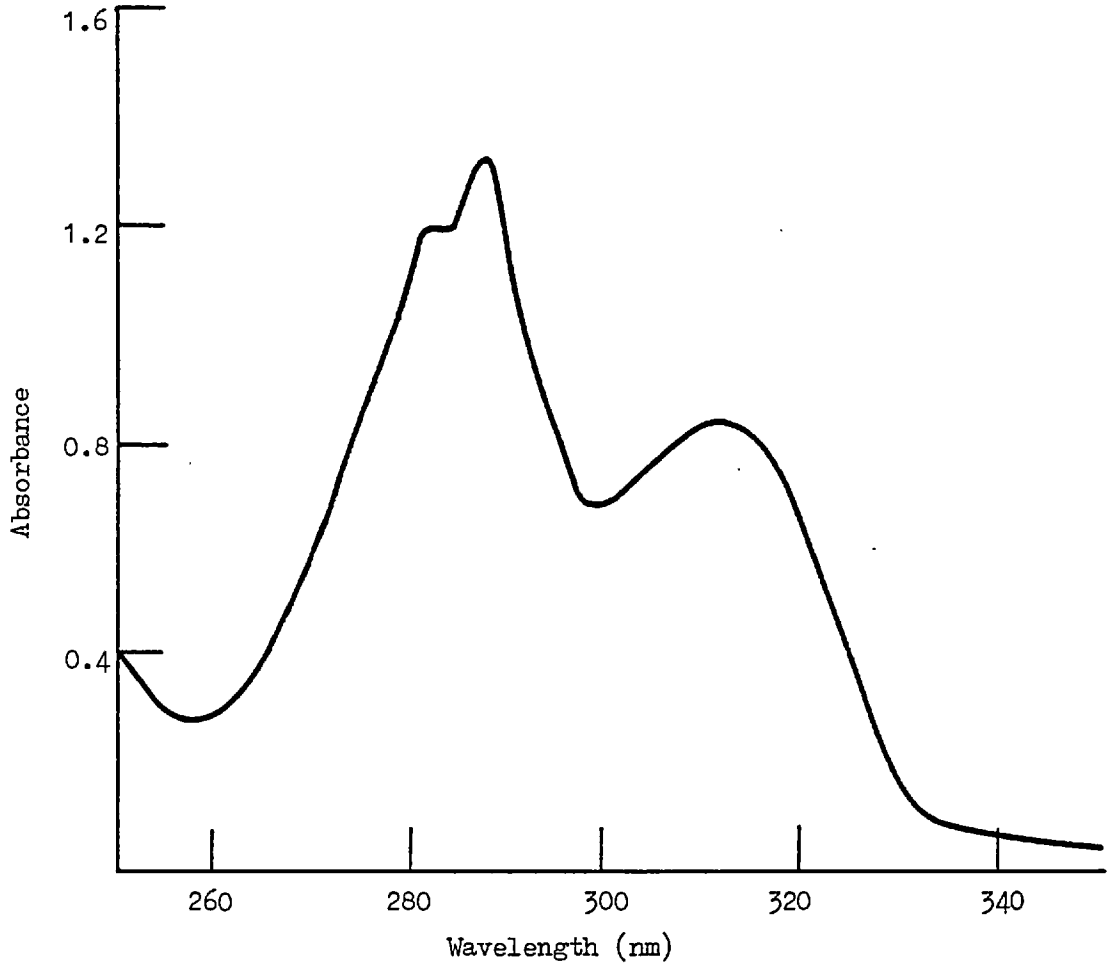
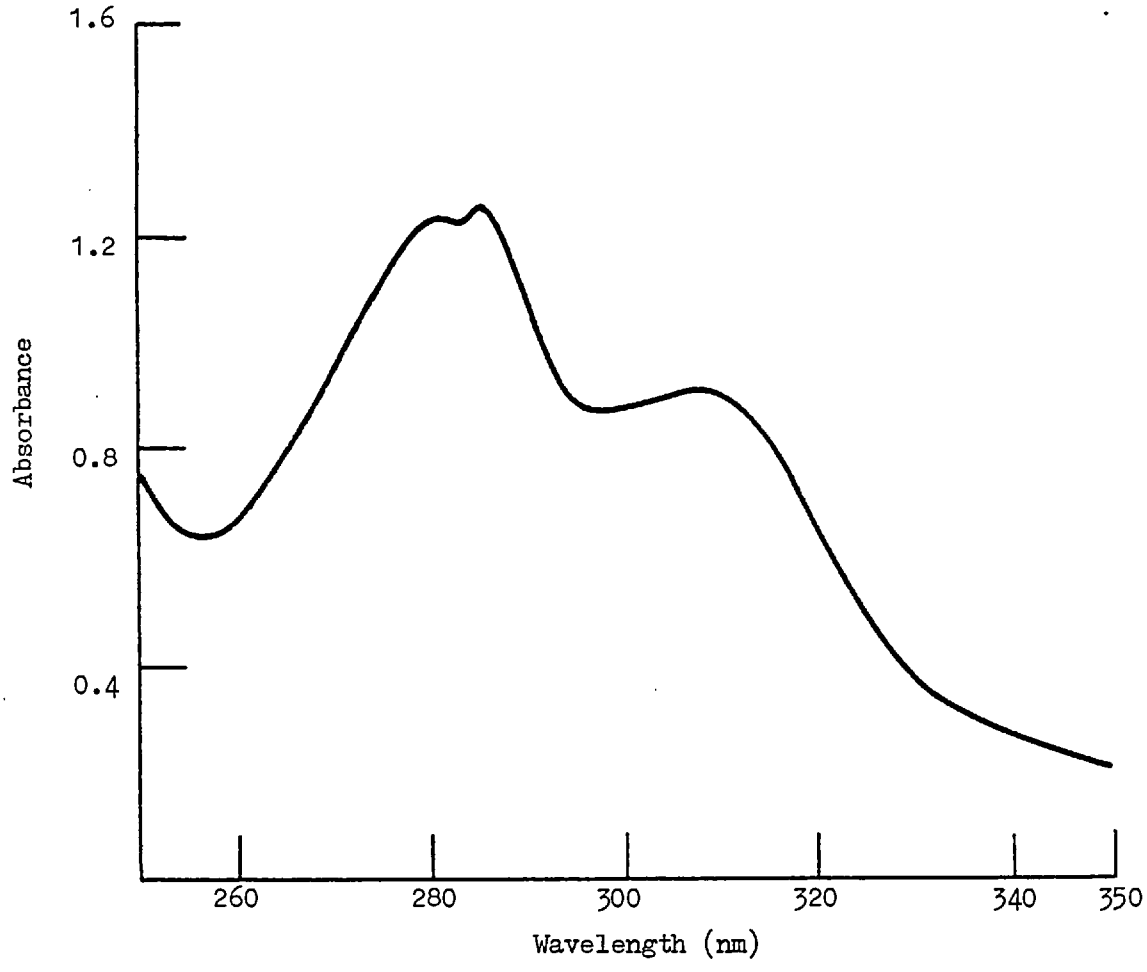


TABLE 2. Characteristics of maackiain/medicarpin band

R_f values	Colour reactions	Spectral features
0.65 chloroform : methanol (98/2)	Antimony pentachloride $SbCl_5$ - Brick red	EtOH λ nm 282, 287, 310
0.48 petroleum ether : diethyl ether (50/50)	Paranitroaniline - Yellow	EtOH + NaOH λ nm 291
0.40 n-pentane : diethyl ether : acetic acid (75/25/1)	Gibb's reagent - brown Diazotized sulphanilic acid - Yellow brown	EtOH + HCl λ nm 282, 287, 310

Figure 7. UV absorption spectrum of a mixture of 6a-hydroxymaackiain and 6a-hydroxymedicarpin from extracts of S. trifoliorum inoculated tissue



recorded in Table 3.

These results are consistent with the phenolic nature of these compounds and with data described in the literature.

Under UV light (254 nm), the area of R_f 0.07 was not clearly defined. When this area was eluted with ethanol and scanned in a spectrophotometer, the spectrum obtained was obscured by contamination with other compounds. On further purification in chloroform : methanol (90 : 10, v/v) x 3, this region containing many overlapping bands, separated into several bands. However none of these bands gave any clear UV spectrum when scanned. Possibly the compound of R_f 0.07 which was responsible for the antifungal activity on Cladosporum TLC bioassay, was lost during the successive purification process.

2. Preparation and characterisation of red clover phytoalexins

Since maackiain and medicarpin run together on TLC, and were therefore very difficult to separate (Debnam, 1975; Higgins, 1972) other sources were used for the independent production of large quantities of these phytoalexins for in vitro studies.

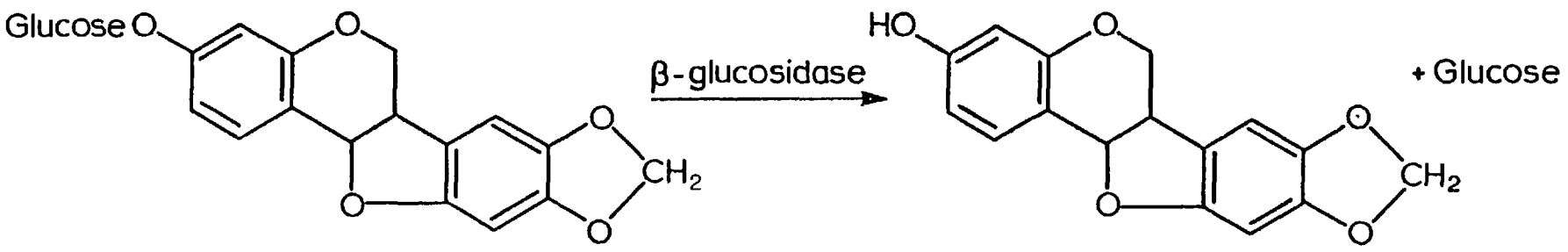
Maackiain was obtained from clover roots by homogenising in water and allowing a host β-glucosidase enzyme to hydrolyse the preformed maackiain glycoside, trifolirhizin to the aglycone, maackiain (Fig. 8).

Thus maackiain was produced not by de novo biosynthesis from remote precursors, but by enzymic release from its glycoside precursor. After hydrolysis, extraction with diethyl ether and TLC was carried out as described in Materials and Methods. Previous workers have allowed the hydrolysis to continue for 24 - 72 h. However, an experiment in which hydrolysis was allowed to proceed for 6 h produced twice the quantity (359 µg/g fresh wt.) as when hydrolysis continued for 72 h, when only 194 µg maackiain/g fresh wt. of roots was recovered. It seems likely that the reduction in phytoalexin

TABLE 3. Characteristics of 6a-hydroxymaackiain/6a-hydroxymedicarpin band

R_f value	Colour reactions	Spectral features
0.16	Antimony pentachloride	EtOH
chloroform : methanol	$SbCl_5$ - Yellow-brown	λ nm 282, 287, 310
98/2		
	paranitroaniline p NA - Yellow	EtOH + NaOH
		λ nm 250, 290
	Gibb's reagent - brown	EtOH + HCl
	diazotized sulphanilic acid -	λ nm 282, 287, 339, 353
	Golden yellow	

Figure 8. Enzymic hydrolysis of trifolirhizin to maackiain



Trifolirhizin (glucoside)

Maackiain (aglycone)

concentration on further incubation up to 72 h, was due to degradation by the soil and root microflora, or by host enzymes.

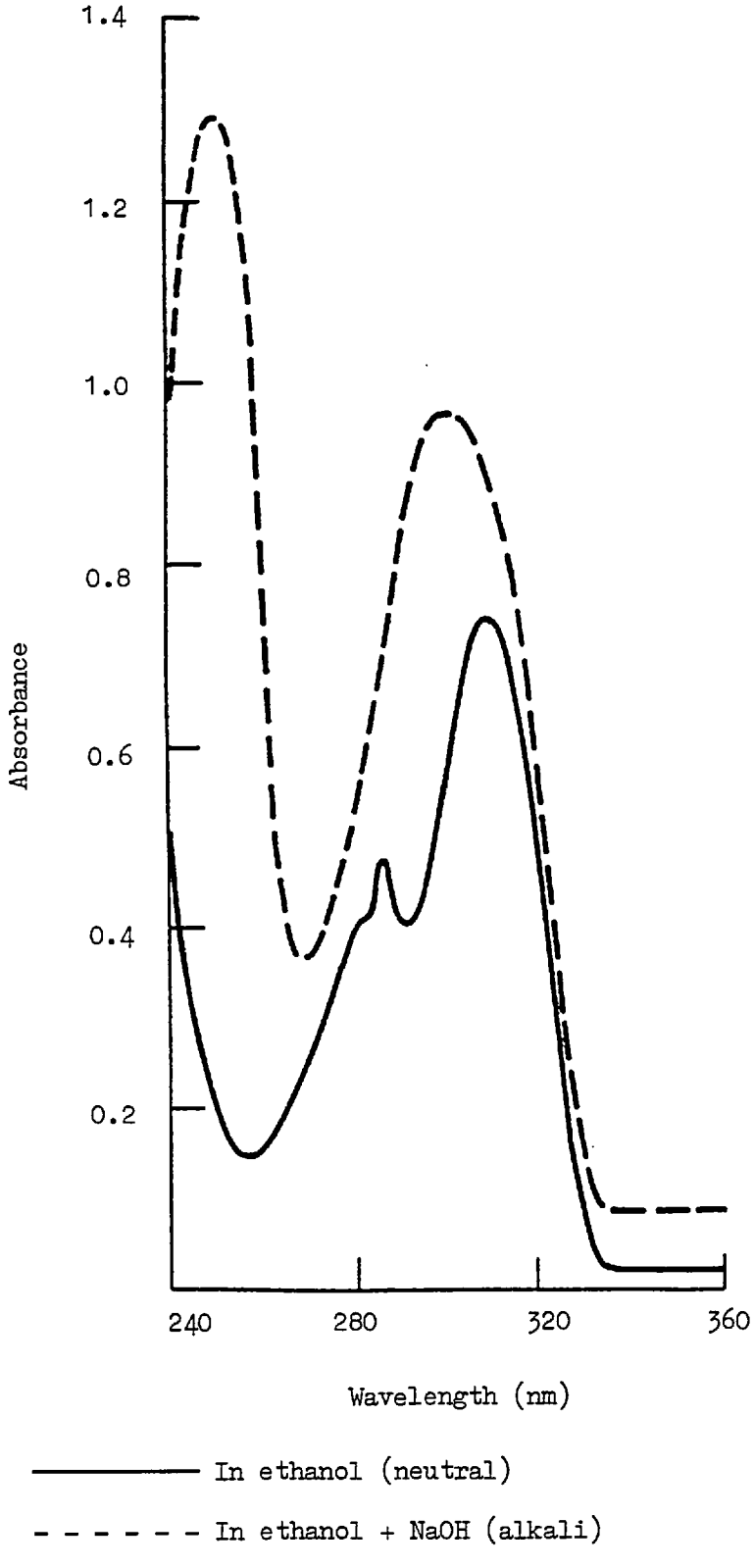
Medicarpin (Fig. 1) is the only reported phytoalexin in Jack beans (Canavalia ensiformis L.), therefore this phytoalexin was obtained from Jack bean seeds by the method of Keen (1975 a). Debnam's (1975) adaptation of this method was too time consuming and yields were low, so seeds were allowed to inbibe water for 24 h and sliced pieces soaked in KH_2PO_4 (pH 7.5) for 4 - 5 days for the natural microflora to elicit the biosynthesis of medicarpin, during rotting. When signs of rotting did not appear by four days, blended mycelium of Botrytis allii was added to speed up the rotting process and the elicitation of the synthesis of the phytoalexin. Extraction with ethyl acetate, and dehydration with anhydrous sodium sulphate were carried out as in Materials and Methods.

TLC of maackiain or medicarpin was first carried out in chloroform : methanol (98 : 2, v/v). The dark absorbing band under UV light (254 nm) with R_f 0.65 was eluted and rechromatographed in diethyl ether : petroleum ether (50 : 50, v/v). R_f in this solvent system was 0.48. If after elution and scanning in a UV spectrophotometer the spectrum did not appear very clear, bands were further chromatographed in n-pentane : diethyl ether : acetic acid (75 : 25 : 1), to obtain a sample of sufficient purity. R_f in this solvent system was 0.40. This band was the only inhibitory band in Cladosporium bioassay.

Purified maackiain in 95% ethanol gave a UV spectrum typical of pterocarpans i.e. the shape of the peak between 280 - 290 nm. A peak occurred at 287 nm, a shoulder at 282 nm and a larger peak at 310 nm. When a drop of 1 M sodium hydroxide (NaOH)was added to a 3 ml ethanolic solution of maackiain, a bathochromic shift took place which resulted in a single peak at 300 nm. (Fig. 9). No change in maackiain spectrum occurred when a drop of concentrated hydrochloric acid (HCl) was added to a 3 ml ethanolic solution.

Purified medicarpin in 95% ethanol was similarly pterocarpanoid in shape, but unlike maackiain the peak at 310 nm was absent, but both the peak

Figure 9. UV absorption spectrum of maackiain from red clover roots.



at 287 nm and the shoulder at 282 nm were present. A bathochromic shift resulting in a single peak at 295 nm and a loss of shoulder was obtained when NaOH was added (Fig. 10).

Purified phytoalexins on TLC plates were visualized after spraying with various reagents and the colours which developed at zones corresponding to the phytoalexins were recorded in Table 4.

The characteristics of both phytoalexins were consistent with their phenolic nature and with published data (Bredenberg and Hietala, 1961; Smith et al., 1971).

The hydroxylated derivative of maackiain (6a-hydroxymaackiain) was first produced by allowing mycelial homogenate of Sclerotinia trifoliorum to degrade maackiain in vitro for 6 h, as described for degradation experiments in Materials and Methods. However, yields were very low so another method was adopted involving the incubation of clover roots homogenised in water, with comminuted S. trifoliorum mycelium. After 48 h incubation, the aqueous medium was extracted with ethyl acetate and chromatographed in chloroform : methanol (98 : 2, v/v) solvent system. When TLC plates were viewed under UV light (254 nm) several overlapping bands were observed around R_f 0.16, therefore the whole area was eluted and rechromatographed in petroleum ether : diethyl ether (50 : 50, v/v) solvent system (x 3). Under UV light (254 nm) several bands were observed, but only the one at R_f 0.10 gave a UV spectrum which was pterocarpanoid in shape. The spectrum was very similar to that of maackiain, with a large peak at 310 nm and a smaller one at 287 nm, containing a shoulder at 282 nm. When NaOH was added a bathochromic shift resulted so that the 287 nm and 310 nm peaks coalesced to form a single peak at 300 nm . Alkali shifts were therefore identical to that of maackiain. When a drop of concentrated hydrochloric acid was added to an ethanolic solution of 6a-hydroxymaackiain a prominent peak at 340 nm appeared with a loss of the peaks at 287 nm and 310 nm (Fig. 11).

Figure 10. UV absorption spectra of medicarpin from Jack bean seeds

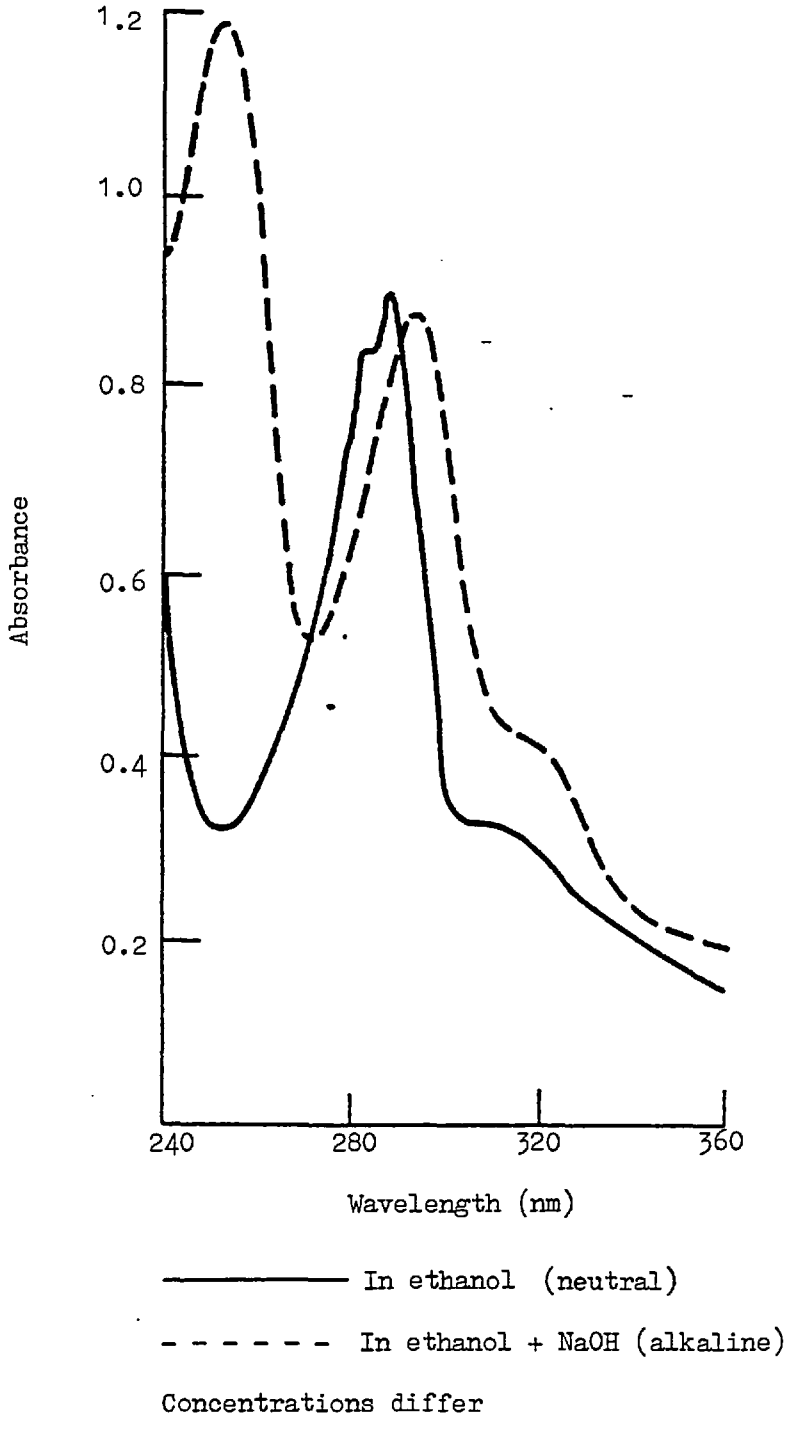
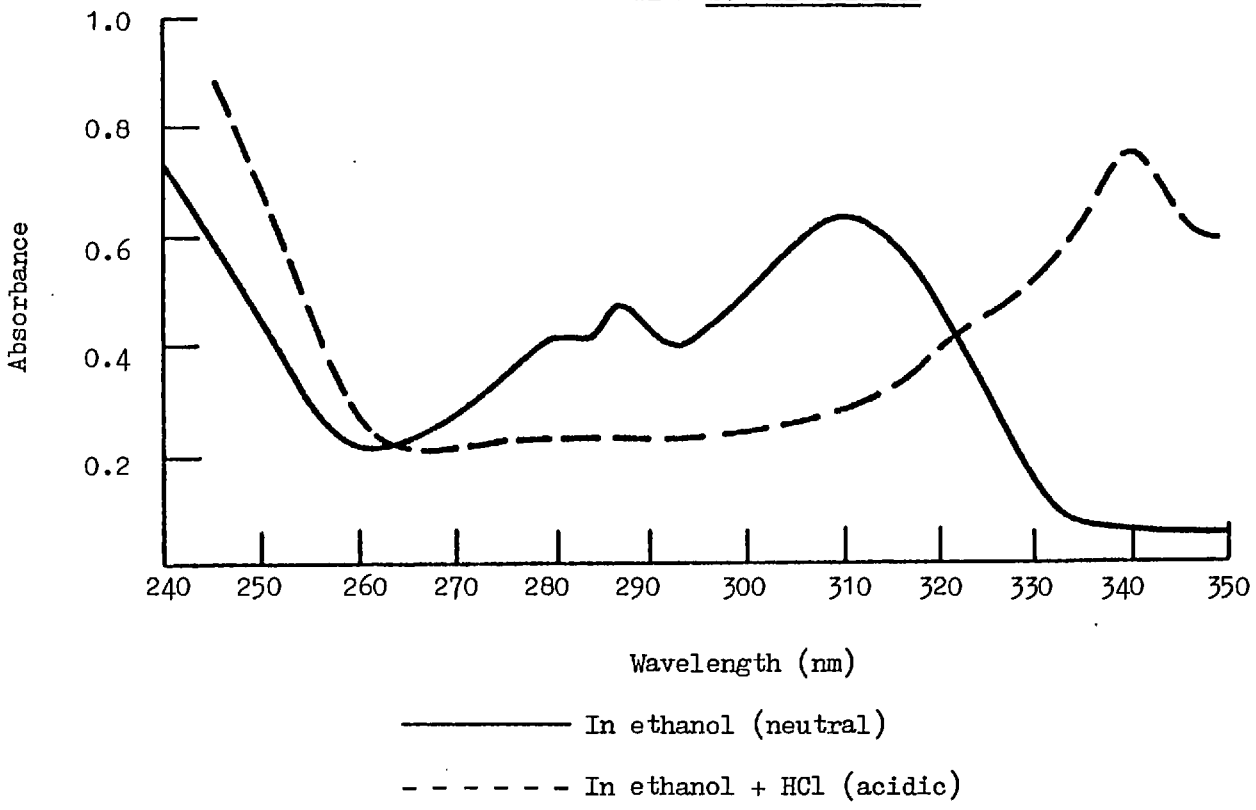


TABLE 4. Visualization of phytoalexins with spray reagents on TLC plates

Reagent	Maackiain	Medicarpin
Antimony pentachloride $SbCl_5$	Reddish brown	Yellow
Paranitroaniline pNA	Yellow	Yellow
Gibb's reagent	brown	brown
diazotized sulphanilic acid	Yellow	Yellow

Figure 11. UV absorption spectra of 6a-hydroxymaackiain from clover roots incubated with S. trifoliorum



3. Differential reactions on detached clover leaves inoculated with fungi

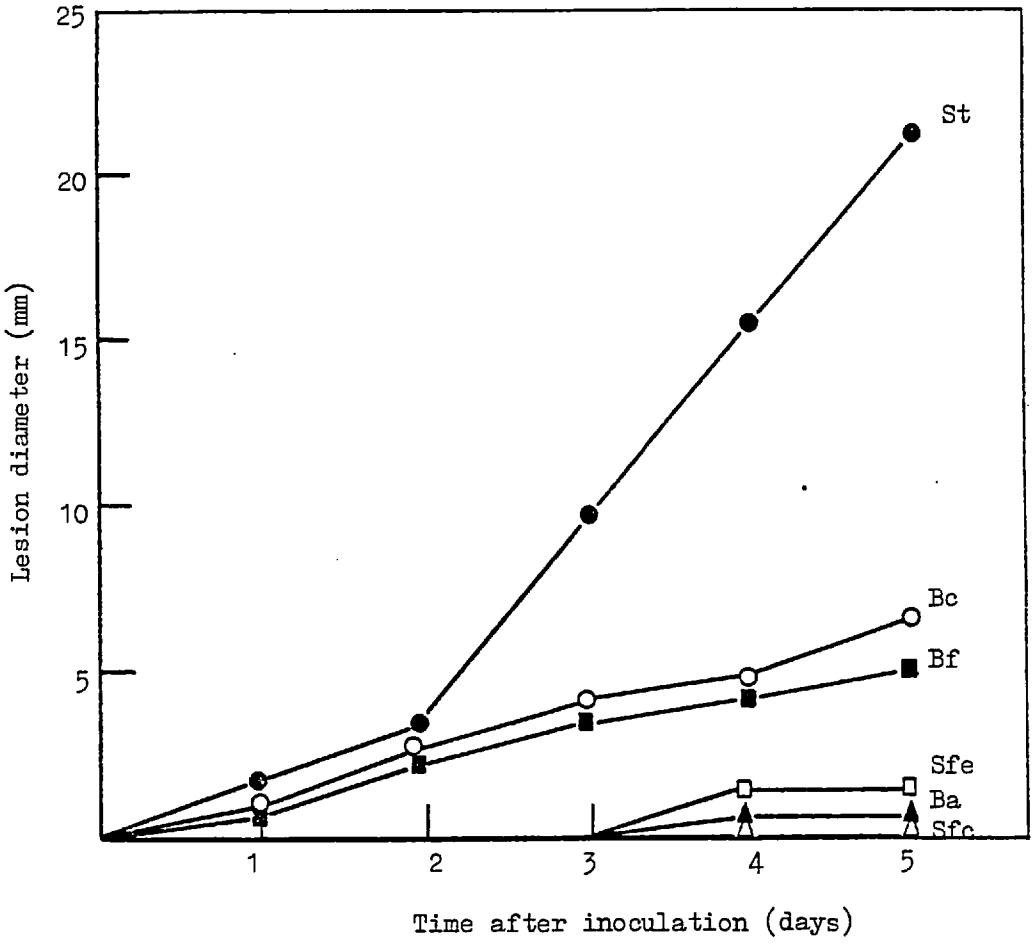
The pathogenicity of six species of fungi in the Sclerotinia and Botrytis genera was compared on red clover leaves. Six replicate leaflets were used for each treatment and each leaflet was inoculated with a 20 µl drop of mycelial suspension of the fungus (inoculation sites 3 mm diameter). The lesion development was observed and the average lesion diameter recorded at daily intervals (Fig. 12).

After 24 h incubation, S. trifoliorum, B. cinerea and B. fabae had produced small lesions (1 - 1.5 cm in diameter) at the infection sites. By 48 h, the upper leaf surface underneath the inoculation drop was completely necrotic. The necrotic lesions formed by S. trifoliorum and B. cinerea were brown in colouration, but those formed by B. fabae were black. At the lower surface of lesions, minute flecking was observed at first but this surface also became completely necrotic. After 48 h, the slowly spreading lesions of S. trifoliorum rapidly spread, eventually affecting the whole leaf (Plate 2). Lesions of B. cinerea (Plate 2) and B. fabae developed more slowly and eventually became limited in 3 - 4 days, at approximately 5 - 6 mm in diameter. No lesions were observed up to 3 days in S. fructigena and B. allii inoculated leaf tissue, but after this time necrotic flecks up to 1 mm in diameter were observed on day 4 (Plate 3). There was hardly any browning on the lower leaf surface. S. fructicola gave no visible symptoms at any time up to 5 days after inoculation, apart from a rare minute necrotic fleck.

After leaves had been observed for macroscopic symptoms they were then fixed, cleared and stained for histological observations.

Microscopically, S. fructicola mycelium grew over the surface of the leaves to the edge of the inoculum drop, but no growth was observed inside the leaf tissue. S. fructigena and B. allii mycelia grew over the leaf surface and hyphae were observed inside the leaf tissue though to a limited

Figure 12. Pathogenicity of six fungi in the Sclerotinia and Botrytis genera to leaves of red clover as measured by lesion diameter at daily intervals



St = S. trifoliorum; Bc = B. cinerea; Bf = B. fabae;

Sfe = S. fructigena; Ba = B. allii; Sfc = S. fructicola.

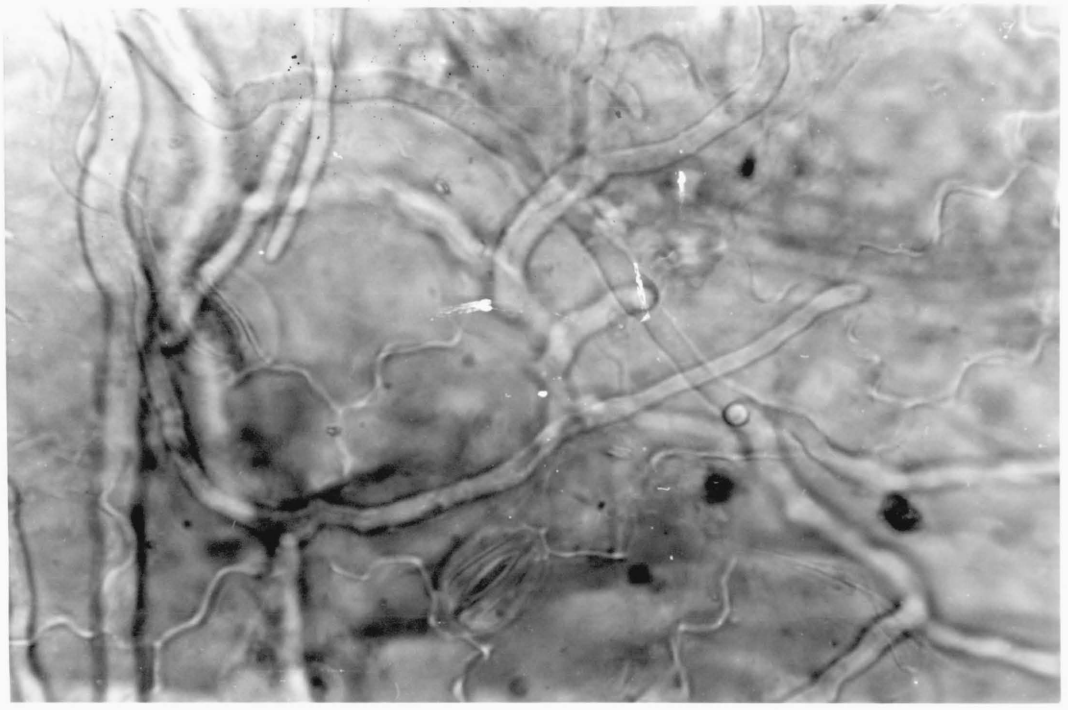
Plate 2. Leaves inoculated with 4 drops homogenised mycelium per leaflet of B. cinerea (Bc) or with a single drop per leaflet of S. trifoliorum (St). Photographed 4 days after inoculation. Note limited and spreading lesions respectively.

Bc St

Plate 3. Leaves inoculated with 4-6 drops homogenised mycelium per leaflet of S. fructigena. Note flecking lesions underneath inoculum droplets.

extent and only in association with flecks. Thus a hypersensitive type reaction was observed. Hyphae observed in tissue inoculated with B. cinerea or B. fabae produced some branches but their growth was stopped as the lesions became limited. S. trifoliorum hyphae in leaf tissue, branched dichotomously and ramified throughout the tissues (Plate 4). Thus there was a very clear differentiation between S. trifoliorum the clover pathogen, B. cinerea and B. fabae which behaved as weak pathogens and the other species which virtually failed to infect clover leaves.

Plate 4. Branched S. trifoliorum hyphae ramifying throughout leaf tissue three days after inoculation x 1500.



4.a. Accumulation of phytoalexins in tissue and diffusates of clover leaves inoculated with Sclerotinia and Botrytis species

To see whether phytoalexins play any role in the differential response of clover leaves to these fungi, it is important to know the amount of phytoalexins accumulating in diffusates and tissue.

Clover leaves were therefore inoculated with mycelial suspension of six fungi in the Sclerotinia and Botrytis genera. After incubation in an illuminated incubator at 20°C for 48 h, the droplets containing the mycelium were removed. The tissue underneath the inoculum drop showed varying degrees of browning. S. fructigena, S. fructicola and B. allii had formed only a slight brown fleck by this time, whereas B. cinerea and B. fabae had formed brown and black lesions respectively about 1 cm in diameter. S. trifoliorum had already formed brown necrotic lesions about 2 cm in diameter. The underlying tissues were removed and both diffusates and tissue extracted separately and subjected to TLC as before. The bands corresponding to maackiain and medicarpin were eluted and the phytoalexins quantitated by UV spectrophotometry. Large quantities of phytoalexins were obtained from the tissues of all interactions (Table 5). However the variation in phytoalexin content had no obvious relation to pathogenicity. In the diffusates from leaves inoculated with the non-pathogenic S. fructigena, S. fructicola and B. allii, fairly large amounts of phytoalexins were present but in those from leaves inoculated with S. trifoliorum, B. cinerea and B. fabae only traces were present (Table 6).

From the above results, the phytoalexin response of red clover appears to be a general response to fungal infection. Different species of Sclerotinia and Botrytis elicit phytoalexin biosynthesis in red clover leaf tissue but the amount accumulated had no obvious variation to pathogenicity. The absence of phytoalexins in diffusates of S. trifoliorum, B. cinerea and B. fabae may be due to degradation of these inhibitors as fast as they are produced. The accumulation of phytoalexins in diffusates of S. fructigena,

TABLE 5. Concentration of maackiain and medicarpin in tissue[a] 48 h after inoculating clover leaves with mycelium of fungi

Species	Maackiain[b]	Medicarpin[b]
S. trifoliorum	93	81
B. cinerea	178	16
B. fabae	72	15
S. fructigena	79	61
B. allii	85	76
S. fructicola	113	65

a) Concentration in µg/g fresh wt.

b) Values calculated from mixed maackiain/medicarpin spectra

TABLE 6. Concentration of maackiain and medicarpin in diffusates[a] 48 h after inoculating clover leaves with fungi

Species	Maackiain[b]	Medicarpin[b]
S. trifoliorum	0	0
B. cinerea	0	0
B. fabae	0	0
S. fructigena	25	27
S. fructicola	25	17
B. allii	33	27

a) Concentration in µg/ml

b) Values calculated from the mixed maackiain/medicarpin spectra

Zero figures signify results below detection level of about 5 µg/ml

S. fructicola and B. allii may contribute to their restriction.

4.b. Changes in concentration of phytoalexin and their degradation products following inoculation of clover leaves with S. trifoliorum and B. cinerea mycelium

To determine the role of phytoalexins in controlling limited lesions it was considered essential to relate changes in phytoalexin concentrations to the development of different types of lesions. These changes in phytoalexins and their degradation products may also give information of the importance of degradation during lesion development. S. trifoliorum and B. cinerea which formed spreading and limited lesions respectively, were used in this study.

Clover leaves were inoculated with mycelial suspension of both fungi and incubated at 20°C. After various time intervals, droplets containing the inoculum were removed and extracted with the underlying tissue plus a 2 mm border of apparently healthy tissue. TLC and quantitation was as in Materials and Methods. The accumulation of the phytoalexins and their degradation products through a time course after inoculation is shown in Table 7 a. Both maackiain and medicarpin were produced in all interactions. Twenty four hours after inoculation, S. trifoliorum had formed small brown lesions beneath the inoculum and had elicited the accumulation of large amounts of phytoalexins. This initial increase was then followed by a decrease as the lesion spreads and the susceptible tissues became completely necrotic and invaded by the pathogen. Medicarpin decreased relatively faster than maackiain and correspondingly more 6a-hydroxymedicarpin was recovered 48 h after inoculation. This level fell presumably due to degradation by 96 h. However, 6a-hydroxymaackiain was maintained at a higher level up to 96 h.

In B. cinerea-inoculated leaves, both maackiain and medicarpin accumulated rapidly as the tissue became necrotic. This initial rate of

TABLE 7a. Concentration of maackiain, medicarpin and their 6a-hydroxylated derivatives in tissue inoculated with mycelium of S. trifoliorum and B. cinerea

	Time (h) after inoculation	Pterocarpans (µg/g fresh wt)[a]			
		Maa	Med	OH-Maa	OH-Med
S. trifoliorum	24	190	212	< 10	< 10
	48	101	130	65	92
	72	88	91	59	81
	96	53	30	70	51
B. cinerea	24	182	225	< 10	< 10
	48	289	447	111	72
	72	198	226	84	56
	96	317	284	49	38

a) Values calculated from mixed maackiain/medicarpin spectra

Maa = maackiain; Med = medicarpin; OH-Maa = 6a-hydroxymaackiain; OH-Med = 6a-hydroxymedicarpin. < 10 µg/g = detection limit

maackiain production was maintained up to two days. Although there was a decline after this time, the final concentrations of maackiain and medicarpin by 96 h were still relatively high, being 3 - 4 times higher than in S. trifoliorum lesions. Both 6a-hydroxymaackiain and 6a-hydroxymedicarpin accumulated in B. cinerea lesions by 48 h, but these decreased to about half this concentration by 96 h. At this time B. cinerea lesions had become limited to about 6 cm in diameter.

In a repetition of this experiment, an essentially similar pattern of phytoalexin accumulation was obtained (Table 7 b).

4.c. Detection of degradation products in diffusates from leaves inoculated with fungi in the Sclerotinia and Botrytis genera

In this experiment, clover leaves were inoculated with mycelial suspension of Sclerotinia and Botrytis species as before. After 48 h incubation, diffusates were collected and extracted three times with ethyl acetate and subjected to TLC as before. Two main absorbing bands (254 nm) were observed in diffusates from S. trifoliorum and B. cinerea-inoculated tissue. These had R_f values in chloroform : methanol (98 : 2, v/v) solvent system of 0.16 and 0.07, which corresponded to the 6a-hydroxymaackiain/6a-hydroxymedicarpin and 6a,7-dihydroxylated derivatives respectively. Diffusates from B. fabae-inoculated tissue had one main band at R_f 0.43 whereas diffusates from S. fructigena, S. fructicola and B. allii-inoculated tissue produced only one main absorbing band. This band had an R_f of 0.65 corresponding to the maackiain/medicarpin band. After elution with ethanol and scanning in a spectrophotometer the bands of R_f 0.07, 0.16 and 0.65 had the characteristic pterocarpanoid peaks (Section 3). The band of R_f 0.43 from the B. fabae-inoculated tissue did not give any characteristic peaks. Thus diffusates from S. fructigena, B. allii and S. fructicola-inoculated leaves contained both maackiain and medicarpin and no hydroxylated derivatives whereas those

TABLE 7b. Concentration of maackiain, medicarpin and their 6a-hydroxylated derivatives in tissue inoculated with *S. trifoliorum* and *B. cinerea*

	Time (h) after inoculation	Pterocarpans (µg/g fresh wt)[a]			
		Maa	Med	OH-Maa	OH-Med
S. trifoliorum	24	105	125	< 10	< 10
	48	93	81	31	84
	96	49	11	61	30
B. cinerea	24	195	169	< 10	< 10
	48	328	112	99	58
	96	149	77	44	29
B. cinerea spores	96	1852	991	< 10	< 10

a) Values calculated from mixed maackiain/medicarpin spectra

Maa = maackiain; Med = medicarpin; OH-Maa = 6a-hydroxymaackiain; OH-Med = 6a-hydroxymedicarpin; < 10 µg/g = detection limit

from S. trifoliorum and B. cinerea-inoculated leaves contained both the 6a-hydroxylated derivatives and the 6a,7-dihydroxylated derivatives. Only traces of maackiain and medicarpin were detected in diffusates from leaves inoculated with S. trifoliorum, B. cinerea and B. fabae.

4.d. Horizontal distribution of phytoalexins and their 6a-hydroxylated derivatives in spreading lesions of S. trifoliorum

The localisation of phytoalexins and their degradation products within and around spreading lesions may give some information relevant to the role of phytoalexins in the infection of clover leaves with S. trifoliorum, e.g. if the centre of the lesion contained no phytoalexins but some or no phytoalexin degradation products and the peripheral zone contained phytoalexins but either some or no degradation products, explanations such as degradation of phytoalexins as the lesion spreads could be invoked. S. trifoliorum lesions (5-days old) were harvested and divided into three regions : the centre (5 mm in diameter); the peripheral 2 mm plus a 2 mm of apparently live cells; and the area between these two zones. These three areas were separately extracted as in Materials and Methods. Chromatography and quantitation of phytoalexins were as previously described.

TABLE 8.

Leaf tissue analysed	Concentration of phytoalexins in µg/g fresh wt.			
	Maa	Med	OH-Maa	OH-Med
Centre	< 10	< 10	<10	<10
Middle	20	12	52	21
Periphery	35	18	57	33

Legend as in Table 7.

As shown in Table 8, only trace amounts of phytoalexins and their 6a-hydroxylated derivatives were detected in the centre of the lesions.

However both phytoalexins and their 6a-hydroxylated metabolites were detected in the middle region and in the periphery. Also present in this peripheral zone were the preformed isoflavones, formononetin and biochanin A, these being absent in the centre and middle zones.

4.e. Accumulation of phytoalexins in clover leaves inoculated with spores of B. cinerea and S. fructicola

From a previous section it was observed that clover leaves inoculated with B. cinerea mycelium accumulated 100 - 400 μg/g fresh weight of the clover phytoalexins. This is in contrast to the high levels detected by Debnam and Smith (1976) who used B. cinerea spores in 5%, w/v glucose solution. Therefore a thorough time course of phytoalexin accumulation in clover leaves inoculated with B. cinerea spores was investigated. S. fructicola was included for comparison. Clover leaves were inoculated with a spore suspension (10^6 spores/ml in 5% w/v glucose solution) of B. cinerea or S. fructicola. After various times leaf tissue were excised and the phytoalexins extracted and quantified. As shown in Table 9 leaves inoculated with B. cinerea spores accumulated large quantities of phytoalexins. Lesion size was limited to about 3 mm in diameter at day 4. S. fructicola on the other hand, caused the accumulation of large quantities of phytoalexin initially, but this level decreased by 4 days. At this time there was very little damage done apart from a rare minute necrotic fleck. This decrease in phytoalexin as time progressed may be due to metabolism by the host tissue, since S. fructicola did not degrade the phytoalexins in vitro (Section 6).

4.f. Accumulation of phytoalexins in clover leaves inoculated with B. cinerea spores suspended in SCA medium or 5% glucose solution

Since B. cinerea mycelium in SCA medium induced less phytoalexins than B. cinerea spores in 5% glucose solution, it was of interest to investigate what level of phytoalexin B. cinerea spores in SCA medium would

TABLE 9. Concentration of maackiain and medicarpin in tissue[a] inoculated with spores of *B. cinerea* and *S. fructicola*

	Time (h) after inoculation	Maackiain[b]	Medicarpin[b]
B. cinerea	24	298	380
	48	522	205
	72	565	480
	96	1028	856
S. fructicola	24	106	151
	48	97	70
	72	55	86
	96	67	78

a) Concentration in µg/g fresh wt.

b) Values calculated from the mixed maackiain/medicarpin spectra

induce. An experiment was therefore set up in which B. cinerea spores (10^6/ml) were either suspended in 5% glucose solution or in SCA medium. Leaves were inoculated and incubated as before. Extraction and quantitation of phytoalexins were as already described. As shown in Table 10, larger amounts of phytoalexins were recovered from tissue inoculated with B. cinerea spores in glucose solution than from tissue inoculated with spores suspended in SCA medium. With regard to the 6a-hydroxylated derivatives, only traces were present in leaves up to three days after inoculation with spores in glucose solution. In this experiment, however, there were some of the 6a-hydroxylated products 4 days after inoculation. However, when this experiment was repeated, no 6a-hydroxyderivatives were present at any time up to 4 days. B. cinerea spores in SCA medium caused the accumulation of large quantities of the 6a-hydroxylated derivatives by 48 h, but this level declined by 96 h. Lesion development showed that larger lesions were formed by B. cinerea spores in SCA medium than by spores in glucose solution. Lesion diameters 4 days after inoculation were 4.5 mm and 3.0 mm respectively.

4.g. Effect of SCA and glucose on germination and germ tube length of B. cinerea spores

Since B. cinerea spores suspended in SCA medium produced larger lesions and less phytoalexins than spores suspended in 5% glucose solution it was decided to investigate whether factors like germination and germ tube growth may contribute to these differences. SCA medium and 5% glucose solution were used in spore germination tests on glass slides. Germination and germ tube growth were stopped after 24 h. Percentage germination was measured by counting 100 spores/drop and germ tube length was given as the average of 30 germ tubes in each drop. Germination was the production of a germ tube at least the length of the spore diameter.

TABLE 10. Concentration of maackiain and medicarpin in tissue inoculated with spores of B. cinerea suspended in SCA medium or 5% glucose solution

	Time (h) after inoculation	Pterocarpans (µg/g fresh wt)[a]			
		Maa	Med	OH-Maa	OH-Med
B. cinerea spores in SCA	24	128	229	< 10	< 10
	48	172	190	258	228
	72	161	111	195	149
	96	298	95	147	80
B. cinerea spores in glucose	24	298	205	< 10	< 10
	48	522	380	< 10	< 10
	72	565	480	< 10	< 10
	96	1203	721	40	48

a) Values calculated from mixed maackiain/medicarpin spectra

Maa = maackiain; Med = medicarpin; OH-Maa = 6a-hydroxymaackiain; OH-Med = 6a-hydroxymedicarpin. < 10 µg/g = detection limit

Results for the effect of SCA medium and glucose on conidia of B. cinerea are summarized in Table 11.

A students t test was carried out to compare the germ tube length for control and treated conidia and an analysis of variance of the split plot design type carried out to find out if different concentrations of the SCA medium and glucose solution affect the germ tube lengths and also if there was any interaction between the type of suspending medium and the concentrations.

Germ tube length for B. cinerea conidia germinated in SCA medium or 5% (w/v) glucose solution, was significantly greater than those of conidia germinated in water, however, B. cinerea conidia germinated equally well in water as it does in SCA medium or glucose.

The effect of the suspending medium was highly significant, but the effect of the concentration was not significant. The interaction was just significant. For the effect of medium on conidial germination, neither SCA medium nor glucose had any significant effect.

From these results it appears as if germination and germ tube growth had no part to play in the fact that lesions of B. cinerea in SCA were larger than those of this fungus in glucose. In fact germ tube growth was greater in glucose than in SCA medium, so other factors must be involved in the differential reaction.

Thus, as with other studies failure of B. cinerea to form spreading lesions does not reside in an inherently poor capacity for growth on media.

TABLE 11a. The effect of SCA and glucose on germination and germ tube length of B. cinerea conidia

Medium	Dilutions							
	0		1 : 1		1 : 4		1 : 8	
	GTLμ	G%	GTLμ	G%	GTLμ	G%	GTLμ	G%
SCA	319.8 ± 7.61	100	242.7 ±10.67	100	211.6 ± 8.98	100	192.6 ± 2.07	100
5% Glucose	488.13 ±28.84	100	569.13 ±66.84	100	553.77 ±103.65	98	492.57 ±82.50	100
H_2O	64.57 ±6.19	95						

GTL = germ tube length μ with standard error.

G% = % germination.

Results are means of three replicates.

TABLE 11b. t test comparing germ tubes length for conidia of *B. cinerea* in water and in glucose or SCA medium

Medium	$t\left(\frac{d}{sd}\right)$
5% glucose	28.75***
SCA medium	46.00***

*** = significant at the 0.001 probability level

Analysis of variance of the effect of media and concentrations on the germ tube length of *B. cinerea*

Main effects and concentrations	df	SS	MS	F (observed)	Fp = 0.01 (expected)
Medium	1	484958.94	484958.94	1991.0954***	31.33 P = 005
Error (a)	4	974.28	243.57		
Concentration	3	15560.55	5186.85	1.9430NS	3.49 P = 0.05
Interaction	3	28362.1930	9454.0643	3.5415*	(3.49 (P = 0.05 ((5.95 (P = 0.01
Error (b)	12	32034.28	2669.5233		
Total	23				

*** = significant at the 0.005 probability level

* = " " " 0.05 " "

NS = not significant

4.h. Factors affecting B. cinerea lesion development

Some factors which may affect lesion development were investigated to see whether these may play any role in B. cinerea lesion development and possibly lesion spread. Factors studied were spore concentration, temperature, and the effect of incubating in light or dark.

The effect of spore concentration on B. cinerea lesion development

In this experiment different concentrations of B. cinerea spores in 5% glucose were prepared and applied as 20 µl drops to clover leaves. Leaves were incubated at 20°C under high humidity and lesion development was observed daily.

After 24 h, necrotic lesions were observed underneath the inoculum drops of B. cinerea at concentrations of 1×10^6 and 1×10^7 spores/ml. No lesions were observed underneath inoculum drops containing B. cinerea spores at any of the lower concentrations. However as time progressed flecks and/or lesions developed. After 6 days incubation, B. cinerea at concentrations of 10^6 and 10^7 spores/ml had formed lesions of about 3mm in diameter. At a concentration of 10^5 spores/ml lesions were about 2mm in diameter, whereas at concentrations of 10^4 and 10^3 spores/ml flecking lesions were present. At a concentration of 10^2 spores/ml only very slight flecks were present even after 6 days. Thus with decreasing spore concentration, there was a delay in lesion formation. The optimum spore concentration was 10^6 - 10^7 spores/ml (Table 12).

The effect of temperature and light on B. cinerea lesion development

Clover leaves were inoculated with B. cinerea at a concentration of 1×10^6 spores/ml. Clover leaves in sandwich boxes were incubated in illuminated incubators at 18°C, 20°C and 30°C. One box was incubated in

the dark at 20°C, and the lesion development was observed at daily intervals. After 24 h small necrotic lesions were observed in leaves incubated at 18°C, 20°C, and in those incubated in the dark (20°C). No lesion was observed on leaves incubated at 30°C at this time or any time up to 6 days. Lesions on leaves held at 18°C or 20°C in the light or dark increased in diameter to about 3 mm on day 4, after which they became limited. Thus lesion formation at 18°C and 20°C (dark or light) were similar. No lesion developed at 30°C. (Table 13).

Further experiments could be designed to study the effect of environmental stresses such as light and temperature on phytoalexin accumulation. It would be hoped that any correlation between resistance and phytoalexin accumulation will be established.

4.i. Accumulation of phytoalexins in clover leaves inoculated with mycelium of B. fabae, S. fructigena, B. allii and S. fructicola

This experiment was set up to see whether high phytoalexin concentrations are maintained over a period of 4 days by fungi which do not degrade clover phytoalexins in vitro and also to see whether the 6a-hydroxylated products accumulate in these tissue. B. fabae which degraded maackiain and medicarpin in vitro to unknown products was also included in this study.

Mycelial inoculum was used and after various times lesions were excised and the phytoalexins extracted and quantitated as previously described. The accumulation of maackiain and medicarpin in leaf tissue inoculated with the four fungi is shown in Table 14. In most cases high phytoalexin concentrations were maintained throughout a 4 day period. In no case was the 6a-hydroxylated derivatives detected in tissue.

TABLE 12. The effect of spore concentration on lesion development

Spore concentration	Lesion development Days after inoculation					
spores/ml	1	2	3	4	5	6
10^2	-	-	-	±	±	±
10^3	-	-	+	+	+	++
10^4	-	-	+	++	++	++
10^5	-	+	++	++	++	+++
10^6	+	++	+++	++++	++++	++++
10^7	+	++	+++	++++	++++	++++

- No lesion; ± few flecks; + some flecks; ++ many flecks; +++ lesions - 2 mm diameter; ++++ lesions - 4 mm diameter

TABLE 13. The effect of temperature and light on lesion development

Temperature	Lesion development Days after inoculation					
	1	2	3	4	5	6
18°C	+	++	+++	+++	++++	++++
20°C	+	++	+++	++++	++++	++++
30°C	-	-	-	-	-	±
20°C (dark)	+	++	+++	++++	++++	++++

- No lesion; ± few flecks; + some flecks; ++ many flecks; +++ lesion - 2 mm diameter; ++++ lesion - 4 mm diameter

TABLE 14. Concentration of maackiain and medicarpin in tissue[a] inoculated with mycelium of *B. fabae*, *S. fructigena*, *B. allii* and *S. fructicola*

	Time (h) after inoculation	Maackiain[b]	Medicarpin[b]
B. fabae	24	56	114
	48	92	122
	72	154	118
	96	110	98
S. fructigena	24	132	53
	48	109	90
	72	111	120
	96	223	200
B. allii	24	166	179
	48	132	103
	72	116	99
	96	98	79
S. fructicola	24	182	106
	48	167	142
	72	150	167
	96	-	-

a) Concentration in µg/g fresh wt.

b) Values calculated from the mixed maackiain/medicarpin spectra

- = not determined; no hydroxylated derivatives were present

5. Antifungal activity of phytoalexins against species of Botrytis and Sclerotinia

The antifungal activity of purified maackiain and medicarpin was estimated by mycelial growth tests, to see whether any differential sensitivity exists between the pathogen and non-pathogens of clover. Essentially, known quantities of phytoalexins in DMSO were added to a mycelial suspension of the fungi in 50 ml Erlenmeyer flasks. After 24 h, the dry weights were estimated and compared with a control which had only DMSO added to the mycelial suspension.

a) Antifungal activity of maackiain

Table 15(a) shows the effect of varying concentrations of maackiain on the mycelial dry weight of three Sclerotinia and three Botrytis species. Maackiain was antifungal towards all six fungi tested. All three non-pathogens of clover, S. fructigena, S. fructicola and B. allii were more sensitive to 20 µg/ml maackiain than S. trifoliorum and the weakly pathogenic B. cinerea and B. fabae. When the concentration was reduced to 5 µg/ml for the non-pathogenic species there was still a significant growth inhibition of the fungi. S. trifoliorum was the least sensitive when 50 µg/ml or higher was used. Treatment of B. cinerea with 100 or 200 µg/ml caused a loss of mycelial dry weight. Only treatment with 200 µg/ml caused any loss of S. trifoliorum mycelial dry weight.

b) Antifungal activity of medicarpin

As with maackiain, the fungi exhibited a differential sensitivity towards medicarpin. The non-pathogens of clover, S. fructigena, B. allii and S. fructicola were more sensitive to 20 µg/ml medicarpin than S. trifoliorum B. cinerea and B. fabae. S. fructicola was the most sensitive and at this concentration (20 µg/ml) there was a loss of mycelial dry weight (Table 15b)

TABLE 15.

a) Sensitivity of fungal mycelial to maackiain

Phytoalexin concentration	Mycelial growth[a]					
µg/ml	St	Bc	Bf	Sfe	Ba	Sfc
5	-	-	-	40	50	59
20	55	56	46	39	42	24
50	23	2	-	-	-	-
100	2.5	-9.6[b]	-	-	-	-
200	-4.4[b]	-10.8[b]	-	-	-	-

b) Sensitivity of fungal mycelium to medicarpin

Phytoalexin concentration	Mycelial growth[a]					
µg/ml	St	Bc	Bf	Sfe	Ba	Sfc
20	48	56	43	9	34	-38[b]
50	12	1	-	-	-	-

St = S. trifoliorum Bc = B. cinerea Bf = B. fabae

Sfe = S. fructigena Ba = B. allii Sfc = S. fructicola

a) Mycelial growth as percentage of control

b) Indicating a loss in dry wt.

Each figure is based on two replicates. - = not determined. Standard errors up to 6.

6. Studies on the metabolism of clover phytoalexins by fungi

a) Metabolism by fungal mycelium and spores

It has been shown in a previous section that the concentrations of phytoalexins in *B. cinerea*-inoculated leaves with limited lesions were higher than in *S. trifoliorum*-inoculated leaves with spreading lesions. This low level of phytoalexins recovered from *S. trifoliorum*-inoculated leaves may be due to less induction by *S. trifoliorum*, to suppression of phytoalexin biosynthesis or to metabolism of the phytoalexins in *S. trifoliorum*-inoculated leaves.

In this same section, it was also shown that diffusates from *S. fructicola*, *S. fructigena* or *B. allii*-inoculated leaves contained quantities of the phytoalexins, maackiain and medicarpin, but no phytoalexins were recovered from diffusates of leaves inoculated with *S. trifoliorum*, *B. cinerea* or *B. fabae*. This absence of phytoalexins may also be due to the reasons given above.

In tissues from *S. trifoliorum* inoculated leaves there was an initial increase in phytoalexins which then decreased as the lesions spread by the fungus ramifying throughout the tissue. *B. cinerea* on the other hand formed limited lesions which contained higher concentrations of phytoalexins.

Debnam and Smith (1976) have already shown that *S. trifoliorum* and *B. cinerea* can degrade maackiain and medicarpin *in vitro*. The degradation products for *S. trifoliorum* were the 6a-hydroxylated derivatives. These compounds also accumulated to high concentrations in *S. trifoliorum*-inoculated tissues forming spreading lesions, but they accumulate to low concentrations in leaves inoculated with *B. cinerea* spores in glucose which formed limited lesions (Tables 7 and 10).

These results suggest that metabolism of clover phytoalexins in tissue may be a factor in the greater pathogenicity of *S. trifoliorum* to red clover leaves.

In view of the above results, experiments were designed to study the degradation of phytoalexins by S. trifoliorum and B. cinerea to critically compare the rates of degradation; formation of degradation products, and to identify degradation products and test their fungitoxicity. It was hoped that these comparisons might provide some information which could explain the difference in pathogenicity of S. trifoliorum and B. cinerea to clover leaves. Degradation by B. fabae, B. allii, S. fructicola and S. fractigena was also investigated.

The method used is described in Materials and Methods. Phytoalexins in DMSO was added to 5 ml aliquots of homogenised mycelium and incubated for either 24 h or for various times after phytoalexin addition depending on the experiment. The mycelium from replicate flasks was removed by centrifugation and the supernatant partitioned twice with CCl_4 and then twice more with ethyl acetate. The less polar compounds were extracted in the CCl_4 layers whilst the more polar compounds were partitioned into the ethyl acetate phases. CCl_4 and ethyl acetate extracts were evaporated and taken up in ethanol for U.V. spectroscopy. Absorbance at 287 nm was recorded as an index of phytoalexin recovery. Controls were phytoalexin in SCA medium and phytoalexin in autoclaved S. trifoliorum and B. cinerea. The validity of this procedure was checked by running chromatograms of the extract and a similar profile was obtained.

(i) Metabolism of maackiain by homogenised mycelium of six fungi

Samples (50 µl) of DMSO containing 100 µg maackiain was added to replicate flasks of six fungi containing 5 ml mycelial homogenate. After 24 h incubation, the supernatants were extracted and the UV absorption spectra of the CCl_4 and ethyl acetate extracts in ethanol recorded. The absorbance at 287 nm was compared with control (SCA + phytoalexin) which was taken as an index of maximum phytoalexin recovery (Table 16).

TABLE 16. Metabolism of maackiain by fungi

Species	% recovery[a] Maackiain	
	20 µg/ml	5 µg/ml
S. trifoliorum	23	-
B. cinerea	39	-
B. fabae	34	-
S. fructigena	86	100
B. allii	94	84
S. fructicola	88	78
Autoclaved S. trifoliorum	85	-
Autoclaved B. cinerea	100	-

a) % recovery after 24 h, based on U.V. absorbance of CCl_4 extracts. Each figure is the mean of two replicates.

- = not determined.

Over 60% of the maackiain had disappeared from media containing mycelium of S. trifoliorum, B. cinerea or B. fabae (Table 16). In media containing B. allii, S. fructigena or S. fructicola there was only a slight loss or no loss at all at this concentration or even at a lower concentration of 5 µg/ml. Autoclaved mycelium of B. cinerea or S. trifoliorum did not cause any significant loss of maackiain in 24 h. None of the fungi produced substantial amounts of CCl_4 or ethylacetate soluble UV-absorbing substances in SCA medium in the absence of maackiain.

To test the efficiency of the above system in measuring phytoalexin degradation rather than adsorption by the fungi, 5 ml homogenised mycelium with and without maackiain (100 µg) were incubated in 50 ml flasks. The UV absorption spectra of the CCl_4 and ethyl acetate extracts of the supernatants (aqueous media) were taken after 24 h. The mycelium was taken up in 5 ml CCl_4 and incubated for 30 min. This process was repeated once and the two CCl_4 fractions evaporated to dryness and referred to as the mycelial washings. Hyphae in 5 ml aliquots of ethanol were then macerated twice with a ground glass hand homogeniser. The suspension was then centrifuged at 1800 *g* for 30 min and the supernatant evaporated to dryness. All extracts were then taken up in ethanol for spectrophotometry, and the optical density (O.D.) at 287 nm recorded in Table 17.

TABLE 17. Recovery of UV Absorbing Substances (O.D. @ 287 nm) after 24 h incubation of *S. trifoliorum* and *B. cinerea* with 100 µg maackiain

	St	Bc	Medium control
Supernatant	0.16	0.14	0.46
Mycelial washings	0.00	0.00	0.00
Mycelial macerate*	0.10	0.13	0.00
Total	0.26	0.27	0.46
Total recovery as % control	57%	59%	100%

St = *Sclerotinia trifoliorum* Bc = *Botrytis cinerea*

* O.D. of macerate may be due to non-specific background absorbance

As shown in Table 17, the maackiain lost from the medium of both *B. cinerea* and *S. trifoliorum* in 24 h, could not be recovered from the mycelial washings or macerate. Therefore both fungi metabolized maackiain *in vitro*. A compound with a UV spectrum similar to maackiain appeared in the ethyl acetate extracts of both *B. cinerea* and *S. trifoliorum*.

Recovery of phytoalexin at time zero

Debnam and Smith (1976) reported a loss of up to 40% in phytoalexin recovery immediately after phytoalexin was added to fungal mycelium, but no explanation was given for this low recovery. Therefore experiments were designed to investigate whether the loss was dependent on the amount of fungus or not.

In this experiment maackiain (20 μg/ml) in DMSO was added to different masses of homogenised mycelium of S. trifoliorum and B. cinerea. Immediately after phytoalexin addition, three replicate samples were extracted with CCl_4 and assayed as described in Materials and Methods.

TABLE 18.

S. trifoliorum		B. cinerea	
Fungal mass (mg)	O.D. @ 287 nm	Fungal mass (mg)	O.D. @ 287 nm
1.91	0.49	2.22	0.46
5.72	0.47	5.63	0.39
6.87	0.50	10.84	0.34
25.00	0.56	-	-
Control phytoalexin in SCA medium	0.50	Control phytoalexin in SCA medium	0.50

- = not determined.

As shown in Table 18, increase in mycelial mass of S. trifoliorum did not significantly affect the phytoalexin recovery at time zero. With B. cinerea on the other hand, there was a decrease in phytoalexin recovery with increase in mycelial mass. This differential effect of S. trifoliorum and B. cinerea on phytoalexin recovery shows that loss of phytoalexin at time zero may be due to the amount of mycelium in the case of B. cinerea. This was not so for S. trifoliorum.

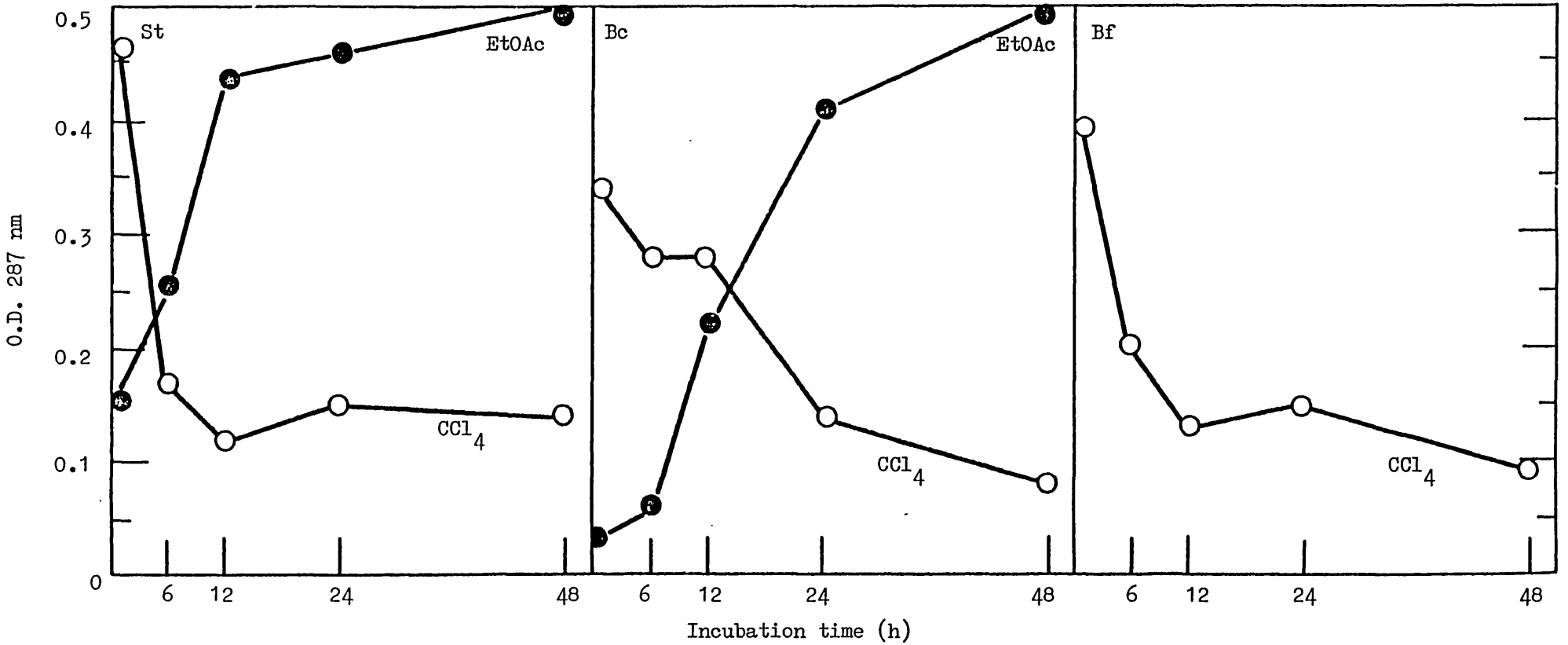
Time course metabolism of maackiain by S. trifoliorum, B. cinerea and B. fabae

Results in Tables 16 indicate that these three fungi can metabolize clover phytoalexins in vitro. This confirmed work by Debnam and Smith (1976) in which S. trifoliorum and B. cinerea were shown to metabolize clover phytoalexins. In this present study standardised inocula (homogenised mycelium) were used to compare critically rates of degradation and production of degradation products. These results can provide useful information about the greater pathogenicity of S. trifoliorum to clover leaves in view of the suggestion that pathogenicity may depend on the ability to degrade the host's phytoalexin in a particular manner.

In this experiment 20 µg/ml maackiain was used and samples from replicate flasks extracted at various times as before. As shown in Fig. 13 there was a progressive fall in phytoalexin recovery with time. S. trifoliorum degraded maackiain at a significantly faster rate than B. cinerea but at only a slightly faster rate than B. fabae. This was evident by the decrease in O.D. of the CCl_4 fractions with time. Less than 40% of the amount at zero time was left in the CCl_4 extracts after 6 h incubation with S. trifoliorum. After 12 h, apart from the background absorbance, there was virtually no phytoalexin recovered from the medium. Concurrent with the loss in maackiain, there was an increase in the O.D. of the ethyl acetate soluble compounds indicating an increase in degradation product(s).

B. cinerea also degraded maackiain (Fig. 13). However, after 6 h more than 70% of the phytoalexin recovery at time zero remained in the CCl_4 extract. Between 12 h and 24 h there was a large loss in maackiain to low levels (comparable to those obtained from S. trifoliorum degradation experiments at 24 h). There was also an increase in the O.D. of the ethyl acetate extracts, indicating an increase in degradation product(s). This O.D. reached a value of 0.50 after 48 h, much less than the O.D. of the corresponding S. trifoliorum extract which at this time was 1.0. Also evident was the difference in the O.D. values for maackiain at time zero 0.38 for B.cinerea

Figure 13. Time course degradation of 20 µg/ml maackiain by S. trifoliorum, B. cinerea and B. fabae



O.D. at 287 nm of CCl_4 and ethyl acetate (EtOAc) extracts from S. trifoliorum (St) B. cinerea (Bc) and B. fabae (Bf) mycelial suspension to which maackiain (20 µg/ml) was added at time 0. Scale for (St) ethyl acetate extract (0 - 1.0) No discernible compound in EtOAc extract of B. fabae.

and 0.47 for S. trifoliorum, although the same amount of maackiain was added. The lesser value for B. cinerea compared to S. trifoliorum may be due to a faster phytoalexin transport system or a higher binding constant of the former fungus (more phytoalexin receptors).

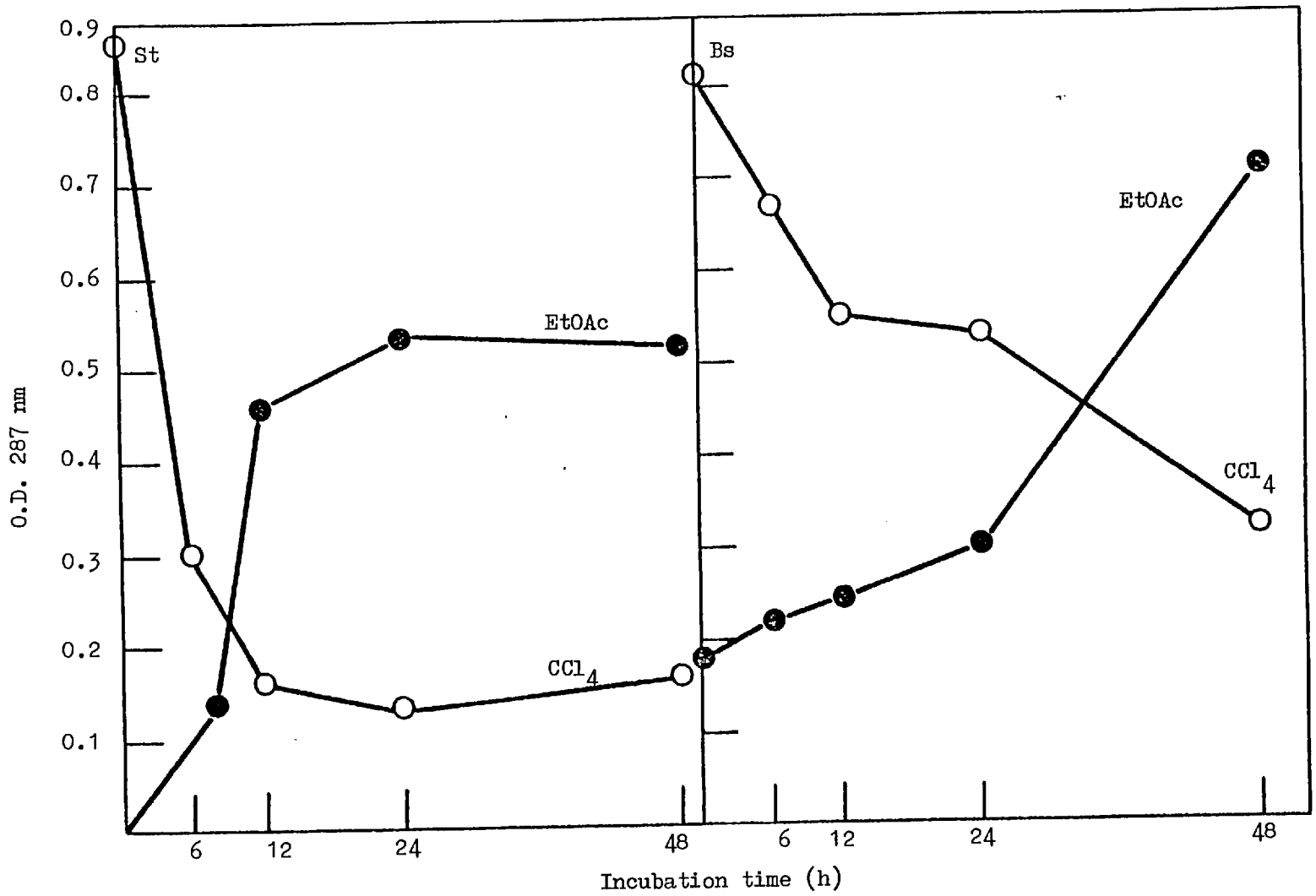
B. fabae also degraded maackiain as time progressed. By 6 h 45% had disappeared and by 12 h the loss was 66% (Fig. 13). There was a slight accumulation of UV-absorbing ethyl acetate soluble compound(s).

When the phytoalexin concentration was increased for S. trifoliorum and B. cinerea, a similar profile with 50 µg/ml was obtained (Fig. 14). S. trifoliorum degraded this higher maackiain concentration at a faster rate than B. cinerea. B. cinerea was also able to degrade maackiain at a concentration of 100 µg/ml (Fig. 15), but at a reduced rate.

Identification and characterisation of maackiain degradation products

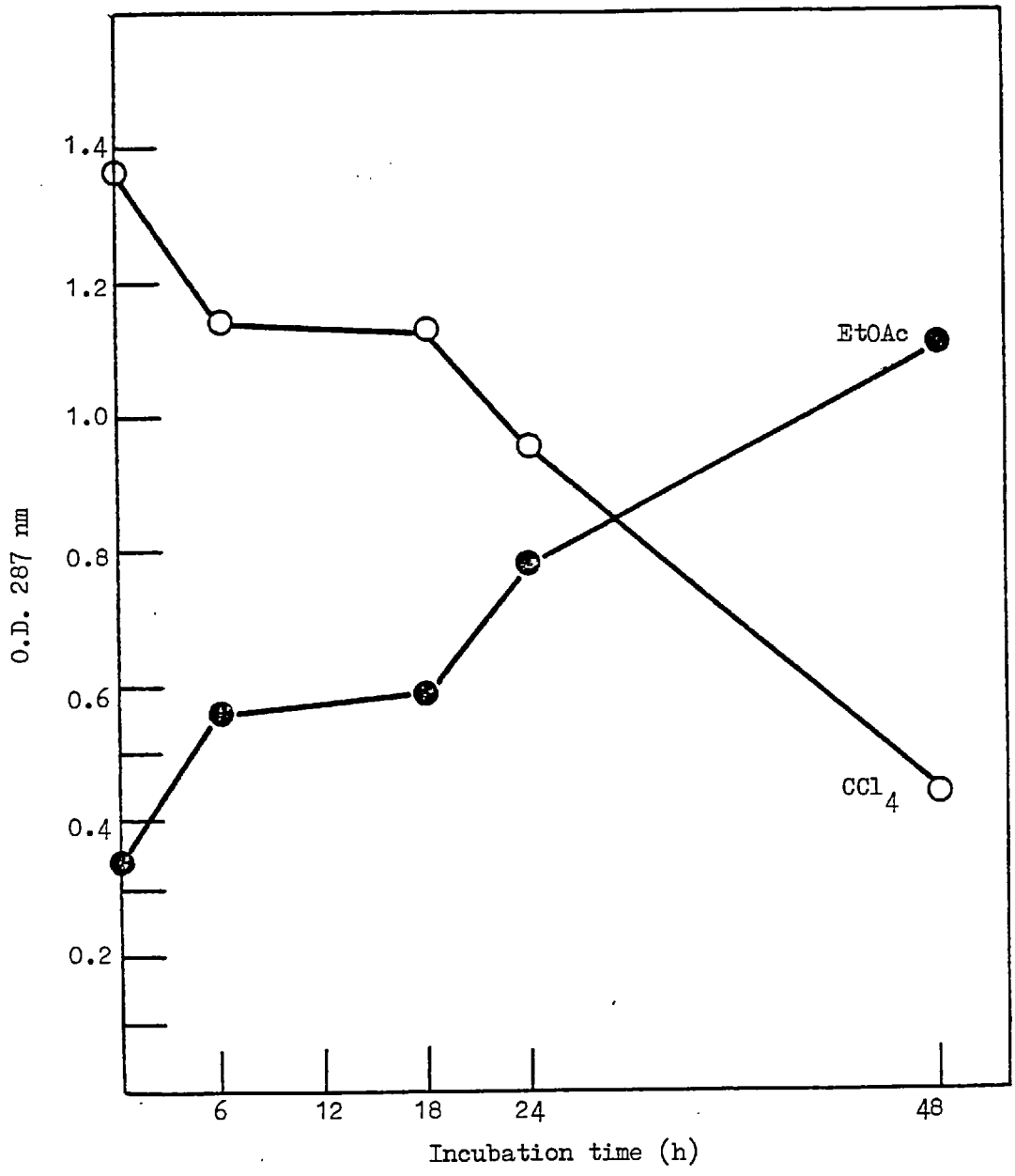
A degradation experiment with 20 µg/ml maackiain was set up as already described. After 6h incubation, three replicate flasks were harvested together with three control flasks containing fungal mycelium + DMSO. The supernatant were either extracted with CCl_4 followed by ethyl acetate or extracted directly with ethyl acetate. The ethyl acetate extracts were then dried down and subjected to TLC with a 2% v/v methanol in chloroform solvent system. Under UV light (254 nm), several bands were observed. These were then eluted and scanned in a UV spectrophotometer. The three main absorbing bands in the S. trifoliorum and B. cinerea extracts gave UV spectra similar to that of maackiain with a peak at 287 nm, a shoulder at 282 nm and a larger peak at 310 nm. Their R_f values were 0.65, 0.16 and 0.07, which corresponded to those of maackiain, 6a-hydroxymaackiain and 6a,7-dihydroxymaackiain respectively. The two latter compounds were absent in the control. When NaOH (1 drop, 1 M) was added to a 3 ml ethanolic solution of either of the two latter compounds, a bathochromic shift occurred which resulted in a single peak at about 300 nm. Both compounds gave a yellow colouration with p-nitroaniline. The compound of R_f 0.07 gave a blue colouration with Gibbs reagent. The compound of

Figure 14. Time course degradation of 50 µg/ml maackiain by S. trifoliorum and B. cinerea



O.D. at 287 nm of CCl_4 and ethyl acetate (EtOAc) extracts from S.trifoliorum (St) and B. cinerea (Bc) mycelilial suspension to which maackiain (50 µg/ml) was added at time 0.

Figure 15. Time course degradation of 100 µg/ml maackiain by B. cinerea



O.D. at 287 nm of CCl_4 and ethyl acetate (EtOAc) extracts(in 5 ml ethanol) from B. cinerea mycelial suspension to which maackiain (100 µg/ml) was added at time 0.

R_f 0.16 was antifungal to Cladosporium in TLC bioassay. This compound had an R_f value of 0.38 on polyamide with methanol : water (75 : 15, v/v) solvent system. The compound of R_f 0.07 was not tested in this respect. The identity of the degradation product(s)were further checked by their UV spectra in acid conditions (Fig. 11) and by their solubility in CCl_4 and ethyl acetate. Both compounds were insoluble in CCl_4 but soluble in ethyl acetate. These properties were consistent with those of 6a-hydroxymaackiain and 6a,7-dihydroxy-maackiain. The former has been confirmed as 6a-hydroxymaackiain by mass spectrometry (Bilton et al., 1976) but the identity of 6a, 7-dihydroxymaackiain (Fig. 17) was not confirmed by this technique. Large quantities of 6a-hydroxymaackiain were also present in lesions caused by S. trifoliorum and B. cinerea. The structure of this compound is shown in Fig. 16. It can also be called 6a-hydroxyinermin or 3,6a-hydroxy-8,9-dioxymethylene pterocarpan. This compound is also a product of pisatin degradation by Fusarium solani f. sp. pisi (Van Etten, Pueppke and Kelsey,1975) and Ascochyta pisi (Land, Wiersma-Van Duin and Fuchs, 1975a). The extinction coefficient is log $\mathcal{E}$ = 3.64 at 286 nm (Van Etten et al., 1975), therefore an absorbance of 1.0 = 69 µg/ml.

When the ethyl acetate extract from the B. fabae degradation experiment was subjected to TLC with chloroform : methanol (98 : 2, v/v) a prominent band at R_f 0.43 was present. However when this band was eluted and scanned in a UV spectrophotometer no clear peaks were obtained. The B. fabae degradation product was therefore ostensibly different from those of S. trifoliorum and B. cinerea but not enough compound was isolated for any attempt at characterization.

Thus in cultures of both S. trifoliorum and B. cinerea to which maackiain was added, two degradation products of this phytoalexin were present. These were the 6a-hydroxylated derivative (6a-hydroxymaackiain) and the 6a, 7-dihydroxylated product (6a, 7-dihydroxymaackiain). These have been brought about by successive hydroxylation of the 6a position and at the 7 position. Such hydroxylations increase the polarity of the compound, so

Figure 16. Structure of 6a-hydroxymaackiain

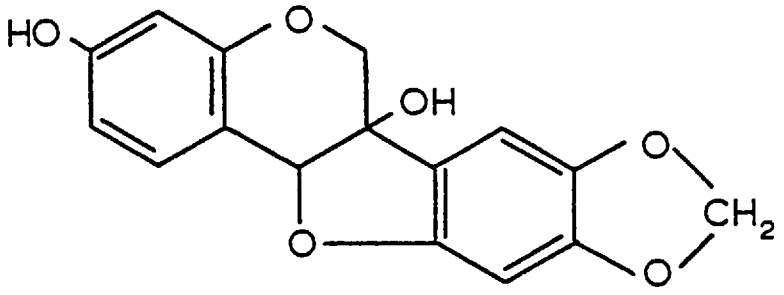
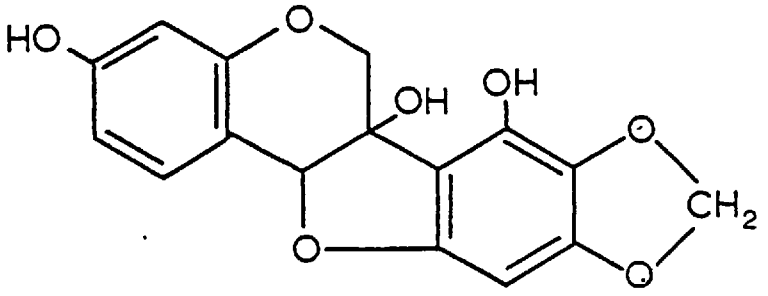


Figure 17. Structure of 6a,7-dihydroxymaackiain



that the products are insoluble in CCl_4 but soluble in ethyl acetate.

Since no 6a-hydroxymaackiain was present in the control the first degradation step from maackiain to 6a-hydroxymaackiain must be due to the fungi. To determine whether this degradation process was a detoxification step the fungitoxicity of the 6a-hydroxylated product must be reduced. This was examined by the mycelial growth test using 20 µg/ml 6a-hydroxymaackiain. After exposure of S. trifoliorum and B. cinerea to this compound for 24 h the percentage growth compared with controls were 105% and 65% respectively. So 6a-hydroxymaackiain had no inhibitory effect on S. trifoliorum, but inhibited B. cinerea by 35%.

Metabolism of 6a-hydroxymaackiain by S. trifoliorum and B. cinerea

The capacity of S. trifoliorum and B. cinerea to degrade 6a-hydroxymaackiain was tested in vitro. This maackiain degradation product (100 µg) in 50 µl DMSO was added to replicate flasks containing 5 ml homogenised mycelium of both fungi or to 5 ml SCA medium. The flasks were then incubated for 24 h. After this time, samples were harvested and extracted three times with ethyl acetate. Thin layer chromatography of the ethyl acetate extracts in 2% methanol in chloroform showed the presence of two main UV absorbing bands. One at R_f 0.16 and the other at R_f 0.07, corresponding to 6a-hydroxymaackiain and 6a, 7-dihydroxymaackiain respectively. The % recovery of 6a-hydroxymaackiain from S. trifoliorum and B. cinerea cultures were 20% and 80% respectively. This latter was close to that of the control (82%). Thus it appears as if S. trifoliorum but not B. cinerea can degrade 6a-hydroxymaackiain to 6a, 7-dihydroxymaackiain.

Metabolism of maackiain by sporeling of B. cinerea

This experiment was carried out to see whether conidia of Botrytis cinerea could degrade maackiain in a similar way to mycelium. For this purpose, a spore suspension (0.5 ml) of B. cinerea (10^6 spores/ml) was suspended in 5 ml SCA medium in 50 ml Erlenmeyer flasks, and left for 24 h

to allow spore germination. Maackiain (100 µg) dissolved in 50 µl DMSO was added to each flask, and replicate flasks harvested immediately and at various times thereafter. Extraction and quantification of phytoalexins were as in Materials and Methods. Figures 18 and 19 show the scan of the carbon tetrachloride and ethyl acetate extracts in ethanol corresponding to various incubation times. As shown in the scan of the CCl_4 extract, maackiain disappeared from the medium as time progressed. By 24 h all the maackiain had almost completely disappeared from the medium. The scan of the ethyl acetate extracts show the appearance of spectra resembling 6a-hydroxymaackiain, with λ max at 310 nm and 287 nm and a shoulder at 282 nm. When the combined ethyl acetate extracts were subjected to TLC in a chloroform : methanol (98 : 2, v/v) solvent system, two main bands were observed under UV (254 nm), with R_f values 0.16 and 0.07. The UV spectrum of the former was the same as that of 6a-hydroxymaackiain and that of the latter though not very clear, resembled that of 6a,7-dihydroxymaackiain. This latter compound produced a blue colouration with Gibb's reagent.

So germinating spores of B. cinerea degraded maackiain to 6a-hydroxy-maackiain and this in turn is degraded to 6a,7-dihydroxymaackiain.

(ii) Metabolism of medicarpin by homogenised mycelium of six fungi

The experimental procedure was as for maackiain degradation. Medicarpin (100 µg) in 50 µl DMSO was added to 5 ml homogenised mycelium of six fungi. After 24 h incubation replicate samples were extracted with CCl_4 and the phytoalexin quantitated by spectrophotometry (Table 19). A significant disappearance (more than 80%) of the phytoalexin was caused by S. trifoliorum, B. cinerea and B. fabae. The other fungi, B. allii, S. fructigena and S. fructicola caused very slight reductions in medicarpin. Autoclaved mycelium of B. cinerea and S. trifoliorum caused the disappearance of very little medicarpin or none at all.

Metabolism of maackiain by B. cinerea conidia (1)

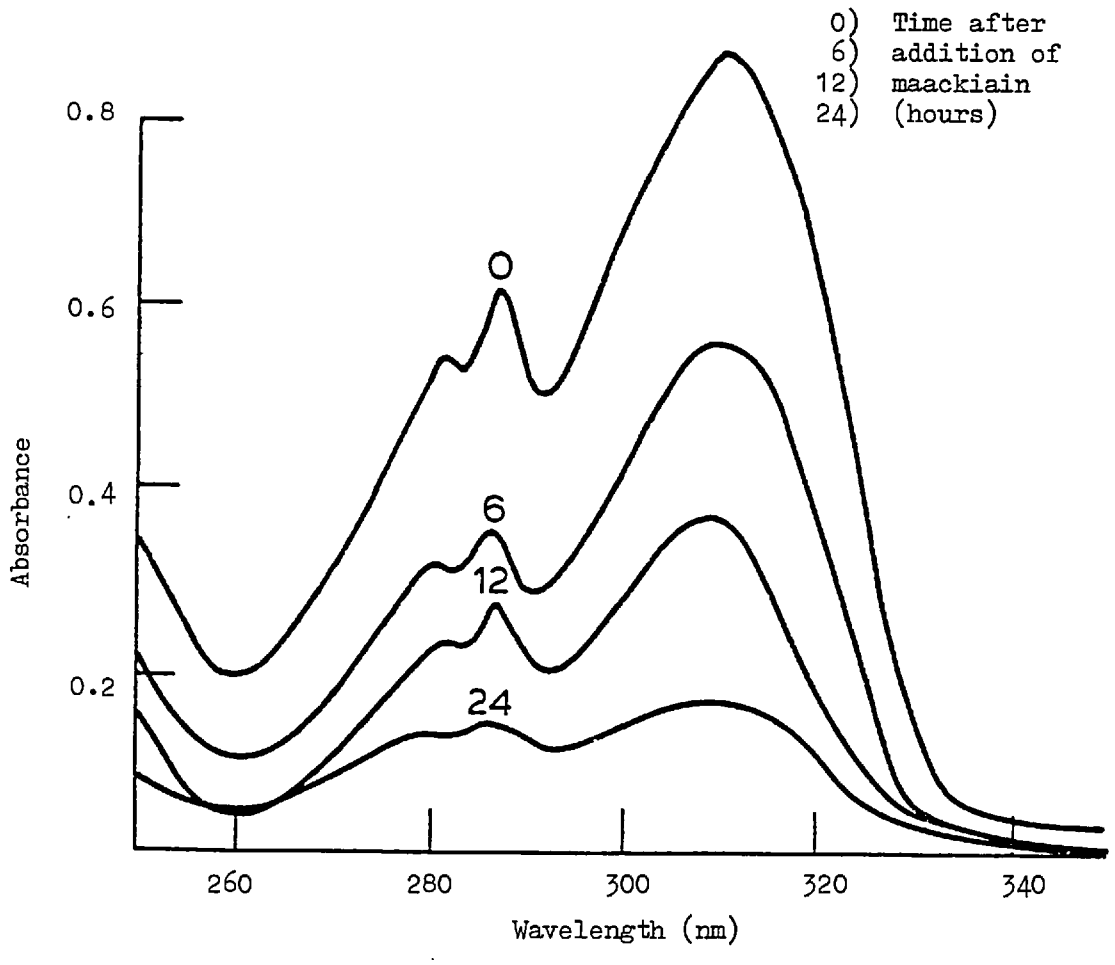


Figure 18. UV absorption spectra of dried down CCl_4 extracts redissolved in ethanol

Metabolism of maackiain by B. cinerea conidia (2)

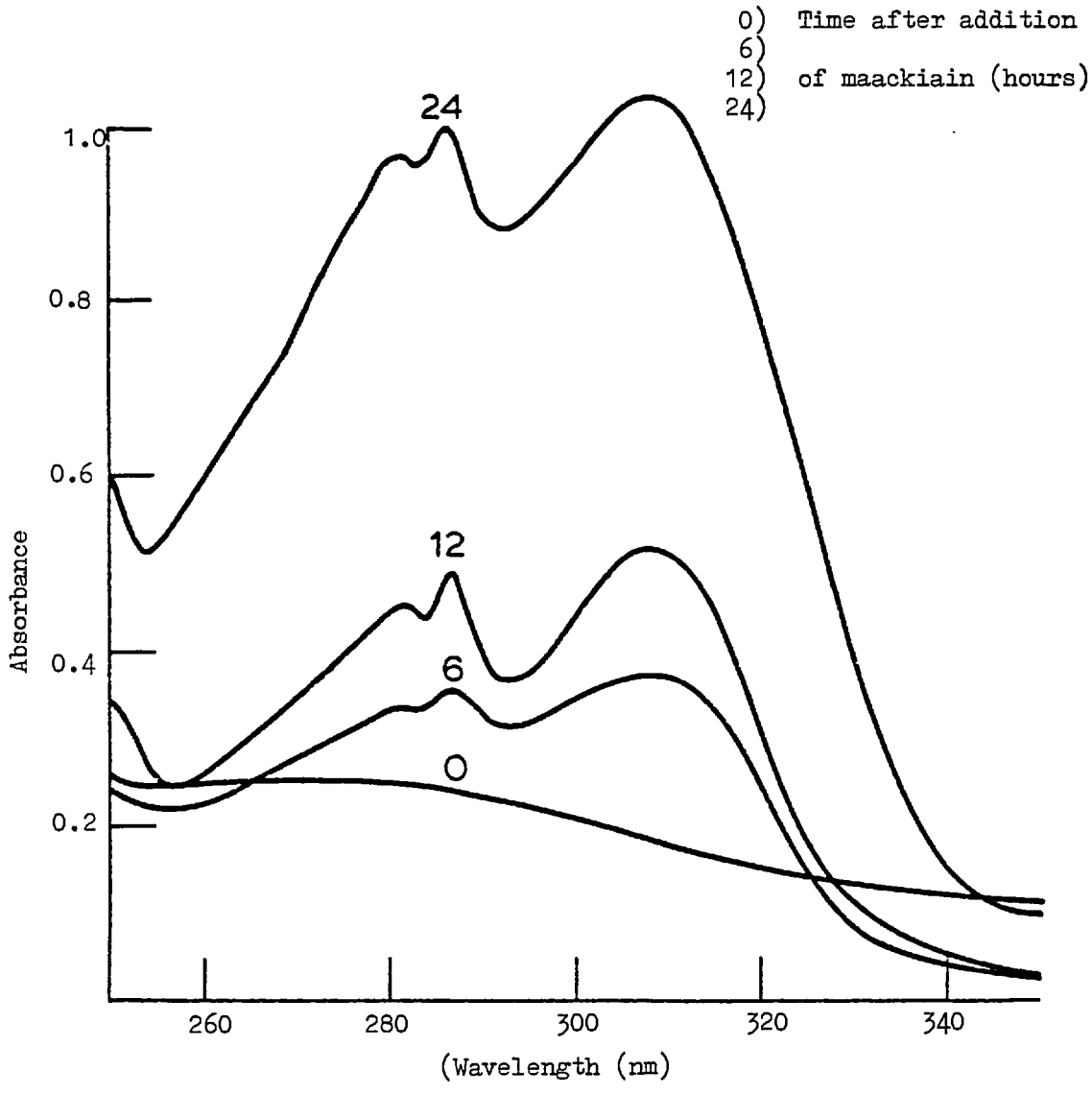


Figure 19. UV absorption spectra of dried down ethyl acetate extracts redissolved in ethanol.

TABLE 19. Metabolism of medicarpin by fungi

Species	% recovery[a] Medicarpin 20 µg/ml
S. trifoliorum	15
B. cinerea	17
B. fabae	17
S. fructigena	83
B. allii	76
S. fructicola	75
Autoclaved S. trifoliorum	84
Autoclaved B. cinerea	100

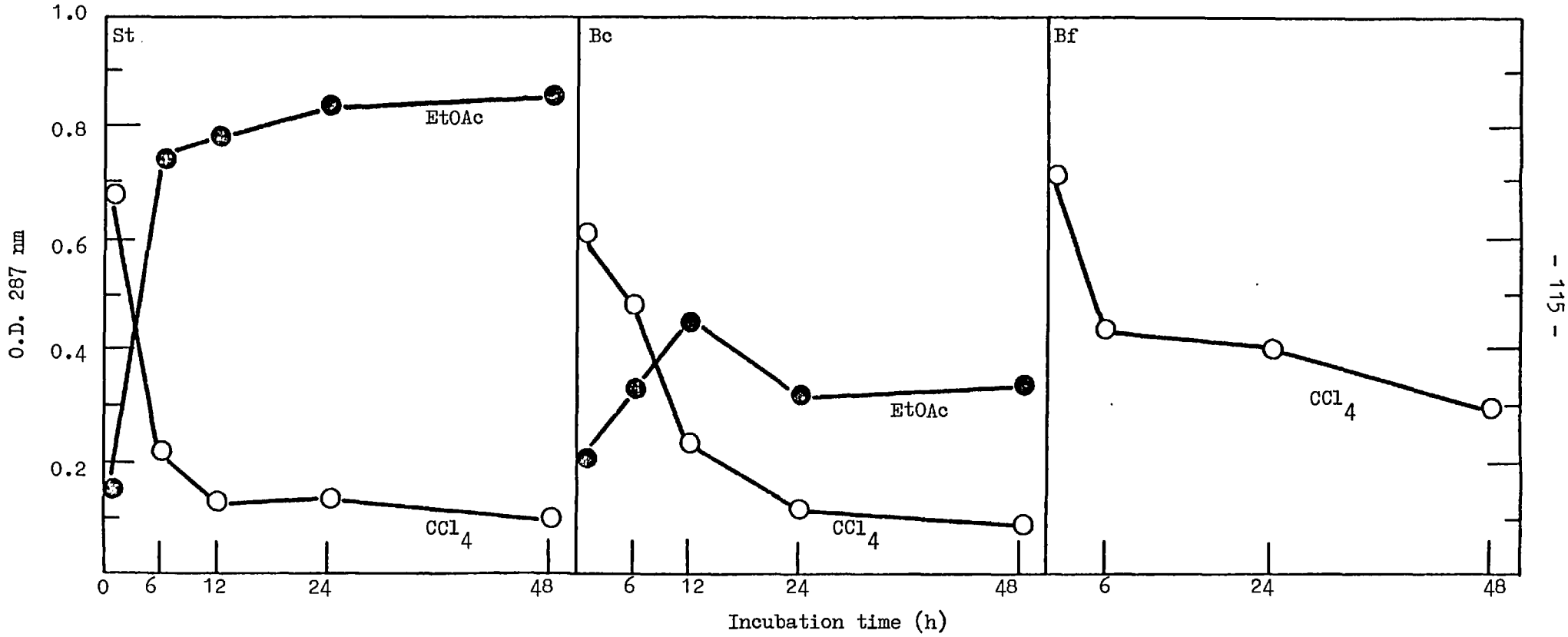
a) % recovery after 24 h, based on UV absorbance of CCl_4 extracts. Each figure is the mean of two replicates.

Time course metabolism of medicarpin by S. trifoliorum, B. cinerea and B. fabae

Similar procedures as for maackiain degradation were employed, with 20 µg/ml medicarpin. All three fungi progressively degraded medicarpin with time. This was evident by the disappearance of medicarpin in the CCl_4 extracts. S. trifoliorum degraded medicarpin rapidly, so that by 6 h over 70% of the phytoalexin at time zero had disappeared. By 12 h almost all the phytoalexin had disappeared from the medium. Simultaneous with this fall in phytoalexin, was the accumulation of ethyl acetate soluble compounds, which increased rapidly during the first six hours when most phytoalexin was lost. Thereafter the O.D. remained essentially constant (Fig. 20).

B. cinerea also degraded medicarpin in time. About 20% had disappeared by 6 h and by 12 h, 63% had disappeared. However by 24 h virtually no phytoalexin was recovered. Concurrent with the loss in phytoalexin there was

Figure 20. Time course degradation of 20 µg/ml medicarpin by S. trifoliorum, B. cinerea and B. fabae



O.D. at 287 nm of CCl_4 and ethyl acetate (EtOAc) extracts from S. trifoliorum (St), B. cinerea (Bc) and B. fabae (Bf) mycelial suspension to which medicarpin (20 µg/ml) was added at time 0. No discernible compound in EtOAc extract of B. fabae

an increase in ethyl acetate soluble compound(s). This increased to a maximum by 12 h after which a slight decrease occurred. B. fabae likewise degraded medicarpin in time. At 6 h about 40% had disappeared but by 48 h 40% of the phytoalexin was recovered from the medium. There was also an increase in ethyl acetate soluble compound(s), but when analysed no peaks characteristic of pterocarpans were observed.

From the above results, it therefore appears that S. trifoliorum degraded medicarpin at a faster rate than B. cinerea and B. fabae. Degradation products also accumulated at a faster rate.

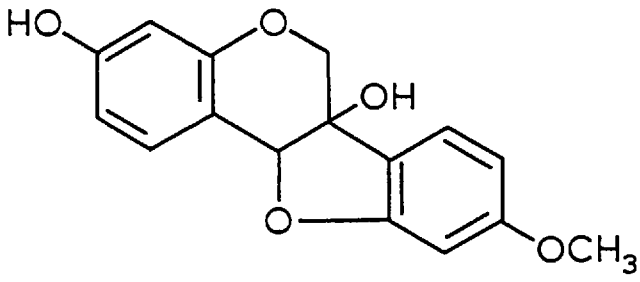
Identification and characterisation of medicarpin degradation product(s)

Medicarpin (100 µg) was added to S. trifoliorum and B. cinerea mycelium as in previous experiments. After 12 h, three replicate flasks were harvested and the combined supernatants extracted three times with ethyl acetate. Control flasks with mycelium + DMSO were also similarly extracted. After drying the extracts down, they were then subjected to TLC in chloroform : methanol (98 : 2, v/v) solvent system. Under UV (254 nm) light several bands were observed, which were then eluted and scanned with a spectrophotometer. Only two bands gave the typical pterocarpan peak; their R_f values were 0.65 and 0.16 which corresponded to medicarpin and 6a-hydroxymedicarpin respectively. Purified 6a-hydroxymedicarpin had a UV spectrum similar to medicarpin. It produced a yellow colour with diazotized p-nitroaniline and was antifungal in Cladosporium TLC bioassay. When NaOH (1 drop, 1 M) was added to a 3 ml solution, a bathochromic shift identical to that of medicarpin resulted with peaks at 290 nm and 250 nm (Fig. 10). So 6a-hydroxymedicarpin (Fig. 21) was the first degradation product, of S. trifoliorum and B. cinerea. The identity of 6a-hydroxymedicarpin has already been confirmed with mass spectroscopy by Bilton et al. (1976).

A faintly absorbing band under UV (254 nm) at R_f approximately 0.07 gave no clear peaks. Due to the unavailability of medicarpin and 6a-hydroxy-medicarpin, it was not possible to verify whether this latter compound was

indeed degraded to 6a,7-dihydroxymedicarpin.

Figure 21.



6a-hydroxymedicarpin

Metabolism of medicarpin by ~~conidia~~ sporeling of B. cinerea

To see whether conidia of B. cinerea could also degrade medicarpin in a similar way, an experiment using spores instead of mycelium, was set up. A 0.5 ml aliquot of a 1 x 10^6 spores/ml conidial suspension was suspended in 5 ml SCA medium in 50 ml Erlenmeyer flasks and left for 24 h to allow spore germination. Medicarpin at a concentration of 20 µg/ml was then added and replicate flasks harvested at various times. Extraction and quantitation of phytoalexins was as in Materials and Methods. Figures 22 and 23 show the scan of the carbon tetrachloride and ethyl acetate extracts dissolved in ethanol. Figure 22 clearly indicates a loss of medicarpin with time. By 24 h most of the medicarpin had completely disappeared from the suspending medium. Figure 23 shows the appearance of a UV absorbing substance resembling

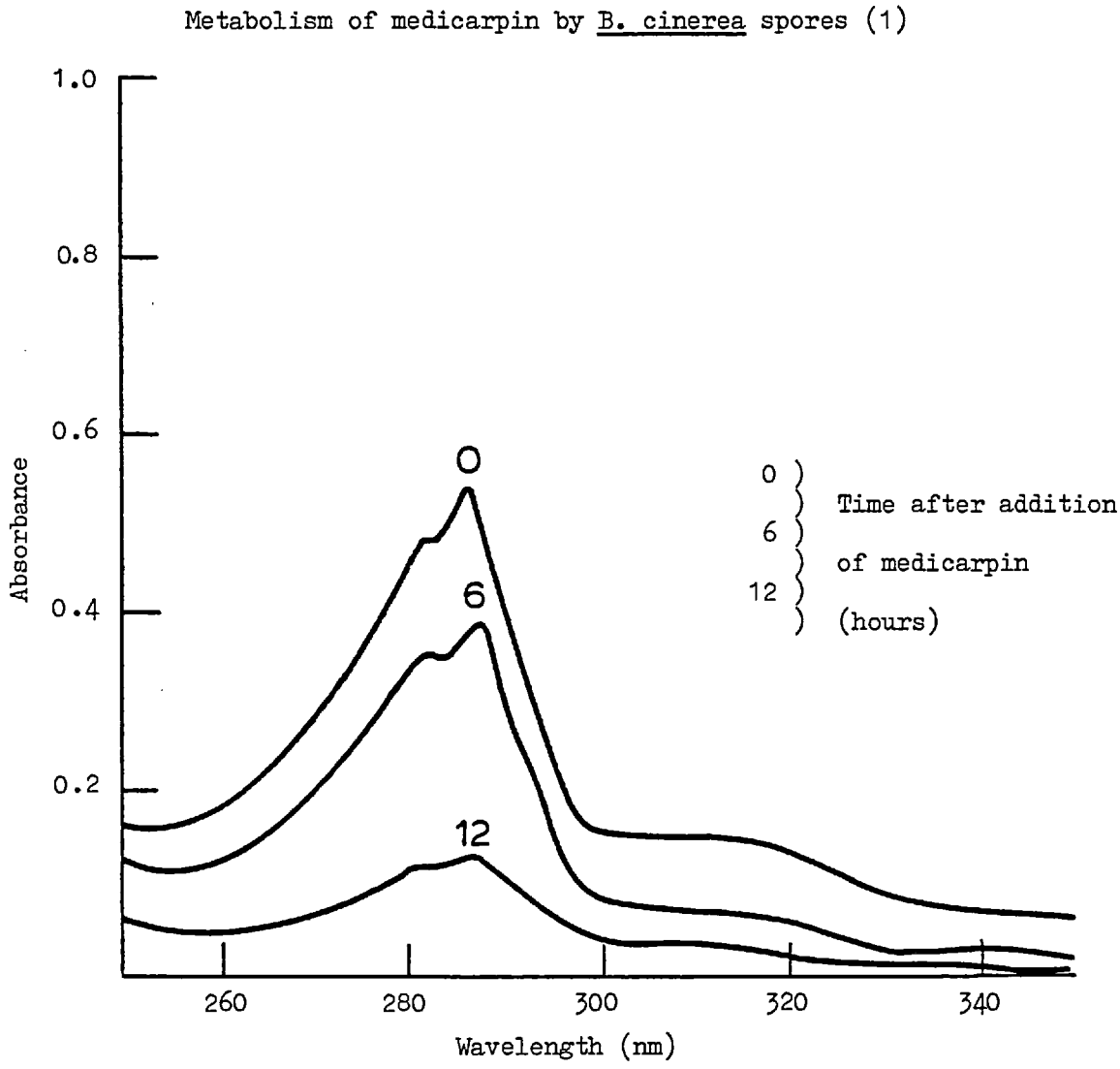


Figure 22. UV absorption spectra of dried down CCl_4 extracts redissolved in ethanol.

Metabolism of medicarpin by B. cinerea spores (2)

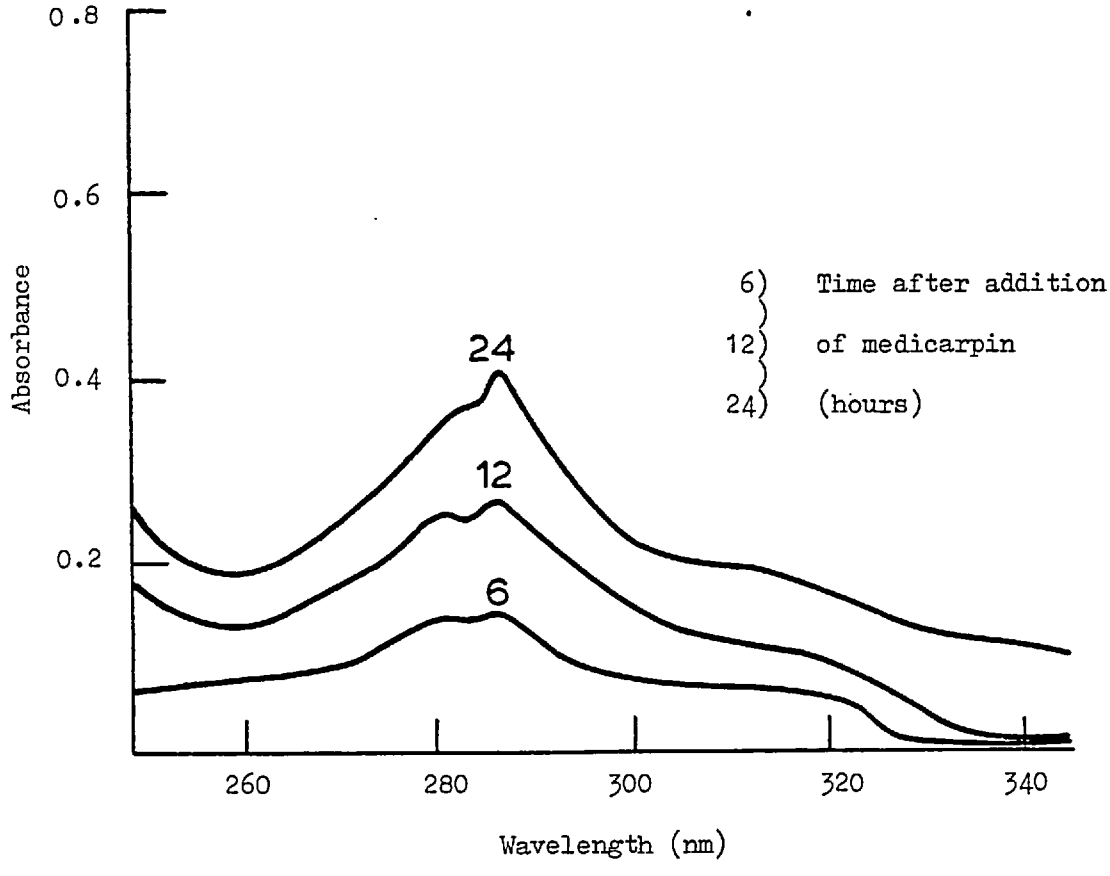


Figure 23. UV absorption spectra of dried down ethyl acetate extracts redissolved in ethanol.

medicarpin in spectral characteristics. When the combined ethyl acetate extracts were subjected to TLC in chloroform : methanol (98 : 2, v/v) solvent system a very prominent band at R_f 0.16 was obtained. This is the R_f of the 6a-hydroxylated derivative , 6a-hydroxymedicarpin,the same product obtained by B. cinerea and S. trifoliorum mycelium. Other bands from the TLC did not give any clear spectrum. Thus B. cinerea conidia degraded medicarpin by hydroxylation at the 6a-position to form 6a-hydroxymedicarpin, the same degradation product as S. trifoliorum and B. cinerea mycelium.

(iii)

Time course of maackiain metabolism and fungal growth by S. trifoliorum and B. cinerea

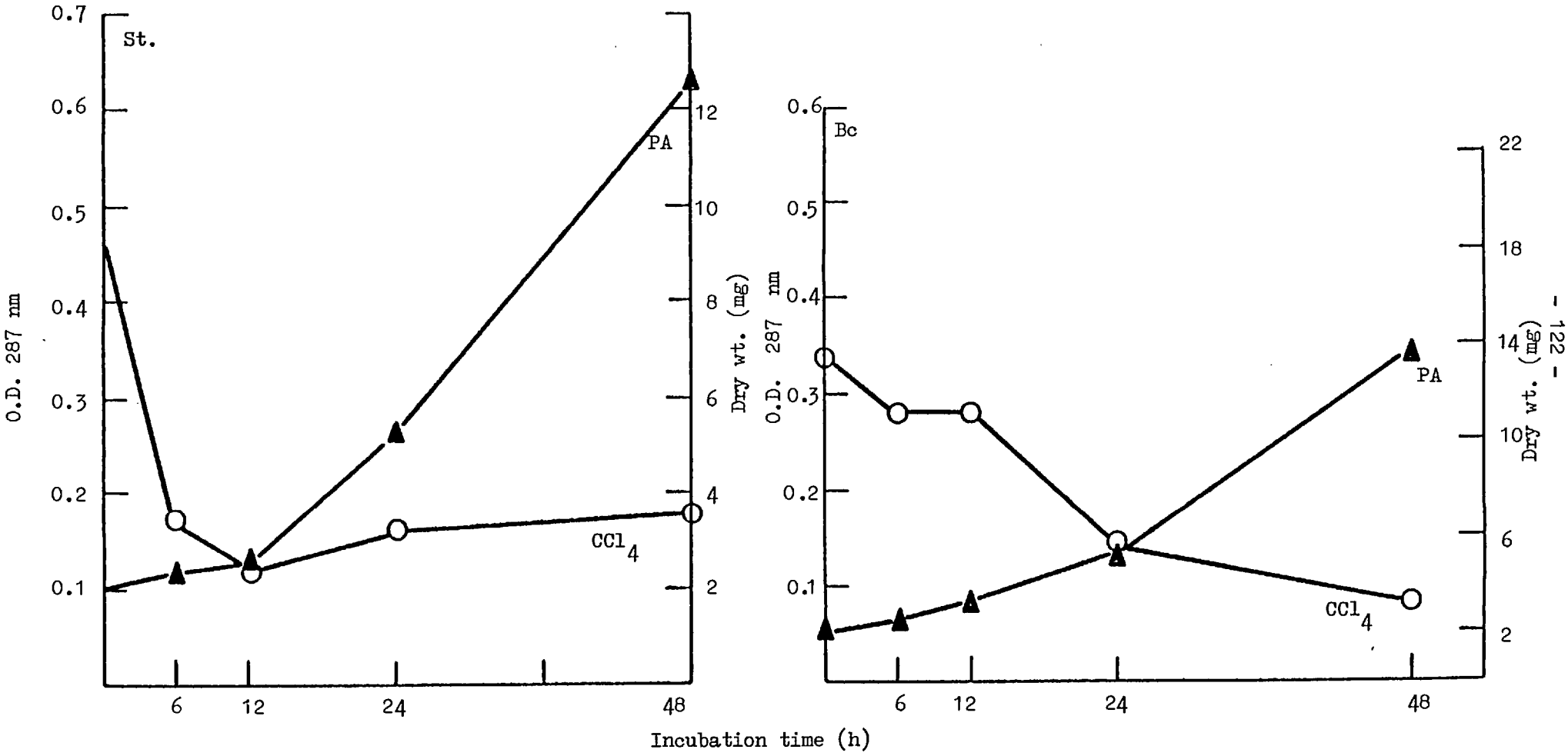
Evidence for equating the ability of fungi to overcome inhibitory concentrations of phytoalexins in vitro, to metabolism of the phytoalexins to non-toxic products is scarce. However, an analysis of the kinetics of mycelial growth versus the rate of degradation of phytoalexins to non-inhibitory compounds may elucidate the above processes.

In these experiments, a typical time-course degradation experiment was set up. At each time interval experimental flasks containing mycelium with phytoalexin and sometimes control flasks with DMSO were harvested. The phytoalexin was extracted with CCl_4 as before, and the dry weights of the mycelium determined by heating at 80°C for 24 h.

In Fig. 24 the degradation of 20 µg/ml maackiain versus the growth profile is shown. Both S. trifoliorum and B. cinerea exhibited a lag phase, before a constant growth rate occurred. However with S. trifoliorum this phase was about 12 h whereas with B. cinerea it was longer. In both cases, a significant degradation occurred before growth had taken place.

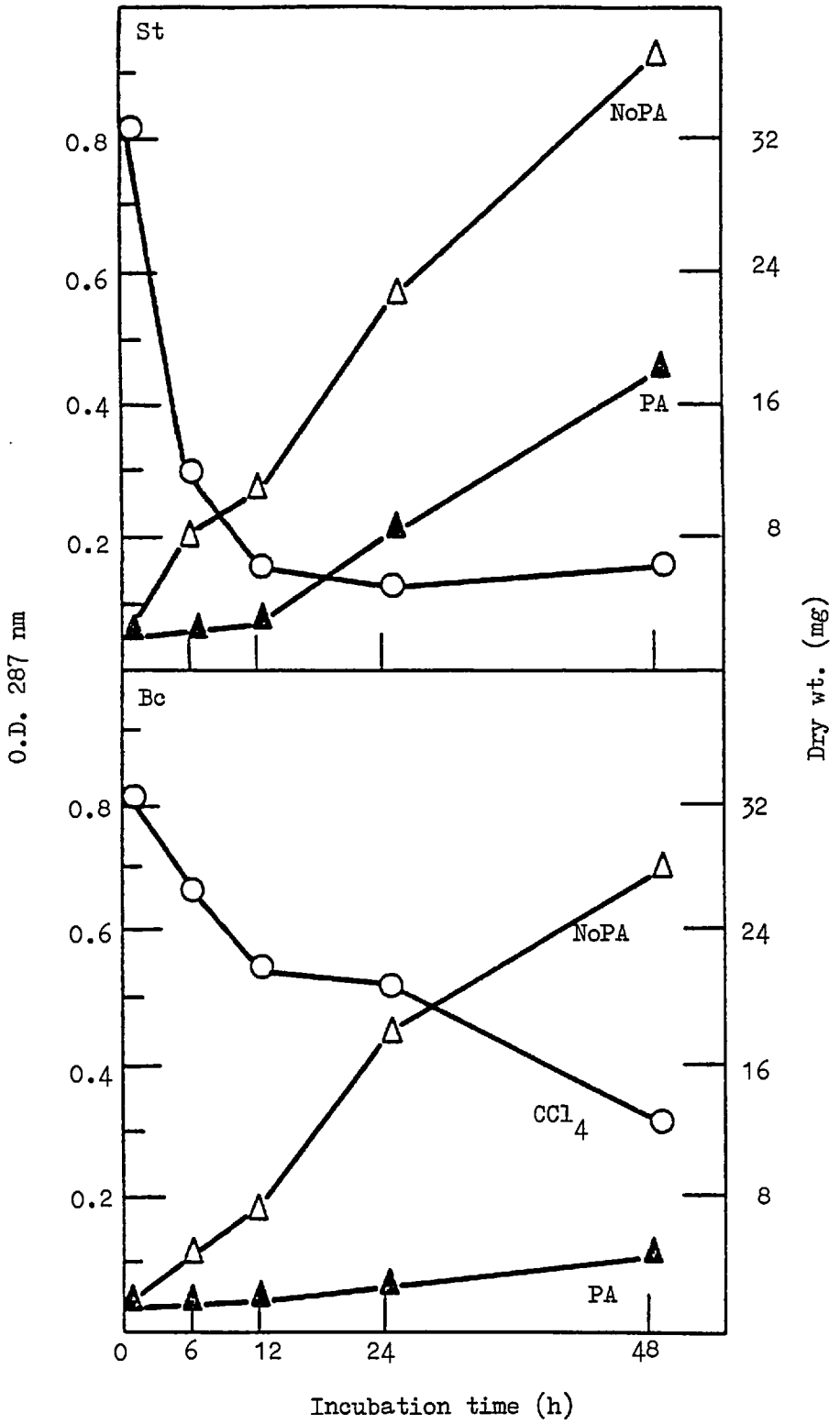
Figure 25 shows the degradation of 50 µg/ml maackiain versus the growth of S. trifoliorum or B. cinerea. With both fungi, mycelial growth did not occur before significant phytoalexin degradation had taken place. With S. trifoliorum, the eventual growth rate approached that of the control. When the concentration was increased to 100 µg/ml for B. cinerea a similar profile was obtained (Fig. 26). In this case however, there was a loss in mycelial weight by 24 h but the mycelium recovered to about the original weight by 48 h. Degradation was correspondingly slower at this concentration.

Figure 24. Relationship between the growth of fungi in liquid culture and degradation of 20 µg/ml maackiain



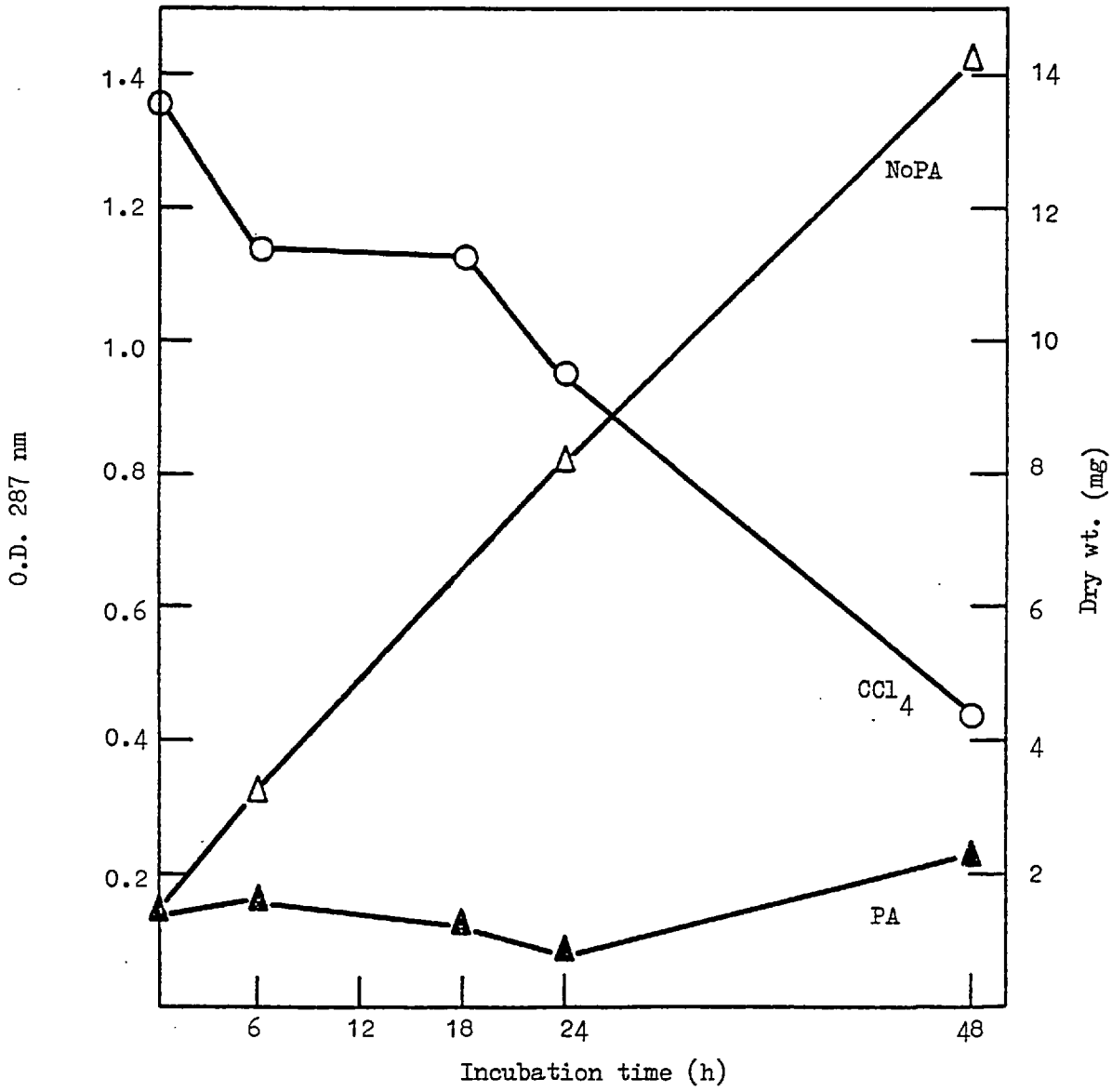
St = S. trifoliorum; Bc = B. cinerea PA = Dry wt. increase in presence of 20 µg/ml maackiain in dimethyl-sulphoxide added at time 0. CCl_4 = O.D. at 287 nm of CCl_4 extract in ethanol.

Figure 25. Relationship between the growth of fungi in liquid culture and the degradation of 50 µg/ml maackiain.



St = Sclerotinia trifoliorum; Bc = Botrytis cinerea; PA = Dry wt. increase in presence of 50 µg/ml maackiain in dimethylsuphoxide added at time 0. NoPA = Dry wt. increase in presence of 1% Dimethylsulphoxide added at time 0. CCl_4 = O.D. at 287 nm of CCl_4 extract in ethanol.

Figure 26. Relationship between the growth of B. cinerea in liquid culture and the degradation of 100 µg/ml maackiain



PA = Dry wt. increase in presence of 100 µg/ml maackiain in dimethylsulphoxide added at time 0.

NoPA = Dry wt. increase in presence of 1% dimethylsulphoxide added at time 0.

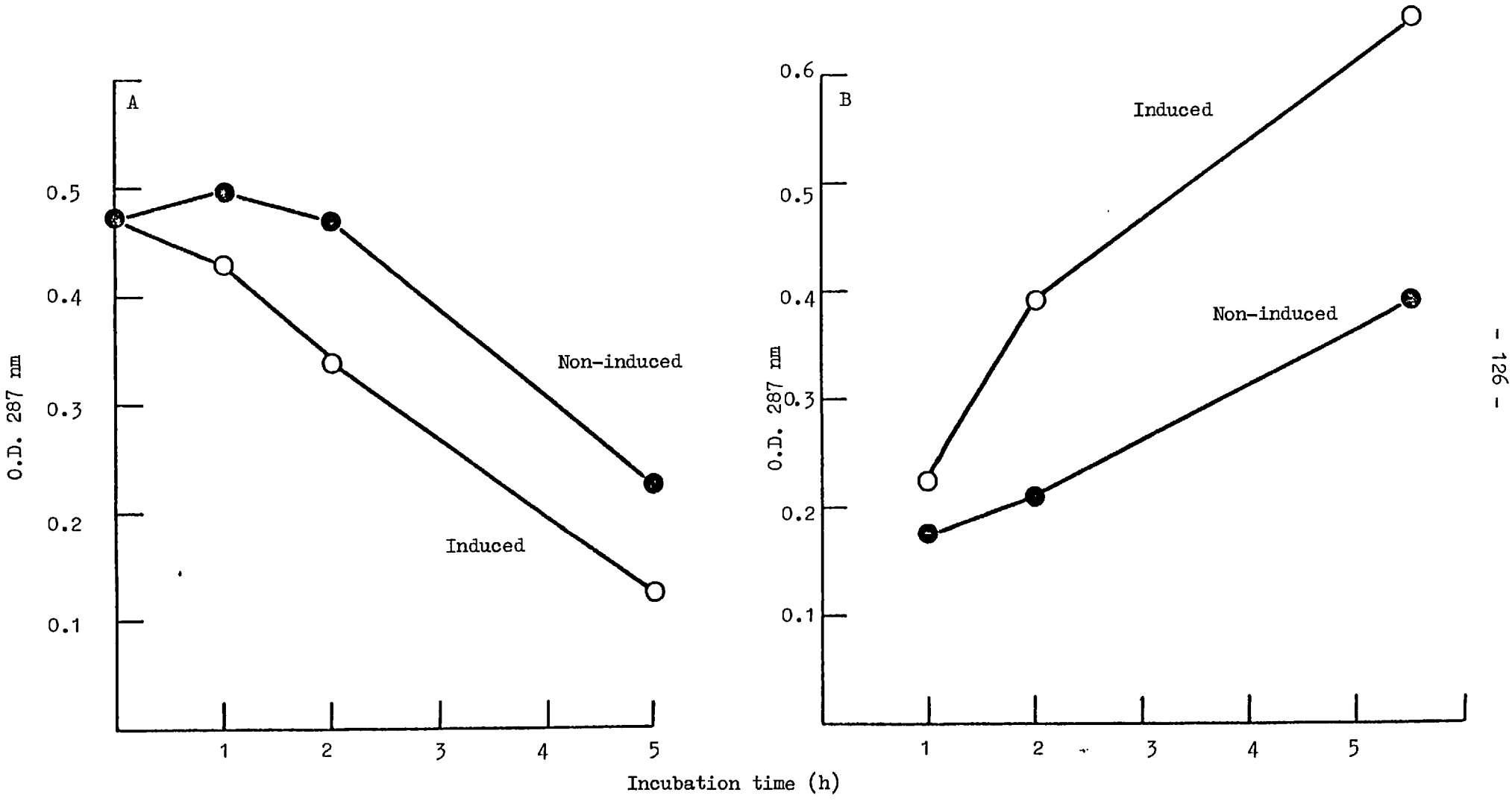
CCl_4 = O.D. at 287 nm of CCl_4 extract in ethanol.

b) Inducibility of maackiain degradation system

In section 6.a it was shown that maackiain could be degraded by S. trifoliorum, B. cinerea and B. fabae. However, no attempt was made to study the nature of the degradation system. It was important to establish whether the degradation system was inducible or constitutive because if it was the former, an induction process would be necessary for activating the degradation system, prior to any attempt at producing a cell-free preparation capable of degrading phytoalexins. There have been reports of phytoalexins inducing the degradation system in some fungi (Higgins, 1975; Heuvel and Van Etten, 1973) so experiments were designed to see whether the maackiain degrading system in S. trifoliorum and B. cinerea was inducible or constitutive.

Homogenised mycelium as for degradation experiments was used. Mycelium (5 ml) in 50 ml Erlenmeyer flasks was either induced with 100 µg maackiain in DMSO or not induced (DMSO only). After 6 h incubation, the supernatant was removed and the mycelium aseptically washed twice with sterile distilled water. Mycelium was then taken up in 5 ml SCA medium and 100 µg maackiain added to induced and non-induced mycelium. After the earlier times of 1 h, 2 h, 3.5 h and 5.5 h maackiain recovery and degradation products were estimated by extraction with CCl_4 and ethyl acetate as for degradation experiments, and quantitated by UV spectrophotometry. The results (Figure 27) show that in the non-induced control there was a lag phase of 1 - 2 h before maackiain disappearance was evident. In the induced system, no lag phase was apparent. Maackiain disappearance was observed by the first sampling time (1 h), in this induced system. However, after about 2 h, the rates of maackiain disappearance in both induced and non-induced systems were similar. With regard to product formation, a similar pattern of greater rate of product accumulation in the induced than in the non-induced system was observed (Figure 27). At 2 and 5 h after incubation the OD of the ethyl acetate extracts of the non-induced mycelium was only 54 - 60% of

Figure 27. Induction of maackiain degrading system in S. trifoliorum



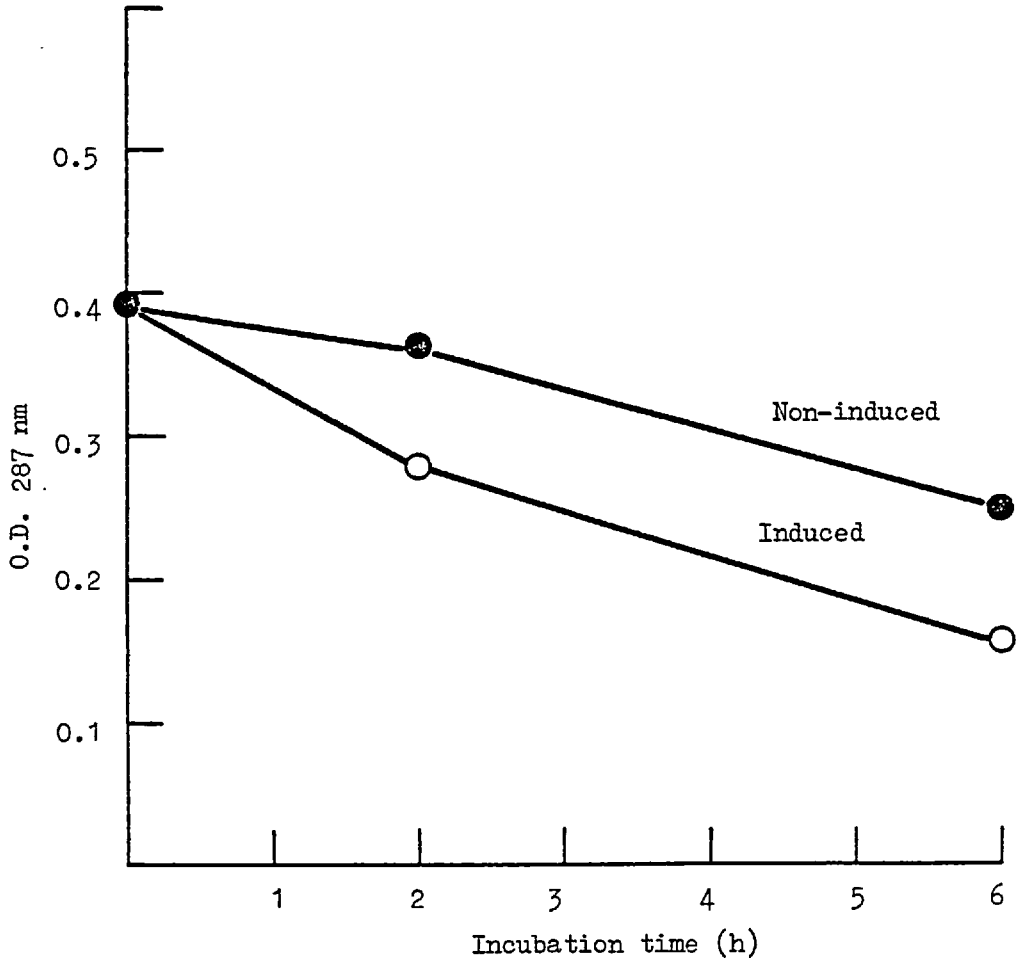
A - O.D. of CCl_4 extracts in ethanol; B - O.D. of ethyl acetate extracts in ethanol

that of the induced mycelium.

A similar experiment was carried out with B. cinerea to see whether there was any difference between this fungus and S. trifoliorum with regard to the inducibility of the degradation system. As shown in Fig.28, non-induced B. cinerea mycelium exhibited a lag phase (about 1 - 2 h) before maackiain degradation was evident. No lag phase was present in the mycelium of B. cinerea induced with maackiain.

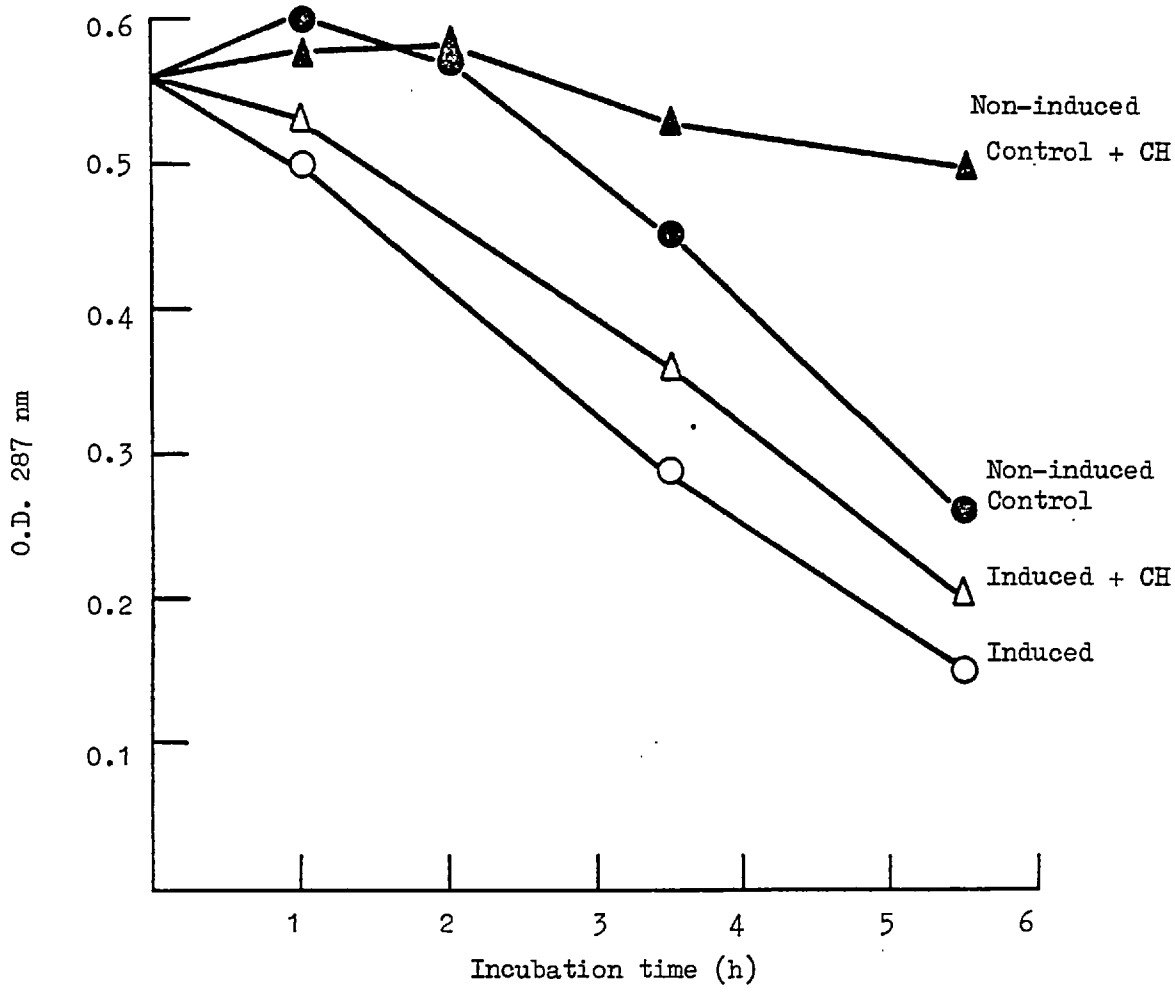
The inducibility of the maackiain degrading system was further confirmed by investigating the ability of cycloheximide (CH) to prevent degradation of maackiain. Maackiain (20 µg/ml)and cycloheximide (2 µg/ml) were added to induced and non-induced S. trifoliorum mycelium. As shown in Figure 29, there was a definite lag phase of about 2 h in the control (without CH) before maackiain disappearance was apparent. After 5.5 h incubation over 50% of the maackiain had disappeared from the medium. In the control (+ CH) very little loss of maackiain was evident at this time (12%). In treatments induced with maackiain (without CH) there was an immediate decrease in the amount of maackiain. Induced treatments (with and without CH) behaved very similarly in that there was no lag phase. In these treatments there was an immediate decrease in the amount of maackiain. The rates of degradation in both treatments were essentially similar to each other and to the control (- CH) after the lag phase. These results show that the maackiain-degrading system in both S. trifoliorum and B. cinerea is an inducible system which could be induced by the presence of maackiain. The protein synthesis inhibitor, cycloheximide, inhibits this induction but not the activity of the maackiain-degrading system as such.

Figure 28. Induction of maackiain degrading system in B. cinerea mycelium



O.D. of CCl_4 extracts in ethanol

Figure 29. Effect of cycloheximide (CH) on the induction of the maackiain degrading system



O.D. of CCl_4 extract in ethanol

c) Development of a cell-free degradation system

Preliminary studies

These experiments were carried out before the inducibility of the degradation system was established. Both constitutive and inducible enzyme systems have been claimed to exist for phytoalexin degradation (Christenson and Hadwiger, 1973; Higgins, 1972, 1975). Therefore in the following experiments fungal mycelium was either induced by prior exposure to maackiain or was not induced at all.

In search of an extracellular enzyme 'system' capable of degrading maackiain, a degradation experiment with homogenised mycelium of S. trifoliorum was set up as described previously. Maackiain (100 µg) in DMSO or DMSO alone was added to 5 ml aliquots of mycelial homogenates. After 6 h incubation, the contents of the flasks were harvested and the mycelium centrifuged at 1800 g in the cold (4°C). After checking for maackiain remaining in a sample of the supernatant, 100 µg maackiain was added to 5 ml aliquots of supernatant and incubated for 12 h at 25°C. A control of phytoalexin (100 µg) in 5 ml SCA medium was also included. After 12 h the phytoalexin was extracted with CCl_4 and quantitated as described in Materials and Methods. No loss of maackiain compared with control was observed in either the supernatant from induced mycelium or that from non-induced mycelium.

One of the possible reasons for the lack of activity could be dilution of the enzyme in the large volume of supernatant. Therefore a large scale degradation experiment was set up as follows : Agar plugs of S. trifoliorum (10) were inoculated into 100 ml aliquots of SCA medium in 250 ml Erlenmeyer flasks. After 6 days, the cultures were aspetically homogenised in an Omnimixer at setting 6 for 20 s and the homogenised mycelium allowed to recover for 24 h. After recovery, mycelium was then centrifuged at 1800 g in the cold (4°C) and the mycelium from two flasks taken up in 40 ml SCA medium. Maackiain (10 µg/ml) in DMSO or DMSO alone was added to each flask. After a 6 h induction period the supernatants were concentrated to $\frac{1}{4}$ the

original volume (20 ml) by ultrafiltration as described in Materials and Methods. The phytoalexin remaining in the supernatant of the induced mycelium was checked and 100 µg maackiain added to 5 ml aliquots of concentrated and unconcentrated supernatants. After 12 h incubation there was no apparent loss of phytoalexin compared with control, nor was there any loss with the ultrafiltrate.

From the above results it was clear that no extracellular enzymes capable of degrading maackiain were present in the culture filtrates of induced or non-induced mycelium of S. trifoliorum.

If indeed the degradation enzymes were not extracellular they must be intracellular, either wall or membrane-bound. To confirm this, the following experiment was set up : Induced and non-induced mycelium was prepared as in the last experiment. Mycelium was suspended in 60 ml 0.1 M sodium phosphate buffer, pH 7.2 and disrupted using a pestle and mortar and acid-washed sand at 4°C. The homogenate was then centrifuged at 26,500 g for 30 min and the supernatant concentrated by ultrafiltration to ¼ the original volume (15 ml). Both the concentrated supernatant, the ultrafiltrate and the unconcentrated control were used in the experiment. After addition of maackiain (100 µg) to 5 ml unconcentrated and concentrated supernatants or 30 ml ultrafiltrate, flasks were incubated for up to 20 h, after which phytoalexins were extracted and analysed. There was no significant loss of phytoalexin in any of the treatments compared with the control (maackiain in buffer). The absence of activity could be due to the following factors : (1) proteolysis or oxidation of the enzymes causing maackiain degradation. (2) enzyme levels too low to give significant degradation. (3) inadequate extraction procedures for degradation enzymes. (4) a co-factor requirement. Experiments were therefore designed to eliminate some of these factors. Also since the inducibility of the degradation system had previously been established, in all subsequent experiments, mycelium was initially subjected to a period of induction by exposure to maackiain for 6 h.

Cell-free degradation of maackiain

In this experiment an attempt was made to inhibit any proteolytic enzyme activity. Alternative, more efficient extraction techniques were used.

Flasks (2) each containing 10 agar plugs were harvested and the mycelium induced with maackiain as before. The maackiain remaining in the supernatant and the degradation products were estimated after extraction with CCl_4 and ethyl acetate respectively. The extracts from the CCl_4 fraction showed a loss in phytoalexin and the extract from the ethyl acetate showed an increase in the degradation products. This indicates an induction of the degradation system. The mycelium was then given four successive washings with 10 ml of a 1 M KCl solution on each occasion to release all extracellular proteolytic enzymes. Washed mycelium was then transferred to a sonicator tube immersed in ice. After 5 min, 20 ml 0.1 M sodium phosphate buffer, pH 7.2, was added followed by 0.3 ml 1.6%, w/v, phenyl methyl sulphonyl fluoride (a protease inhibitor) in isopropyl alcohol. Mycelium was then sonicated with six 20 s pulses at 70% maximal power, care being taken to ensure that the temperature remained below 6°C. Microscopic examination revealed that cellular breakage was very low. Sonicated mycelium was therefore transferred to a fairly loose fitting, medium coarse ground glass hand homogeniser (Wickland homogeniser; 130 ml volume of ground glass stem and 230 ml total volume) and homogenised for 10 min at 5°C. Homogenised mycelium was then centrifuged at 26,500 *g* for 30 min and the supernatant called "cell-free extract". This extract was assayed directly for maackiain degrading ability as shown below :

1. Cell-free extract (4 ml) + 40 µl DMSO

2. Cell-free extract (4 ml) + 80 µg maackiain in 40 µl DMSO

3. Sodium phosphate buffer (4 ml) + 80 µg maackiain in 40 µl DMSO (0.1 M, pH 7.2)

After 12 h incubation at 25°C, replicate samples were extracted twice with CCl_4 followed by two further extractions with ethyl acetate. The extracts were evaporated to dryness *in vacuo* and the residue taken up in ethanol for spectrophotometry. Results (Table 20) of the CCl_4 extract show that the cell free extract caused an approximate 73% loss of maackiain in 12 h. The optical background of the ethyl acetate extract was very high so even though there was an increase in the ethyl acetate soluble compounds compared with control no definite peaks of maackiain degradation products were discernible. When this extract was subjected to TLC in $CH Cl_3$: MeOH (98 : 2, v/v) solvent system no bands corresponding to the known maackiain degradation products were eluted.

TABLE 20.

	O.D. at 287 nm	
	CCl_4	EtOAc
Cell-free extract	0.05	0.66
Cell free extract + maackiain	0.18	1.78
Sodium phosphate buffer + maackiain	0.52	0.12

A cell-free extract from *S. trifoliorum* capable of degrading maackiain was therefore obtained.

Methods for increasing the efficiency of the system were further pursued e.g. to increase the enzyme levels the mycelial mass must be increased, so a method for producing large quantities of mycelium was developed.

Scaled up production of *S. trifoliorum* mycelium

It is essential that a large mass of fungal mycelium is used for production of a cell free extract. A method to scale up the production of *S. trifoliorum* mycelium was therefore developed. This method involved the culturing of *S. trifoliorum* as for *in vitro* degradation studies and the aspetical homogenisation of the mycelium in a Sorvall Omnimixer at setting 6

for 20 s. Aliquots (50 ml) of this homogenised mycelium were then inoculated into 500 ml SCA medium in 2 litre flasks. These were then incubated in a Griffin orbital incubator at 70 r.p.m. and maintained at 23° - 27°C for 8 days. With this method 20 - 30 g wet weight mycelium could be produced in 8 days. This method was used in subsequent experiments.

Comparison of the efficiency of (1) hand homogeniser (2) mickle disintegrator in breaking fungal cells

In this experiment, the efficiency of two methods of disrupting fungal cells was compared. This was to investigate the possibility of increasing the enzyme levels.

A time course of protein release from disrupted mycelium of S. trifoliorum was first investigated as follows : disruption was accomplished by use of (1) ground glass homogeniser (2) a mickle disintegrator. Mycelium (5 g fresh wt) was initially homogenised in an Omnimixer with 20 ml 0.1 M sodium phosphate buffer at 4°C. Mycelium was then disrupted by either (1) or (2) above. At various times, 2 ml aliquots were withdrawn and diluted with 2 ml of the above buffer. The mixture was then centrifuged at 26,500 g and the protein content of the supernatant estimated at 280 nm as described in Materials and Methods. Results are given in Table 21, which shows that the ground glass hand homogeniser released slightly more protein from S. trifoliorum up to 20 min, than the mickle disintegrator.

TABLE 21. Time course of protein release from disrupted *S. trifoliorum* mycelium

Time (min)	Protein (mg/ml)	
	Hand homogeniser	Mickle disintegrator
5	1.76	1.60
10	2.28	2.00
15	2.34	2.08
20	2.72	2.40
30	2.48	2.60

Maximum protein release occurred after 20 min. The Mickle disintegrator was used for further investigations.

Cell-free degradation of maackiain

In this experiment, the procedure was further improved by inhibiting proteolysis and oxidation of the degradation enzymes and by chelating heavy metal ions which could also inhibit degradation, with EDTA.

Induction of the degradation system and production of the cell-free extract has already been described in Materials and Methods. Essentially 5 g *S. trifoliorum* was induced with 10 µg/ml maackiain for 6 h. Induced mycelium was then disrupted in buffer containing EDTA, a chelating agent, mercaptoethanol, an antioxidant and phenyl methyl sulphonyl fluoride, a proteolytic enzyme inhibitor. The supernatant obtained after centrifugation was then referred to as the cell-free extract.

In a preliminary experiment, this extract was diluted 1 : 1 with the same buffer and 100 µg maackiain added to 2 ml of this extract in a cuvette. The O.D. at 310 nm was recorded at intervals over a time course (Table 22).

TABLE 22.

Time (min)	O.D. at 310 nm
0	1.27
15	1.26
30	1.24
45	1.21
60	1.19
75	1.17
90	1.13
120	1.13
180	1.13
720	1.14

In a second experiment, 75 µg maackiain was added to 1 ml of the undiluted cell free extract and incubated for various time intervals. In one treatment $^{M}/_{100}$ $HgCl_2$ was added to investigate whether this compound would exert any inhibitory effect on the degradation process. Phytoalexins were extracted three times with CCl_4 and thrice more with ethyl acetate.

TABLE 23. Reaction profile of CCl_4 extracts in ethanol

	O.D. at 310 nm
Buffer control after 12 h incubation	.76
$HgCl_2$ ($^{M}/_{100}$) after 12 h incubation	.48
Untreated extract + PA after 1 h incubation	.54
" " " 3 h "	.36
" " " 12 h "	.25
Untreated extract + DMSO	.10

As shown in Tables 22 and 23 there was a continuous fall in phytoalexin recovery as time progressed. By 1 h 50% of the maackiain had disappeared (Table 23). Mercuric chloride caused the inhibition of the degradation process to some extent. The failure of this compound to inhibit this process by 100% may be due to chelation of some of the $HgCl_2$ by EDTA present in the medium.

Effect of various factors on the activity of the cell-free extract

In this experiment the effect of boiling, dialysis and storage on the activity of the cell-free extract was investigated. The cell-free extract was obtained as in the last experiment.

a) Boiling - a sample of the cell-free extract was boiled for 5 min and 100 µg maackiain added to 1 ml of boiled and unboiled extract and incubated for various times.

b) Dialysis - a sample of the cell-free extract was dialysed for 12 h against 2 litres of sodium phosphate buffer (0.1 M, pH 7.2) containing EDTA, mercaptoethanol and phenyl methyl sulphonyl fluoride. After this time, 100 µg maackiain was added to 1 ml dialysed extract.

c) Storage at $4^{\circ}C$ - a sample of the cell-free extract was stored at $4^{\circ}C$ for 24 h after which 100 µg maackiain was added to 1 ml extract.

After various incubation times, the phytoalexin recovery was determined as before, by extracting with CCl_4 and scanning in a spectrophotometer. The O.D's at 310 nm are recorded in Table 24.

TABLE 24. The effect of various factors on the activity of the cell-free extract

Treatment	Time of incubation (h)	O.D. at 310 nm
Buffer control	3 h	0.67
Boiled extract	3 h	0.64
Unboiled extract	1 h	0.55
Unboiled extract	3 h	0.34
Dialysed extract	2 h	0.78
Undialysed extract	2 h	0.48
Extract after storage at $4^{o}C$	2 h	0.62
Extract before storage at $4^{o}C$	2 h	0.48

The results show that boiling, dialysis and storage of the cell-free extract resulted in a loss of activity.

Effect of lyophilization on the activity of the cell-free extract

As before, cell-free extract was obtained as described in Materials and Methods. The crude extract was then subjected to lyophilization by using an Edwards Modulyo freeze dryer. After freeze drying, the residue was taken up in sodium phosphate buffer (0.1 M, pH 7.2) to form either 1-fold or 3-fold concentrated extract. Maackiain (100 µg) was added to a 2 ml extract and incubated for 2 h, after which maackiain recovery was determined by extracting with CCl_4 and scanning in a spectrophotometer. Results (Table 25) show that there was no activity in the cell-free extract after lyophilization, even when the concentration of the extract was increased 3-fold.

TABLE 25. The effect of lyphilization on the activity of the cell-free extract

Treatment	O.D. at 310 nm
Buffer control	.76
Concentrated extract 3 x	.82
Unconcentrated extract 1 x	.84
Unlyophilized extract	.53

7. Induction of synthesis of phytoalexins by elicitors from fungi

The capacity of S. trifoliorum and B. cinerea to induce the synthesis of phytoalexins in clover leaves was compared by using mycelial extracts and fungal culture filtrates from both fungi.

a) Cell wall elicitor

Cell wall from S. trifoliorum and B. cinerea were extracted and an elicitor preparation from this wall was made as described in Materials and Methods. S. trifoliorum produced 45.62 µg mycelial wall/g fresh wt.mycelium whereas B. cinerea produced 78.85 µg/g. After dialysing, the elicitor preparation was filtered through a 0.22 µm millipore membrane and the carbohydrate content determined by the anthrone method. B. cinerea elicitor preparation had 323 µg glucose equivalents/ml whereas S. trifoliorum had 540 µg/ml. A solution containing 500 µg glucose equivalents/ml was applied as 6 - 8, 20 µl drops on to the adaxial side of clover leaves. Even after 4 days only slight necrotic flecks were observed under the inoculum of both treatments. After various incubation periods the phytoalexin present in the leaf tissue was extracted and quantitated as in Materials and Methods. Results are given in Table 26.

TABLE 26.

	Phytoalexin (µg/g fresh wt.)	
	Maackiain	Medicarpin
S. trifoliorum		
24 h	42	81
48 h	66	98
B. cinerea		
24 h	50	73
48 h	80	118

Results in Table 26 show that there was only a small difference in the amount of phytoalexin induced by S. trifoliorum and B. cinerea on clover leaves. However, S. trifoliorum produced more elicitor than B. cinerea.

b) Crude culture filtrate

Culture filtrates were obtained from S. trifoliorum and B. cinerea which were grown as described in Materials and Methods. The carbohydrate content as determined by the anthrone method was 16.43 mg/ml glucose equivalents for S. trifoliorum and 16.96 mg/ml for B. cinerea. SCA medium gave 20.00 mg/ml. Culture filtrates were then applied as 6 - 8, 20 µl drops on to clover leaves as before. After 48 h incubation there was total necrosis underneath the inoculum droplets. The phytoalexin was then extracted and quantitated as already described. Results obtained are as follows.

	Phytoalexin (µg/g fresh wt.)	
	Maackiain	Medicarpin
S. trifiolrum		
Expt. 1	25.60	14.00
Expt. 2	46.23	21.47
B. cinerea		
Expt. 1	22.86	10.78
Expt. 2	33.29	17.63

When leaves were inoculated with SCA medium, larger amounts of phytoalexins were present after 48 h incubation (65 µg/g maackiain and 50 µg/g medicarpin). This may be due to microbial contamination.

Autoclaved culture filtrate also elicited phytoalexin accumulation in clover leaves. Very slight browning was observed after 48 h incubation of clover leaves inoculated with autoclaved culture filtrates. The phytoalexins present at this time were as follows :

	Phytoalexin (µg/g fresh wt.)	
	Maackiain	Medicarpin
S. trifoliorum	14.11	11.59
B. cinerea	12.73	12.74

Generally more phytoalexins were recovered from leaves inoculated with culture filtrates which were not autoclaved than from culture filtrates which were autoclaved.

c) Ethanol soluble and insoluble fractions of culture filtrate

i) Ethanol-soluble fractions from culture filtrates

Ethanol-soluble fractions from culture filtrates were obtained as described in Materials and Methods. The carbohydrate content of the fraction from S. trifoliorum was 16.43 mg/ml glucose equivalents, whereas that from B. cinerea was 12.14 mg/ml. After treatment of clover leaves as above, the phytoalexin present was extracted and quantitated as before. By 48 h slight necrosis was already visible underneath the inoculum droplets.

TABLE 27.

		Phytoalexin (µg/g fresh wt.)		
		Maackiain	Medicarpin	Total
S. trifoliorum	Day 2	208	290	498
	Day 4	169	391	560
B. cinerea	Day 2	145	313	458
	Day 4	120	214	334

and by 96 h the whole area underneath the inoculum was totally necrotic. In response to the above treatments, maackiain and medicarpin accumulated to high concentrations in both treatments. There was only a slight difference in the phytoalexin levels from leaves inoculated with fractions from both fungi. Control leaves inoculated with

sterile distilled water contained no phytoalexins. Thus S. trifoliorum and B. cinerea produce ethanol-soluble compounds capable of eliciting the accumulation of phytoalexins in red clover leaves. After 4 days fractions from S.trifoliorum induced more phytoalexins than those from B. cinerea.

ii) Ethanol insoluble fraction from culture filtrates

Ethanol insoluble fraction from culture filtrates were obtained as already described in Materials and Methods. The carbohydrate content of the fraction from S. trifoliorum culture filtrate was 40.2 µg/ml glucose equivalent, whereas that of B. cinerea culture filtrate was 35.7 µg/ml. Fresh SCA medium contained 20 mg/ml glucose equivalents. Clover leaves were treated with fractions from S. trifoliorum and B. cinerea as above. After various times the phytoalexin present was extracted and quantified as already described. There was no apparent necrosis 48 h after inoculation but by 96 h slight browning was observed in leaves treated with the fraction from S. trifoliorum culture filtrate. Only a rare transluscence was observed underneath the inoculum of leaves treated with the fraction from B. cinerea culture filtrate. Moreover, in response to both treatments small amounts of maackiain and medicarpin accumulated in leaves treated with the fraction from both fungi.

		Phytoalexin (µg/g fresh wt.)	
		Maackiain	Medicarpin
S. trifoliorum	Day 2	48	33
	Day 4	28	37
B. cinerea	Day 2	15	21
	Day 4	11	24

Leaves inoculated with fractions from S. trifoliorum culture filtrate accumulated slightly more phytoalexins than those treated with fractions from B. cinerea culture filtrate. In contrast, no phytoalexins were present in leaves inoculated with sterile distilled water. Thus S. trifoliorum and B. cinerea produce ethanol-insoluble compounds capable of inducing the accumulation of small amounts of phytoalexins in red clover leaves.

8. Effect of phytoalexins on fungi

Studies on how phytoalexins exert their antimicrobial action on fungi may provide more information regarding the significance of such compounds in disease resistance. In this series of experiments, fungi with different degrees of sensitivity in mycelial growth tests were chosen to study the effect of phytoalexin on fungal respiration and on electrolyte transport.

a) Respiration

The effect of maackiain on the respiration of S. trifoliorum, B. cinerea and S. fructicola was investigated with an oxygen electrode. The experimental procedure has already been described in Materials and Methods. Oxygen uptake by S. trifoliorum and B. cinerea was similarly affected by the addition of 50 µg/ml maackiain (Table 28). The rate of oxygen uptake fell to about 60% of the original value in the first hour and tc about 50% in the second hour. The rate increased again by the third hour, returning to the original value by the sixth hour. Both fungi thus recovered from maackiain treatment after an initial phase of inhibition. By contrast, oxygen uptake by S. fructicola was immediately inhibited by about 60% on addition of 50 µg/ml maackiain solution and the rate fell to about 6% of the original value in the first hour and remained at this low level for the remaining period of the experiment. When the concentration was reduced to 20 µg/ml maackiain, oxygen uptake by S. fructicola was inhibited by about 60% 2 h after phytoalexin addition. However, by the 5th hour respiration recovered to about 88% of the initial rate (Fig. 29).

TABLE 28. Effect of maackiain (50 μg/ml) on oxygen uptake by *S. trifoliorum*, *B. cinerea* and *S. fructicola* (n mole/min for 5 ml mycelial suspension)

Time (h)	*S. trifoliorum*		*B. cinerea*		*S. fructicola*	
	Control	Maackiain	Control	Maackiain	Control	Maackiain
Original rate	17	19	17	15	29	26
0*	17	19	17	15	26	10
1	19	12	15	9	26	5
2	15	7	15	7	22	5
3	19	7	17	7	22	3
4	17	7	17	12	23	3
5	19	19	15	17	29	3
6	19	19	17	15	33	3
7	-	-	-	-	29	3
8	-	-	-	-	33	5

*Time of addition of DMSO, without maackiain in control, with maackiain in experimental. Oxygen uptake determined with an oxygen electrode over periods of 15 - 20 min.

TABLE 29. Effect of maackiain (20 µg/ml) on oxygen uptake by S. fructicola (n mol /min for 5 ml mycelial suspension)

Time (h)	S. fructicola	
	Control	Maackiain
Original rate	12	17
0*	10	10
1	19	10
2	17	7
3	19	10
4	19	12
5	19	15
6	19	15
7	19	15

* Time of addition of DMSO, without maackiain in control, with maackiain in experimental. Oxygen uptake determined with an oxygen electrode over periods of 15 - 20 min.

b) Electrolyte transport

In this experiment, 20 µg/ml maackiain was added to rinsed mycelium of S. trifoliorum and S. fructicola. Control flasks had DMSO added to the mycelium. Immediately after addition three replicate flasks of each treatment were harvested and the electrolyte content of the supernatant determined with a flame photometer and a conductivity meter. Similar analysis was made after 24 h incubation of the mycelium. The results for K^+, Ca^{2+} and total electrolytes are given in Table 30 . With S. trifoliorum

TABLE 30. Effect of 20 µg/ml maackiain on electrolyte transport

	K^+		Ca^{2+}		Conductivity (µmho)	
	Time					
	0 h	24 h	0 h	24 h	0 h	24 h
S. trifoliorum						
DMSO	69	24	6	1	27	57
MAACKIAIN	92	41	9	1	31	51
Autoclaved mycelium	98	60	10	1	26	23
S. fructicola						
DMSO	45	3	6	2	24	19
MAACKIAIN	53	51	7	1	25	18
Autoclaved mycelium	78	69	6	0.6	20	19
Standards						
3 ppm K^+	96	100				
1.5 ppm K^+	44	45				
0.3 ppm K^+	11	12				
H_2O	0	1				
40 ppm Ca^{2+}			41	38		
20 ppm Ca^{2+}			20	18		
4 ppm Ca^{2+}			4	0		
H_2O			1	-3	1.6	1.8

All values are averages of three readings.

mycelium treated with DMSO or maackiain, there was a significant uptake of K^+ ions in 24 h. There was also some uptake of K^+ ions by autoclaved mycelium in this time. Results for Ca^{2+} ions show that in 24 h there was some uptake of these ions from the medium in all treatments. This variation may be due to fluctuations of the instruments. However total electrolytes as measured by conductivity revealed a leakage of electrolytes from both DMSO and maackiain treated mycelium. With S. fructicola mycelium treated with DMSO, there was significant uptake of K^+ ions in 24 h. However, with those treated with maackiain there was no significant uptake in this time. Maackiain at this concentration appeared to prevent the uptake of K^+ ions by S. fructicola. There was also no significant uptake of K^+ ions by autoclaved mycelium of S. fructicola. Results for Ca^{2+} ions show an uptake of this ion in all the treatments. The total electrolytes as determined by conductivity measurements did not differ between treatments at time zero and at time 24 h.

9. Studies on S. trifoliorum toxin

Held (1955) and Debnam (1975) found that S. trifoliorum produced a wilt-inducing toxin in liquid culture. Both workers demonstrated that varieties of clover differing in susceptibility to clover rot did not show differences in resistance to toxin. However, Held (1955) also found that a normal strain of the fungus secreted this toxin, whereas a degenerate strain did not. Hence it was decided to carry out studies on this toxin.

a) Confirmation of the presence of toxin in vitro

Cultures of S. trifoliorum growing in SCA medium for 20 and 30 days were centrifuged at 1800 g for 5 min. The supernatants obtained were dispensed as 20 ml aliquots into ten-50 ml Erlenmeyer flasks. As controls, 20 ml aliquots of SCA medium were also dispensed into 50 ml flasks. Petioles of fully turgid clover leaves were cut to 10 cm in length and one leaf immersed into each flask so that the tip of the petiole was submerged in the liquid. High transpiration was obtained by placing a fan about 150 cm from the flasks so that a continuous air flow is maintained. A lamp (250 v, 100 w) was placed about 90 cm from the top of the leaves. After 24 h incubation, leaves in culture filtrate had wilted. The leaves were flaccid and had curled at the edges. There was no wilting of the leaves in unused SCA medium(Plate 5). When the wilted leaves were transferred to distilled water, the less affected leaves recovered but those which were greatly affected did not. When culture filtrates from S. trifoliorum growing for 6 days were used, flaccidity and leaf curling were also observed. These above results confirmed the presence of a toxic principle in culture filtrates of S. trifoliorum.

Plate 5. Effect of 20-day old S. trifoliorum culture filtrate (F) on turgidity of clover leaves. Photographed after 24 h. C = unused SCA medium control. Note wilting and flaccidity of culture filtrate-treated leaves. Controls remain turgid.

b) Some properties of S. trifoliorum toxin

Culture filtrates from 20-day old S. trifoliorum growing in SCA medium were used. These filtrates containing the toxic principle were exposed to various treatments, after which their capacity to cause wilting was tested on healthy, turgid clover leaves as described above. After autoclaving at 1 kg/cm^2 for 15 min the toxin still retained its activity. Therefore this toxin is thermostable. Culture filtrates were dialysed in 2 litres of water for 24h and the dialysate concentrated to the original strength by evaporating to dryness in vacuo at 55°C, or by freeze drying. After treatment of clover leaves for 24 h, similar wilting symptoms were observed. The culture filtrate in the dialysis bag showed a very much reduced effect. So the toxic principle might be of a low molecular weight capable of passing through a dialysis bag. Filtering through a 0.22 µ membrane did not result in a loss of activity. Leaves which had become wilted as a result of toxin uptake for 24 h, were also capable of absorbing the dye, fuschin red suggesting that wilting is not due to blockage of xylem vessels.

When crude culture filtrates (20-day old) were applied to clover leaves as 20 µl drops, browning was observed underneath the inoculum drop. After 48 h diffusates from lesions were removed and the phytoalexin extracted and quantitated.

		Phytoalexin (µg/ml)	
		Maackiain	Medicarpin
S. trifoliorum	Experiment 1	25.6	21.6
	Experiment 2	16.7	< 10.0

Crude culture filtrate of S. trifoliorum which contained a toxic principle was capable of inducing phytoalexin biosynthesis in clover leaves.

When Botrytis cinerea culture filtrate was similarly investigated 20 and 30-day old filtrates also caused wilting of clover leaves in 24 h though this was to a lesser extent. Flaccidity and leaf curling at the edges were also observed.

Wilting capacity was retained after autoclaving, but was lost after dialysis. Fuschin red was absorbed by wilted clover leaves. Culture filtrates from 6-day old cultures gave no obvious effects. When culture filtrates from 20-day old B. cinerea cultures were applied as 20 µl drops to clover leaves, brown flecks were observed underneath the inoculum drops. This was less apparent than those observed for S. trifoliorum, and they also looked translucent when dry. The phytoalexin content in diffusates after 48 h incubation is as follows.

		Phytoalexin (µg/ml)	
		Maackiain	Medicarpin
B. cinerea	Experiment 1	24.4	< 10.0
	Experiment 2	16.7	13.8

Culture filtrate from B. cinerea containing a toxic principle was capable of inducing phytoalexin biosynthesis in clover leaves.

Results in this section show that both S. trifoliorum and B. cinerea produce a toxin(s) in culture. The toxin from both fungi possess very similar properties. Both were thermostable and dialysable (i.e. low molecular weight). B. cinerea produces oxalic acid in culture (Wood, 1967) as well as high and low molecular weight polysaccharides (Dubourdieu and Kamden, 1978). Therefore, if the organic acid is the cause of the observed effects by B. cinerea culture filtrates it is possible that the toxin produced by S.trifoliorum is also oxalic acid. That this may be so is supported by the fact that a very similar fungus, S. sclerotiorum has been reported to produce oxalic acid in culture and in diseased bean tissue (Maxwell and Lumsden, 1970).

A qualitative chemical test using

diphenylamine as described in Materials and Methods shows that 20-day old S. trifoliorum culture filtrate contained oxalic acid. Thus S. trifoliorum produces oxalic acid — a toxic principle in culture. The production of this acid in culture is also supported by the fact that the pH of the culture filtrate was 4.7 in contrast to a pH of 5.6 for the unused culture medium (SCA).

In the preliminary qualitative test above, 20-day old S. trifoliorum culture filtrate was shown to contain some oxalic acid. Therefore in order to estimate the amount of this acid, serially diluted 20-day old culture filtrate and a range of pure oxalic acid were assayed by the chemical method already described. Culture filtrate was assayed as before and pure oxalic acid was assayed by adding diphenylamine and phosphoric acid directly to the organic acid. After heating and taken up in alcohol, the presence of oxalic acid was indicated by the appearance of a blue colour. The results (Table 30) show a gradation of colour intensity from the highest to the lowest concentration.

TABLE 30. Detection test for oxalic acid

Culture filtrate	Colour development (arbitrary scale)	Oxalic acid	Colour development (arbitrary scale)
Undiluted	+++	2.5 g	+++
1 : 10 dilution	++	0.25 g	++
1 : 100 "	+	0.025 g	+
1 : 1000 "	±	0.0025 g	±

With culture filtrate, the $^{1}/_{1000}$ dilution developed the least intensive colour; this was a very faint blue colour. Likewise with oxalic acid, the lowest concentration gave only a faint blue colour. Since the two shades of blue colouration with culture filtrate and pure oxalic acid could not be compared directly it was difficult to estimate the concentration of this acid in culture filtrate by this semi-quantitative method.

Leaf bioassay for toxin

A leaf bioassay using serially diluted culture filtrate and oxalic acid solution was carried out. Clover leaves were treated with 20 µl droplets of the solutions and incubated in an illuminated cooled incubator held at 20°C as previously described.

After various times the symptoms produced were observed and recorded (Table 31). There was a direct relationship between the lesion produced on the leaf and the amount of oxalic acid applied to it. The 0.25% oxalic acid produced necrosis which completely covered the inoculum area by 24 h. Further incubation did not cause any spread. Thus the lesion became limited by about 24 h (Plate 6) the same time that the undiluted culture filtrate took to completely cover the inoculum area. In view of this, an estimate of the concentration of oxalic acid giving a similar reaction as the undiluted culture filtrate would be between 0.025 - 0.25%.

Plate 6. Leaves treated with 3-6 drops of 20-day old S. trifoliorum culture filtrate (F) or 0.25% oxalic acid (A). Photographed 24 h after treatment. Note necrosis at point of treatment.

F A

TABLE 31. Leaf bioassay

	SYMPTOMS		
Oxalic acid	Incubation time (h)		
concentration in % (w/v)	0.5	1.0	24
5	Little necrosis underneath inoculum droplets	Complete necrosis underneath inoculum droplets	Total necrosis of whole leaf
2.5	"	"	"
0.25	No necrosis	Little necrosis underneath inoculum droplets	Complete necrosis underneath inoculum droplets
0.025	No necrosis	No necrosis	Leaf clearing
0.0025	"	"	No necrosis
Culture filtrate			
Undiluted	No necrosis	No necrosis	Complete necrosis underneath inoculum droplets
$^1/_{10}$ dilution	"	"	Little necrosis underneath inoculum droplets
$^1/_{100}$ "	"	"	No necrosis
$^1/_{1000}$ "	"	"	"

DISCUSSION

The original aim of this research was to investigate the role of phytoalexins in the resistance of red clover to Botrytis and Sclerotinia species. The model pathogen was S. trifoliorum and the other species of fungi used were pathogens of other plants but not of red clover.

A central problem in plant pathology is that of host-pathogen specificity. An aspect of this phenomenon deals with why a pathogen grows and causes disease in some plants but not in others. A thorough discussion of this and other forms of specificity is presented in "Specificity in Plant Diseases" (Wood and Graniti, 1975) and by Wood and Smith (1977).

In the work reported here, both pathogenicity tests and histological studies revealed that only S. trifoliorum formed spreading lesions in clover leaves. B. cinerea and B. fabae also formed lesions on clover leaves but the lesions became limited in size; therefore these two fungi could be regarded as weakly pathogenic on this plant. The other fungi i.e. S. fructigena, B. allii and S. fructicola all formed no more than fleck lesions. Non-host resistance mechanisms must therefore be involved in the restriction of these fungi as in other plant-pathogen interactions such as S. botryosum and H. carbonum on clover leaves (Duczek and Higgins, 1976) and in B. cinerea on broad bean (Hargreaves, Mansfield and Rossall, 1977). All the fungi except S. fructicola appeared to penetrate the leaf tissue, but growth in the tissue itself was stopped after a few cells had been penetrated as in flecking lesions, or after a number of cells had been colonised, as occurred in limited lesions where lesion diameter after five days was 5 - 6 mm. Light and electron microscopy (Higgins and Lazarovits, 1978), showed that S. sarcinaeforme and S. botryosum, a pathogen and non-pathogen of clover respectively, varied in their modes of penetration and colonisation but the host cell response to the two fungi appeared similar. Phytoalexins were produced in leaves inoculated with either of

these fungi or with H. carbonum (a maize pathogen) which did not penetrate the leaf tissue at all (Duczek and Higgins, 1976a).

The same phytoalexins, maackiain and medicarpin (the main red clover phytoalexins) were detected in clover leaves inoculated with all the six fungi studied. Neither the other clover phytoalexins (Debnam and Smith, 1976) nor the preformed inhibitors were studied in this work. These have been regarded as having only a minor contribution to the resistance of clover leaves to B. cinerea (Debnam and Smith, 1976). The preformed isoflavones, formononetin and biochanin A, have also been shown to be of little significance in the resistance of chickpeas to H. carbonum (Ingham, 1976b). Though not regarded as important in these interactions there remains the possibility that these inhibitors may play some role in the resistance of clover to other fungi. They may act synergistically or additively with each other and with maackiain and medicarpin, further corroborating the idea that many plants produce several inhibitory compounds which together create a very antifungal environment, e.g. French beans produce five inhibitory compounds (Burden, Bailey and Dawson, 1972; Van Etten and Smith, 1975). Two of these phytoalexins, kievitone and phaseollin had additive antifungal effects on Rhizoctonia solani (Smith et al., 1975) as did wyerone acid and wyerone epoxide on B. fabae and B. cinerea (Hargreaves et al., 1977) and maackiain and medicarpin on H. carbonum, S. botryosum and S. sarcinaeforme (Duczek and Higgins, 1876a).

In clover leaves, the formation of maackiain during infection could not be accounted for by hydrolysis of the trace amount of the glycoside trifolirhizin (Debnam, 1975; Duczek and Higgins, 1976a), therefore the phytoalexins must be produced by de novo biosynthesis. Even though clover roots contain large quantities of trifolirhizin, leaves contain very little (Hietala, 1960 ; Duczek and Higgins, 1976a). This however contradicts the findings of Chang et al. (1969), who reported high levels of trifolirhizin in clover leaves, but as pointed out by Duczek and Higgins (1976a), Chang's foliage samples may have been contaminated with

some root tissue. In the study reported here, the levels of trifolirhizin in roots were very high, since large quantities of maackiain were produced by homogenising clover roots and allowing a host β-glucosidase to hydrolyse trifolirhizin to maackiain.

During the biosynthesis of maackiain and medicarpin, a divergence takes place quite early in the pathway even though both phytoalexins are closely related. This divergence occurs as early as the formononetin stage (Dewick, 1977). This suggests the possibility of several different control mechanisms which may then account for the differing amounts of maackiain and medicarpin in tissues and diffusates. Despite this biosynthetic divergence, both phytoalexins apparently have similar polarities, since they co-chromatograph on TLC. In Jack bean callus, medicarpin synthesis was induced with Pithomyces chartanum (Gustine and Sherwood, 1978). During this period two of the presumed enzymes in the biosynthesis of this phytoalexin also increased in activity. These were phenylalanine ammonia lyase (PAL) and isoflavonoid o-methyltransferase (OMT). Whether these enzymes are key steps in the regulation of medicarpin biosynthesis remains to be shown. However in soybean and cowpea PAL activation was shown to have no essential role in regulation of phytoalexin biosyntehsis (Munn and Drysdale, 1975; Partridge and Keen, 1977). Two other enzymes, cinnamic acid 4-hydroxylase and hydroxycinnamic acid CoA ligase also increased in inoculated Jack bean callus tissue (Gustine and Sherwood, 1978). Recently Yoshikawa et al. (1978b) showed that the phytoalexin glyceollin requires de novo m-RNA and protein biosynthesis. Thus further supporting the hypothesis of de novo synthesis of phytoalexins.

Using mycelial inoculum, large quantities of maackiain and medicarpin were recovered from tissue inoculated with S. fructigena, B. allii and S. fructicola in spite of the relatively small volume of tissue affected. Both inhibitors were also present in diffusates collected from the infection sites. None of these fungi showed any significant capacity to degrade the

phytoalexins *in vitro* and all were relatively sensitive to these inhibitors in mycelial growth tests at concentrations below those found in tissue. Moreover, oxygen uptake by *S. fructicola* was strongly and irreversibly inhibited by maackiain (50 µg/ml) but at 20 µg/ml, oxygen uptake recovered after an initial inhibition. The failure of these three fungi to infect clover leaves could thus simply be attributed to the accumulation of the phytoalexins, maackiain and medicarpin to which the fungi are sensitive and which they are unable to degrade. These factors would be on the basis of *in vitro* assays, sufficient to cause the inhibition of the growth of the fungi in the fleck lesions. Similar explanations could be invoked for the failure of *H. carbonum* to infect red clover (Duczek and Higgins, 1976a) and sweet clover (Ingham, 1976b). Oxygen uptake by *H. carbonum* was also shown to be temporarily inhibited by maackiain and/or medicarpin. Further support for the involvement of these phytoalexins in resistance came from results which showed that maackiain and medicarpin prevented lesion formation on corn leaves inoculated with *H. carbonum* even when phytoalexins were added 6 h after inoculation, thus acting as a protectant (Duczek and Higgins, 1976b). This protectant action of phytoalexins has also been reported for capsidiol against *Phytophthora infestans* on tomato (Ward *et al.*, 1975) and for rishitin against this same fungus on potatoes (Ersek, 1977).

In the present study, the other three fungi examined all formed lesions on clover leaves inoculated with a mycelial inoculum. *B. cinerea* and *B. fabae* both formed limited lesions but *S. trifoliorum* on the other hand formed spreading lesions. This latter fungus is the only one normally regarded pathogenic to clover. All three fungi caused the accumulation of maackiain and medicarpin in lesions but no accumulation of these phytoalexins was found in diffusates. All three fungi were relatively less sensitive to the phytoalexins in mycelial growth tests than the first group of fungi. Oxygen uptake by *S. trifoliorum* and *B. cinerea* though initially inhibited by maackiain, (50 µg/ml) returned to its original level

after a few hours (B. fabae was not tested in this respect). All three fungi were able to degrade maackiain and medicarpin in vitro. S. trifoliorum and B. cinerea metabolized these phytoalexins to their 6a-hydroxylated derivatives which had reduced antifungal activity to both fungi. The products of degradation by B. fabae were not identified since no distinct new compounds could be recovered from the medium. Therefore the degradation pathway may be different from that of S. trifoliorum and B. cinerea or the intermediates may not accumulate in the medium.

It was thus clearly possible to distinguish the second group of fungi causing the more aggressive infection of clover leaves and to attribute their greater pathogenicity, at least in part, to their relative insensitivity to and/or ability to degrade the phytoalexins of clover.

In preliminary experiments, S. trifoliorum apparently degraded 6a-hydroxymaackiain in vitro to 6a, 7-dihydroxymaackiain. This product was also detected in diffusates from leaves inoculated with S. trifoliorum or B. cinerea. Despite this, B. cinerea did not appear to degrade 6a-hydroxymaackiain in vitro. Probably the enzyme which brings about this conversion is absent in B. cinerea. If so, the presence of the 6a, 7-dihydroxylated derivatives in diffusates may be due to abiotic degradation of 6a-hydroxyderivatives or to conversion by contaminant organisms. Other fungi can bring about this same hydroxylation of pterocarpans. The French bean pathogen, Colletotrichum lindemuthianum (Burden et al., 1974) degraded phaseollin to 6a- and 6a, 7-dihydroxy-phaseollin. Both metabolites were less fungitoxic than phaseollin to C. lindemuthianum and Septoria nodorum. Therefore these hydroxylation processes can be considered detoxification steps (Bailey et al., 1977). The coffee pathogen, Colletotrichum coffeanum (non-pathogen of legumes) also brings about both hydroxylations of medicarpin on sweet clover leaf diffusates (Ingham, 1976a). B. cinerea, on the other hand was unable to further hydroxylate the pterocarpans to 6a, 7-dihydroxylated derivatives (Heuvel, 1976; Ingham, 1976a). Successive hydroxylations of pterocarpans

will render these compounds more polar, will reduce their lipid solubility and increase their water solubility, making them more susceptible to oxidative cleavage of the aromatic rings with eventual formation of CO_2 and H_2O (Harbone, 1977). This further cleavage probably occurs with S. trifoliorum (Bilton, et al., 1976b) and in other systems (Ingham, 1976a).

Apart from hydroxylation of pterocarpans other degradation mechanisms have been reported. S. botryosum, a pathogen of alfalfa degraded phaseollin, maackiain and medicarpin to their respective isoflavan derivatives (Higgins, 1975) and demethylates pisatin to 6a-hydroxymaackiain (Van Etten et al.,1975). With maackiain and medicarpin the metabolites were still equally as fungitoxic as the parent phytoalexins. So this first reaction step could not be considered a detoxifcation step per se. However these metabolites were in turn degraded to other products. Unlike S. botryosum, F. solani f. sp. phaseoli (Heuvel et al., 1974) and Cladosporium herbarum (Heuvel and Glazener, 1975) degraded phaseollin by hydroxylation at position 1a, with concomitant dienone formation in ring A to form 1a-hydroxyphaseollone.

In this context it would be interesting to find out whether B. fabae degraded maackiain and medicarpin by any of these other mechanisms.

Apart from the initial reactions, the remaining steps in pterocarpan degradation are still unresolved. However pisatin (a pterocarpan from peas) was recently shown to be degraded by Ascochita pisi to 6a-hydroxy-maackiain (6a-hydroxyinermin) - a detoxification step (Fuchs, 1978). Fusarium oxysporum f. sp. pisi was able to detoxify this metabolite to the isoflavan derivative which was unstable and breaks down to two heterocyclic compounds with emperical formulae $C_7H_6O_3$ and $C_9H_8O_3$. Another product was the anhydroderivative of the isoflavan which was probably also formed non-enzymically.

It was more difficult to assess the basis of the differential pathogenicity of S. trifoliorum, B. cinerea and B. fabae. In the study reported in this thesis, S. trifoliorum was more aggressive than B. cinerea and B. fabae. The main comparison was however made between the first two species. The degradation studies in vitro supported the existence of a difference between S. trifoliorum and B. cinerea on the rate at which they degrade maackiain and medicarpin. S. trifoliorum degraded both phytoalexins at a faster rate than B. cinerea. This would therefore tip the balance in favour of S. trifoliorum in vivo. From the time course of accumulation of phytoalexins and their 6a-hydroxylated derivatives it was clear that an initial increase in phytoalexin concentration was followed by their disappearance and accumulation of 6a-hydroxylated metabolites as lesions caused by S. trifoliorum spread. This would suggest that S. trifoliorum is able to metabolize the phytoalexins in vivo thereby keeping the concentration at a low level. This degradation is supported by the absence of phytoalexins in the centre of spreading lesions indicating that the fungus degraded the phytoalexins as the lesions spread. Also supporting this degradation was the complete absence of maackiain and medicarpin in diffusates and the presence of the 6a- and 6a,7-dihydroxylated derivatives. These two metabolites were also found in diffusates from B. cinerea-inoculated leaves but were absent in diffusates and tissue from B. fabae, S. fructigena, B. allii and S. fructicola-inoculated leaves. B. cinerea like S. trifoliorum degraded clover phytoalexins in vivo, since the same products as those from degradation in vitro were isolated. B. cinerea however caused the accumulation of more phytoalexins, and the conversion was slower than for S. trifoliorum. The 6a-hydroxylated metabolites also decreased in concentration with time, indicating that there is a turnover of these derivatives as well. B. cinerea was more sensitive than S. trifoliorum to 6a-hydroxymaackiain and probably to 6a-hydroxymedicarpin as well. So, although this degradation step from maackiain to 6a-hydroxymaackiain was a complete detoxification by S. trifoliorum it was not exactly so for B. cinerea. A more antifungal environment will therefore exist in B. cinerea lesions. This would be more than is at first envisaged

if the phytoalexins are not evenly distributed in the lesions, but are located mainly in a zone around lesions. The hyphal tips may then come in contact with a considerably higher concentration of the phytoalexins as was suggested for B. cinerea lesions on French beans (Heuvel, 1978). This worker also detected the phaseollin metabolite, 6a-hydroxyphaseollin in B. cinerea lesions indicating degradation by the fungus in vivo. Degradation of phytoalexin in vivo has also been suggested in view of the isolation of 1a-hydroxyphaseollone, an in vitro fungal metabolite of phaseollin, from Fusarium solani f. sp. phaseoli-infected hypocotyls of Phaseolus vulgaris (Van Etten and Smith, 1975).

Though no 6a-hydroxylated derivatives were recovered from leaves inoculated with the non-pathogenic fungi which did not degrade the phytoalexins in vitro, the possibility that the host plant possess enzyme(s) causing hydroxylations at the 6a-position cannot be ignored. This however was not apparent in tissue inoculated with these non-pathogenic species. Also possible is the presence in host plants of other enzyme systems capable of converting these phytoalexins to other as yet undetermined product(s). In some experiments on the accumulation of phytoalexins in tissue inoculated with the fungi which did not degrade in vitro, there was a fall in the phytoalexin content after an initial rise in concentration. Pterocarpans have indeed been shown to be degraded by plant tissue. Dewick (1975) suggested that the red clover phytoalexins are subjected to turnover in red clover seedlings. In French beans, exogenously applied phaseollin was found to be toxic to plant cell suspension cultures and to be adsorbed or degraded by these cells (Hargreaves and Bailey, 1978; Hargreaves and Selby, 1978; Skipp et al., 1977). Capsidiol from sweet pepper (Stoessl et al., 1977) and rishitin from potato (Murai et al., 1977; Rogerson and Threlfall, 1978) were also shown to be degraded by plant tissue. These results would appear to support the view that phytoalexin accumulation results from the blocking of a functioning pathway which in the healthy cell continuously turns over a small phytoalexin pool, e.g.

biosynthesis equalled by degradation. Glazener and Van Etten (1978) suggested that the high phytoalexin accumulation may be due to their phytotoxicity and inactivation of the phaseollin metabolising system in tissue. Further work in this area is surely needed.

Results in the thesis show that when a spore inoculum of B. cinerea in glucose solution was used, the lesions were smaller than those formed with B. cinerea mycelium or B. cinerea spores in SCA medium. Phytoalexin production was much greater and little or no 6a-hydroxylated degradation products were detected.

These phenomena may be due to one or more of the following : differential induction, differential degradation or differential suppression of phytoalexin biosynthesis. The results with the spores in glucose could be explained inter alia in terms of suppression of the degradation of the phytoalexins. The glucose present may repress synthesis of the degradation enzyme(s) by catabolite repression. Differential sensitivity to phytoalexins may also contribute. B. cinerea spores had previously been shown to be sensitive to maackiain and medicarpin. Maackiain at 10 µg/ml reduced germ-tube growth by 70% of the control. Only very little more inhibition occurred at higher concentrations (Debnam and Smith, 1976). No effect on germination was observed. Medicarpin on the other hand was active on spore germination (ED_{50} 30 µg/ml) and on germ tube growth (ED_{50} 20 µg/ml).

On the induction of phytoalexins there is abundant evidence that phytoalexin formation can be induced by a wide variety of biotic and abiotic agents (Van Etten and Pueppke, 1976). To date, no convincing evidence is available on the biochemical mechanisms involved. However two main hypotheses have been proposed. One is that phytoalexins are produced by live cells in response to the presence of fungal metabolites and the other first proposed by Müller and Borger (1940) that phytoalexin biosynthesis is associated with necrobiosis of host cells. The former hypothesis was

propounded by Cruickshank and Perrin (1968) who isolated a peptide, monilicolin A, from Monilinia fructicola. This peptide induced phaseollin biosynthesis in bean pods without killing host cells (Paxton et al., 1974). Contrary to this,other workers (Bailey and Deverall, 1971; Bailey and Ingham, 1971; Mansfield and Deverall, 1974) have found that phytoalexin began to accumulate at about the same time that necrosis was first noticed. From these studies it seems possible that phytoalexin formation is induced as a consequence of cellular death. Varns and Kuć (1972) supported this by demonstrating that both necrosis and phytoalexin accumulation were suppressed in potato tubers inoculated with a compatible race of Phytophthora infestans.

In the context of the former proposal, molecules of fungal origin have been implicated. These substances called elicitors (Keen, 1972) are glycoproteins (Keen, 1978) and are race specific i.e. they elicit higher levels of phytoalexins in resistant race-cultivar interaction than in susceptible ones. Albersheim and Valent (1978) claimed that elicitors from the same fungus, Phytophthora megasperma var. sojae are polysaccharides and moreover they lack specificity.

In the work reported here, fungal cell wall elicitors and both crude and partially purified culture filtrates from S. trifoliorum and B. cinerea all stimulate phytoalexin production in clover leaves. Generally there were no significant differences in the phytoalexin concentrations between the two treatments. However in a few cases greater amounts of phytoalexins accumulated in leaves treated with fractions from S. trifoliorum than in leaves treated with fractions from B. cinerea. Other fractions from fungi are known to cause accumulation of phytoalexins when applied to plants (Keen, 1975; Lister and Kuc, 1977; Morris and Smith, 1978). Dixon and Fuller (1977) claimed that unlike most of the characterised elicitors, the elicitor from B. cinerea culture filtrate was of low molecular weight. In the work here, both high and low molecular weight elicitors were shown to be present in S. trifoliorum and B. cinerea cultures.

Heuvel (1978) also produced results which suggested that either a diffusible 'elicitor' was produced in B. cinerea-inoculated bean leaves or the phytoalexins were translocated since green areas (including the petiole) as well as lesion areas contained phaseollin.

Thus concerning the relationship between host cell death and phytoalexin formation Deverall (1977) summerized several possible alternatives as follows :-

1) Death of a host cell may cause synthesis of phytoalexin in neighbouring cells.

2) Molecules diffusing from a fungus may induce phytoalexin formation and cell death.

3) Phytoalexin accumulation may cause death of host cells.

Recent work by Hargreaves and Bailey (1978) suggests that phytoalexin formation occurs in living cells of Phaseolus vulgaris in response to metabolites released from dead cells - a constitutive elicitor. Further work in these promising areas of plant pathology would elucidate the cell surface recognition phenomena which are claimed to eventually result in phytoalexin induction as reviewed by Callow (1977).

As previously mentioned, antifungal studies in vitro showed that S. trifoliorum was the least sensitive of the fungi examined. At 50 µg/ml maackiain or medicarpin, B. cinerea was more sensitive than S. trifoliorum. In vivo, both phytoalexins would be expected to act together possibly additively thereby creating a very antifungal environment unfavourable for successful fungal growth. The exact mechanisms by which phytoalexins stop the growth of fungal cells is still not understood. However evidence suggests that these inhibitors act on the plasma membrane or affect some process needed for membrane function (Shiraishi et al., 1975;

Van Etten and Bateman, 1971). Experiments with S. trifoliorum and S. fructicola in which the effect of 20 µg/ml maackiain on electrolyte transport was investigated, showed that at this concentration uptake of K^+ ions was blocked in S. fructicola mycelium. This was not so for S. trifoliorum, in fact this fungi took up K^+ ions from the medium in this time. Similar results for the blocking of K^+ uptake was obtained by Slayman and Van Etten (1974). However disruption of the plasma membrane caused the efflux of the ionic constituents from R. solani (Smith and Bull, 1978) but a very high concentration of kievitone was used, which also caused a loss in mycelial dry wt. (Smith, 1978). So if a higher concentration which could result in dry wt. loss was used in the experiments here leakage of electrolytes may have been evident. Results in this study show that like the inhibition of respiration, the inhibition of mycelial growth of S. trifoliorum and B. cinerea was also temporary. Both fungi metabolised 50 µg/ml maackiain before substantial mycelial growth occurred. This suggests that degradation may play a role in the ability of these fungi to overcome the phytoalexins. S. fructicola which did not degrade maackiain was not tested in this respect. However, since the respiration of this fungus recovered from the inhibition caused by 20 µg/ml maackiain, but not from that caused by 50 µg/ml, resumption of respiration and growth may be dependent on phytoalexin concentration. At a concentration of 100 µg/ml maackiain, there was a loss in mycelial dry weight of B. cinerea by 24 h. However some degradation was occurring during this time. By 48 h when the maackiain concentration had reduced by about 70%, growth of the mycelium had resumed and the dry weight was equivalent to that of the original weight. This indicated that a fungitoxic effect was apparent at this concentration, which resulted in disruption of plasma membrane (Van Etten and Bateman, 1971) with a consequent leakage of cellular components from the mycelium and a decrease in dry weight. As suggested by Bailey and Skipp (1978) probably not all the mycelial cells were killed by the phytoalexin. The survival of these cells may be due to their protection

by the mass of inoculum or to actual differences in behaviour of some cells. For the surviving cells, apparently normal growth can then take place. Whether such survival occurs in infected tissue is not clear.

One other factor which to date has not been studied to any great extent in any system is the capacity to suppress phytoalexin biosynthesis. Suppression of phytoalexin synthesis may prevent the plant from stopping the growth of the pathogens. For example, a pathogen might secrete a toxin(s) which may kill the plant cells before they are capable of synthesizing phytoalexins. Pathogens are well known for their ability to produce phytotoxins (Wood et al., 1972). Also possible, is the inhibition of a metabolic step(s) in the biosynthesis of phytoalexins, by phytotoxins or other compounds. Phaseotoxin from Pseudomonas phaseolicola suppresses phaseollin accumulation in French beans (Gnanamanickan and Patil, 1977). One is however confronted with the alternative view that fungal toxins may induce phytoalexin biosynthesis. This has indeed been demonstrated with ascochytine on pea pod endocarp (Land et al., 1975b). However Varns and Kuć (1972) have shown that susceptibility may be determined by the suppression of a general resistance response including phytoalexin accumulation.

S. trifoliorum was shown to produce a phytotoxin(s) in vitro. This result confirmed work by Held (1955) and Debnam (1975) both of which found no correlation between resistance to S. trifoliorum and resistance to toxin. High toxin treatment produced irreversible wilting and desiccation of leaf tissue. Wilting is regarded as one of the symptoms of clover rot.

In the study reported in this thesis, oxalic acid was identified as a phytotoxic principle produced by S. trifoliorum. This organic acid is also produced by other fungi in vitro and in vivo (Bateman and Beer, 1965; Maxwell and Lumsden, 1970) and has been shown to play a role in pathogenesis. Culture filtrate from S. trifoliorum containing the toxic principle was also found to cause collapse of French bean leaves 24 h after injection into the intercellular spaces (unpublished result).

Studies by Mansfield and Rossall (1978) showed that on broad beans, B. fabae (virulent) causes a more rapid and extensive growth during the early stages of infection than B. cinerea (avirulent). This extensive killing of bean cells suppresses phytoalexin production. If this rapid killing of cells also occurs in S. trifoliorum-inoculated clover leaves, a similar suppression of phytoalexin synthesis could be envisaged. Another interesting result (Oku et al., 1978) was the demonstration that a peptide suppressor from germination fluid of Mycosphaerella pinodes could suppress pisatin induction in pea leaves. Thus phytoalexin suppression of phytoalexin synthesis could occur in some interactions.

Another important aspect of the work reported here concerns the production of a cell-free extract which could degrade maackiain. Once the enzymes involved in phytoalexin metabolism are purified and characterised progress in this area will be greatly enhanced. In this research it was found that unlike Leptosphaerulina briosiana which apparently degraded medicarpin by a constitutive enzyme system (Higgins, 1972), S. trifoliorum and B. cinerea metabolised maackiain by an inducible system. Similar inducible degradation systems in F. solani f. sp. phaseoli have been reported for the conversion of phaseollin to 1a-hydroxyphaseollone (Heuvel et al., 1974) in S. botryosum for the conversion of maackiain to dihydroxymaackiain (Higgins, 1975) and in B. cinerea for the conversion of medicarpin to 6a-hydroxymedicarpin and the conversion of capsidiol to capsenone (Stoessl et al., 1973).

No extracellular enzyme(s) capable of degrading maackiain were detected in S. trifoliorum cultures. Similar results have been obtained for some fungi e.g. Fusarium solani f. sp. phaseoli (Heuvel and Van Etten, 1973). Since the phytoalexin degrading enzymes did not appear to be extracellular i.e. in the culture medium, they must be intracellular either membrane bound or in the cytoplasm. A crude cell free preparation from S.trifoliorum mycelium degraded maackiain as time progressed. For degradation activity

an induction process with maackiain prior to mycelial disruption was necessary as with any inducible enzyme systems. Whether other pterocarpan phytoalexins could also induce the maackiain degrading system would be of interest in view of the results of Higgins (1975) which demonstrated that different pterocarpan phytoalexins could induce the maackiain degradation system of S. botryosum and vice versa. These results suggest that the same induced enzyme systems are involved in the degradation of these phytoalexins to their respective isoflavans.

In the isolation of the cell free preparation from S. trifoliorum, inhibition of proteolysis of the degradation enzymes was necessary. Inhibition of oxidation of the degradation enzymes and chelation of heavy metals were also regarded important and were achieved with mercaptoethanol and EDTA respectively. The cell-free extract from S. trifoliorum was further investigated. It was found that the addition of mercuric chloride, boiling or lyophilization of the extract could reduce or destroy degradation activity. Thus the enzyme protein may have been denatured. Mercuric chloride is known to bind to sulphydryl groups in the active sites of enzyme molecules thereby reducing activity.

Experiments with S. trifoliorum mycelium indicated that cycloheximide inhibited the conversion of maackiain if added with maackiain (inducer) or if added to non-induced mycelium. Cycloheximide did not prevent the degradation if added after induction. This indicated that cycloheximide had its effect on inhibition of protein synthesis and not on the inhibition of enzyme activity. Amino acid incorporation studies would be needed to establish the extent of the inhibition of protein synthesis by cycloheximide. Studies with Stemphylium loti showed that only 8.3% of the amount of leucine incorporated into proteins of controls (no cycloheximide) were present in proteins of cycloheximide (10 µg/ml) treated spores.

The results reported here favour an inducible phytoalexin-metabolising enzyme rather than an induced carrier protein transport system with a constitutive phytoalexin metabolising enzyme. If this is the case, it is

implied that in lesions pathogen and phytoalexin come into direct contact with each other. This is further supported by the fact that the degradation system was inducible, by the absence of extracellular enzymes, and the accumulation in lesions of the same degradation products as those obtained from in vitro experiments.

It must however be emphasized that there is need for further work in this area. The enzyme(s) must be purified and characterised. Once this is achieved progress in the field of phytoalexin metabolism will be greatly enhanced.

It may thus be concluded that maackiain and medicarpin play a role in resistance of clover during the formation of flecking lesions and limited lesions. Other phytoalexins have been implicated in some non-host resistance mechanisms e.g. Glyceollin in the resistance of soybean to various Phytophthora species that are normally non-pathogenic to soybean (Yoshikawa et al., 1978b). Results here and elsewhere suggest that degradation may be a prerequisite for growth of mycelium. Once a high level of phytoalexin was maintained growth was prevented. Thus a capacity for more rapid degradation of clover phytoalexins by S. trifoliorum seems to be an important component in the pathogenicity of this fungus.

Research on the aggressiveness of phytoalexin-tolerant mutants of the non-pathogens should provide a test of the hypothesis that phytoalexins are responsible for the final expression of resistance. Also if the capacity to detoxify the phytoalexins is essential for pathogenicity, then mutants of the pathogen lacking this capacity should be incapable of spreading from the infection sites.

Apart from their role in disease resistance, phytoalexin studies have an inherent potential in other areas of biology, e.g. the regulatory mechanisms in phytoalexin biosynthesis by using biotic and abiotic 'elicitors'; mode of antimicrobial action in studying microbial physiology and the use of

phytoalexins and synthetic analogues in agriculture and medicine.

A very useful approach would be an investigation of naturally occurring 'elicitors' which on application will trigger the plant's natural defence mechanisms in particular phytoalexin production, instead of applying toxic compounds artificially. This may then render the plants less liable to infection by potential pathogens.

Such studies will inter alia help to clarify the important question on the role of phytoalexins in disease resistance.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr. I.M. Smith for supervising this research and for valuable suggestions during the preparation of the manuscript. Thanks also go to Professor R.K.S. Wood for various help rendered during my stay at Imperial College. Further thanks to the following : Dr. Dickerson of the Biochemistry Department for useful suggestions, June Cheston for typing the manuscript with much cheerfulness and to colleagues in the Department for their friendliness.

Finally, I am indebted to my parents, Mr. & Mrs. A.H.B. Macfoy for their moral support and generosity during difficult times.