DISEASE RESISTANCE IN RED CLOVER LEAVES

By

Julian Robert Debnam

B.Sc., A.R.C.S.

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Department of Botany and Plant Technology,
Imperial College of Science and Technology,
London SW7 2BB.

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ABSTRACT

When studying the role of antifungal compounds using *Cladosporium* TLC bioassays, antifungal compounds were found in healthy tissue, but more were induced upon infection by a pathogen, *Sclerotinia trifoliorum*, and a non-pathogen, *Botrytis cinerea*. Some of the antifungal compounds identified were those already reported in red clover; the preformed isoflavones biochanin A and formononetin, and the phytoalexins medicarpin and maackiain. Newly identified antifungal compounds were genistein, an isoflavone that increased upon infection, pisatin and homopisatin, both phytoalexins.

The isoflavones were not very antifungal and did not therefore appear important in disease resistance. The pterocarpans were more antifungal to the non-pathogen, but only medicarpin was active against *S. trifoliorum*, maackiain having no effect.

Low concentrations of the main phytoalexins, medicarpin and maackiain, in *S. trifoliorum*-infected tissue was probably due to degradation. *In vitro* experiments showed that *S. trifoliorum* converted medicarpin to 6a-hydroxymedicarpin, and maackiain to 6a-hydroxy maackiain, which was also found in *S. trifoliorum*-infected tissue.

B. cinerea in *in vitro* experiments also degraded maackiain, firstly to probably 6a-hydroxy maackiain, then to another compound. 6a-hydroxy maackiain was not detected in *B. cinerea*-inoculated tissue.

Differences in resistance to *S. trifoliorum* of different varieties of red clover was not apparently due to the antifungal compounds studied. The only compound inhibitory to *S. trifoliorum*, medicarpin, was in concentrations too low in *S. trifoliorum*-infected tissue to cause any inhibition.

Reactions of varieties to a toxin of *S. trifoliorum* produced in liquid medium was not the same as resistance to disease, the tetraploid resistant variety being most affected. Frosting of the varieties did show a trend

towards the susceptible varieties being more damaged, but this was not conclusive.

Inoculations onto detached leaves with S. trifoliorum caused equal rotting in all varieties. Varietal differences were obtained when attached leaves with artificially limited lesions were detached and put into 100% relative humidity.

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INTRODUCTION

Upon fungal infection many legumes have been shown to produce antifungal compounds (phytoalexins), the most documented of which are the pterocarpans phaseollin from French bean, and pisatin from pea. Smith (1971a, 1971b) found, whilst carrying out a survey on antifungal compounds in pods of several tropical legumes, that a number of different responses took place, and that in at least two species (chickpea and groundnut) antifungal compounds were present in uninfected plants. A similar situation had been reported in red clover, where three antifungal compounds were present in uninfected plants and two phytoalexins were induced upon infection. The preformed compounds were identified as two isoflavones, biochanin A (Bredenberg, 1961) and formononetin (Virtanen and Hietala, 1958), which were both found in leaves, and a pterocarpan glycoside, trifolirhizin (Bredenberg and Hietala, 1961a, 1961b) which was found in roots. The two phytoalexins were also pterocarpans and found to be medicarpin and maackiain (Higgins, 1971; Higgins and Smith, 1972), the latter being the aglycone of trifolirhizin.

Large quantities of the two isoflavones also occur in lucerne and chickpea and in both they occur as glycosides which upon infection appear to be hydrolysed to the aglycones. Aglycones are generally more antifungal than glycosides. However, in both of these plants, antifungal activity had not been reported for the isoflavones, but apparently depended on pterocarpans, the phytoalexin medicarpin in lucerne, and two preformed compounds, medicarpin and maackiain, in chickpea.

Red clover, like chickpea and lucerne, may include preformed compounds, preformed compounds that become more inhibitory upon hydrolysis, and phytoalexins. The aim of the work was to study these different types of antifungal compounds and attempt to assess the significance of them in resistance to a non pathogen, and to see how a pathogen overcomes them. The pathogen chosen was *Sclerotinia trifoliorum*, as this causes the most serious disease of red clover in the United Kingdom. The non pathogen used was *Botrytis cinerea*. Four different varieties of red clover, varying in their

susceptibility to S. trifoliorum were also studied to see if antifungal compounds could explain their differences.

After further investigation into the literature concerned with the disease caused by S. trifoliorum, it was found that the infective process was very complicated, and so some studies were carried out on this aspect. It was hoped that a better knowledge of conditions that favoured lesion spread would give a better understanding of the mechanisms of resistance to the disease.

LITERATURE REVIEW

Clover has been used in British agriculture for the last 300 years because it enhances the growth of plants around it and those sown after it. This is due to a relationship with a bacterium, *Rhizobium*, found in nodules on the roots, which can fix atmospheric nitrogen making it available for plant growth. Recently, clover has been found to be more digestible than grasses to sheep and cattle, and to contain high protein levels. Therefore, clover has become an integral part of pasture mixtures and rotation farming.

There are two species of clover commonly used, white (*Trifolium repens*) and red (*Trifolium pratense*). The disadvantage of red clover is its susceptibility to two serious diseases, stem eelworm (*Ditylenchus dipsaci*) and clover rot (*Sclerotinia trifoliorum*).

Clover Rot caused by Sclerotinia trifoliorum Eriksson

In 1898, clover rot caused by *S. trifoliorum* was reported by Carruthers to be highly destructive to clover crops in many parts of Britain. In America in 1890, Chester first discovered the disease in Delaware, and Gilbert and Myer (1915) reported heavy damage to clover crops in Kentucky. *S. trifoliorum* is now known to be widely distributed in most countries where clover or related forage crops are grown i.e. nearly every country of Europe, including Russia and the North American continent (Loveless, 1951b). Surveys of clover rot in England and Wales by Lester and Large (1958) showed little in Wales but in England it was present in 70% of fields in 1953 - 54 and 50% in 1955, which was below normal. In 1953 - 54, the loss of stand due to clover rot at the end of the winter was between 6 and 24% in about 10% of fields, and over 24% in a further 10% of fields. In 1955 losses were half as much. The disease was most frequent and severe where four or five course rotation was practised and one year leys normal. Clover rot has consistently been the most serious disease of herbage and fodder crops in recent years.

The Pathogen

Sclerotinia species are members of the Helotiales of the ascomycetes, characterised by producing apothecia from sclerotia i.e. complex masses of vegetative hyphae forming a hard structure often covered in a dark coloured rind or cortex. The status of S. trifoliorum as a separate species has been challenged by Purdy (1955), who grouped several Sclerotinia species together with S. sclerotiorum, regarding the differences between them as natural variation. By using an agglomerative classification based on morphological, pathogenicity and enzyme characteristics of 114 Sclerotinia isolates, Morrall, Duczek and Shear (1972) found continuous variation, thus supporting Purdy. But S. trifoliorum produces apothecia in autumn whereas S. sclerotiorum produces apothecia in spring (Henson and Valleau, 1940; Williams and Western, 1965a). Although Willets and Wong (1971) found the ontogeny of sclerotia of both S. trifoliorum and S. sclerotiorum to be the same, Wong and Willets (1973) showed, by comparing soluble proteins, that isolates could be separated into the two different species. Therefore, the taxonomic position is very confused.

The survival of sclerotia of S. trifoliorum in the field, and the production of apothecia from sclerotia both in the field and in the laboratory, have received much attention. Williams and Western (1965b) regard the two main factors which influence the persistence of sclerotia as the development of apothecia and soil moisture. Sclerotia placed in soil in March slowly diminished in numbers over the summer months, and in late autumn there was a rapid decrease in those near the surface due to apothecial development. However, if buried under clover, apothecial development was reduced and more sclerotia survived. Over winter, sclerotia one and two inches deep increased in numbers by the formation of secondary sclerotia. With increasing soil moisture there was greater degeneration of sclerotia but at levels above 30% of moisture holding capacity this was offset by the formation of secondary sclerotia. Halkilahti (1962) showed sclerotia were most readily destroyed in wet and warm summers due to

infection by parasitic fungi and bacteria. In temperate regions, Williams and Western (1965b) thought sclerotial survival doubtful after three years, and Halkilahti found very few after 4½ years. Pape (1937) reported some survival after 7½ years.

Williams and Western (1965a) found apothecial formation most abundant in August, steadily declining until ceasing in November. Broad red clover caused an initial flush but very few apothecia formed subsequently. Apothecia developed from sclerotia down to 2 inches in the soil, the number decreasing with depth. The production of apothecia in the laboratory has proved almost impossible, although Henson and Valleau (1940) described a method based on planting sclerotia in water agar in tightly plugged vials. Sproston and Pease (1957) tried different thermoperiods, and Williams and Western (1965a) a wide range of pretreatments and thermoperiods, without consistent results. Therefore, a complex stimulus is needed to initiate apothecial development and the use of ascospores for experiments throughout the year is impossible as they are only available in autumn.

S. trifoliorum will attack not only broad and late flowering red clover (T. pratense) but also, though to a lesser extent, white clover (T. repens), alsike clover (T. hybridum), lucerne (Medicago sativa), sainfoin (Onobrychis sativa), vetch (Vicia sativa), birds foot trefoil (Lotus corniculatus), pea (Pisum sativum), Plantago and other species (Loveless, 1951b; Barr and Callen, 1963; Frandsen, 1946; Pape, 1937). A separate variety, S. trifoliorum var fabae Keay, has been reported on field beans (Vicia faba) by Keay (1939) and Loveless (1951a), which differs slightly in the dimensions of asci and ascospores.

Frandsen (1946) and Björling (1951) found races showing different aggressiveness to clovers. Held (1955) studied the differences between two strains, one a degenerate strain derived from the other, the degenerate strain being non-virulent. He found the only notable difference between the two strains was the production of a wilt inducing toxin by the virulent strain.

The Disease Cycle

The disease first appears in the autumn soon after apothecial formation, when the leaves become peppered with small brown spots, called by Dijkstra (1966) ascospore lesions. These were overlooked by early workers, most (e.g. Wadham, 1925) believing infection was by mycelium arising from sclerotia. In Britain (Loveless 1951b; Williams and Western 1965b) and Holland (Dijkstra, 1964, 1966) there is no evidence for this, but Frandsen (1946) in Scandinavia considers mycelium from sclerotia an important source of infection. Ascospore lesions are caused by a single windblown ascospore, but normally this lesion does not spread until January to March, when spots spread and coalesce and outer leaves of infected plants turn brown, wilt and become covered in mycelium (Dillon, Weston, Loveless and Taylor, 1946). This spreads through the petioles to the crown. Spread from plant to plant takes place mainly along infected leaf stalks (Justham and Ogilvie, 1950) and, if mild damp weather persists, progressively more plants become diseased to form patches of infected clover which rot away leaving bare areas. If the weather is dry and frosty, disease progress is checked, and even if much of the plant is infected, the crown may escape and resume vigorous growth in the spring to yield a moderate crop. Sclerotia form on dead tissue in the stem bases and around the crown in the spring and eventually fall to the soil where they remain dormant over summer.

Normally, therefore, S. trifoliorum overwinters as latent ascospore lesions, and oversummers as sclerotia.

Factors affecting spread from ascospore lesions

The ascospore lesions do little damage but the spreading rot can cause serious losses, therefore factors that affect the spread from ascospore lesions are important. Valleau, Fergus and Henson (1933) considered that ascospore lesions remained limited as long as the leaves were alive but with death of leaves due to natural causes, then lesions spread.

However, Loveless (1951b) found lesions spread on living tissue if plants were kept at 100% relative humidity at a temperature range of 5 - 20°C. In plants kept outside, lesions spread after night frosts, killing the leaves. Because of the need for a high humidity, Loveless compared estimates of clover rot severity based on the December - January rainfall with observed estimates in East Anglia for the 1930 - 47 period, and found a significant regression between disease severity and rainfall. Dijkstra (1964, 1966) found artificial frosting caused spread from ascospore lesions and that known frost resistant varieties appeared more resistant to the disease. He suggested that frost resistance in a healthy leaf may provide a standard for clover rot resistance in leaves. Bruising of leaves with ascospore lesions caused no spreading rot and therefore, biochemically, the leaf may be more suited to fungus development after frosting than after bruising.

Therefore, a constant high humidity and frosting appear important for spread of ascospore lesions.

Histopathology

Purdy (1955), who regarded *S. trifoliorum* as a form of *S. sclerotiorum*, found that a clover isolate of *S. sclerotiorum* took 36 hours for infection after the formation of a single appressorium at the tip of the germ tube. In hanging drop preparations, the appressoria developed only after contact with the cover slip, the germ tube branching dichotomously until an appressorial mass was formed, darker in colour than the hyaline hyphae. After 60 hours mycelium was observed in epidermal cells beneath germinating ascospores, there was disorganisation of cellular contents of guard cells prior to penetration when the ascospores were one to two cells away from the stomatal opening.

A histopathological comparison of clover rot on four clover varieties and one variety of lucerne by Prior and Owen (1964) found that penetration differed. *S. trifoliorum* infected Crimson and Kenland Red clovers by direct penetration as a result of mechanical pressure from the

appressorium breaking the epidermal cell, or enzymatic action of the appressorium destroying the cuticle and the epidermis, or occasionally growth of the hyphae through stomata, or any combination. In the host the mycelium grew intercellularly, though sometimes intracellularly within parenchyma and occasionally tracheids and vessels. Thick hyphal strands and fine hyphae ramified throughout infected tissues, mostly longitudinally, the smaller hyphae invading more resistant tissues. Penetration of Louisiana S-1 clover was only through stomata and growth in host tissues of Louisiana S-1 and Cal Ladino clovers was similar to Crimson and Kenland Red except that enzymatic disintegration of tissues was only seen in advanced stages.

A similar picture was seen in bean infected by S. sclerotiorum using infested oat kernels as the inoculum (Lumsden and Dow, 1973).

Physiology

In comparing a normal (virulent) strain of S. trifoliorum with a degenerate (non-virulent) strain derived from it by sub-culturing from several-week-old cultures, Held (1955) found that only the normal strain produced a toxin in liquid medium. This toxin caused wilting of Ladino clover leaves under conditions inducing rapid transpiration and was not destroyed by heating to 100°C for 10 minutes. Varieties showing differences in resistance to S. trifoliorum showed no difference with the toxin, all wilting alike. This suggests that production of the toxin affects pathogenicity of the fungus, but resistance of the host is not due to resistance to the toxin. Pectic enzymes seem less important, as the degenerate strain produced more than the normal strain in synthetic media.

In 1957, Virtanen, Hietala and Wahlroos found a strong antifungal affect against Fusarium nivale and S. trifoliorum in press juice of clover before flowering. The antifungal compound inhibited F. nivale at 0.02% and S. trifoliorum at 0.04% in agar. Later work (Virtanen and Hietala, 1957; Bredenberg, 1961) showed this compound to be Biochanin A, a substance

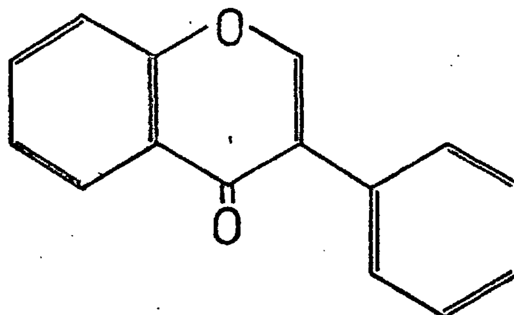
first isolated from Cicer arietinum (chick pea or chana) in 1945 by Siddiqui and its structure elucidated as an isoflavone (Bose and Siddiqui, 1945; Bose, 1954). It was first isolated from red clover by Pope, Elcoate, Simpson and Andrews (1953) and considered quite important because of its oestrogenic properties which have been studied because of their possible effects on sheep and cattle (e.g. Beck, 1964). Another isoflavone, formononetin, was found to be an anti-Sclerotinia factor in red clover by Virtanen and Hietala (1958). This was first found in 1855 by Hlasiwetz in Ononis spinosa, where it occurs as the glycoside ononin, and in Subterranean clover (Trifolium subterranean) by Bradbury and White (1951). The structures of these isoflavones are shown in Figure 1. They are known to occur in healthy cells as glycosides (Francis, Millington and Bailey, 1967; Schultz, 1967), but in diseased tissue, as shown by Olah and Sherwood (1973) in lucerne infected by Ascochyta imperfecta, glycosidase activity probably converts the glycosides to aglycones.

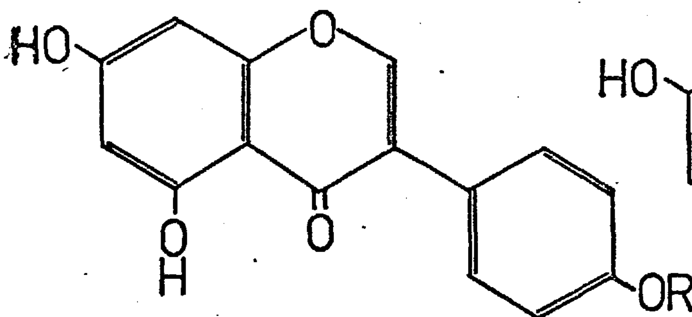
Another antifungal glycoside, trifolirhizin, so called by Bredenberg and Hietala (1961a, 1961b) because it was found in clover roots, was also an isoflavanoid but of a type known as a pterocarpan (Harper, Kemp and Underwood, 1965). It was not found in healthy shoots, and hydrolysed to the aglycone when root homogenates were left for 2 to 3 days in water.

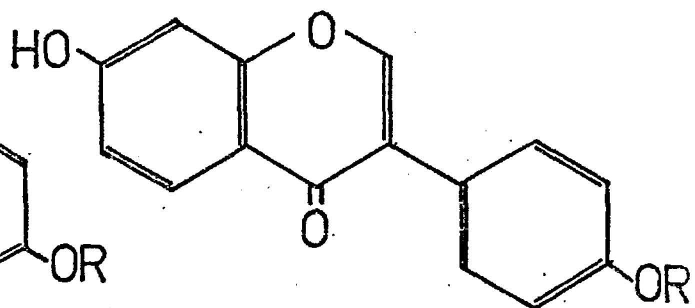
Cruickshank (1965) first reported a phytoalexin in red clover leaves and suggested it might be the aglycone of trifolirhizin, 3-hydroxy-8, 9 methylenedioxypterocarpan, also called maackiain (Suginone, 1962), demethylpterocarpin and inermin (Cocker, Dahl, Carmel Dempsey and Murry, 1962). This was confirmed by Higgins (1971) and Higgins and Smith (1972) who showed another pterocarpan, medicarpin ((-)-3-hydroxy-9-methoxypterocarpan or demethylhomopterocarpin) was also present. This compound was first identified as a phytoalexin from lucerne (Smith, McInnes, Higgins and Millar, 1971). The structures of these compounds are shown in Figure 2. Helminthosporium turcicum Pass was the phytoalexin inducer. No work has been reported on the importance of these phytoalexins in Clover Rot.

ISOFLAVONES.

Skeletal Structure.







Genistein, R=H

Biochanin A, R=CH_3

Daidzein, R=H

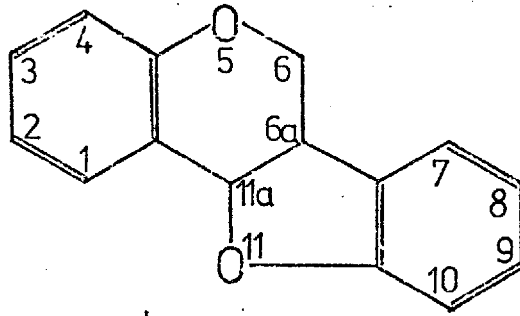
Formononetin, R=CH_3

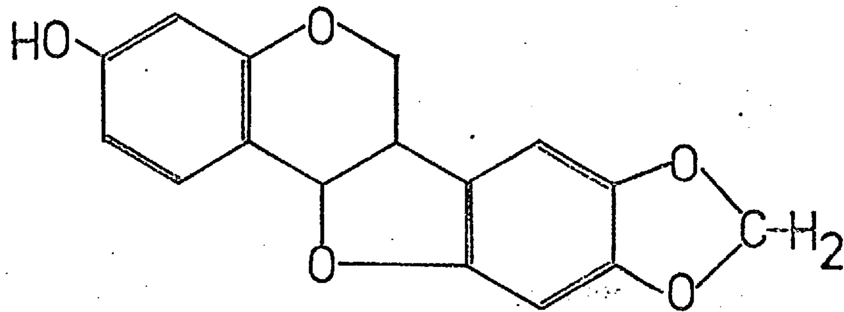
Figure 1

PTEROCARPANS.

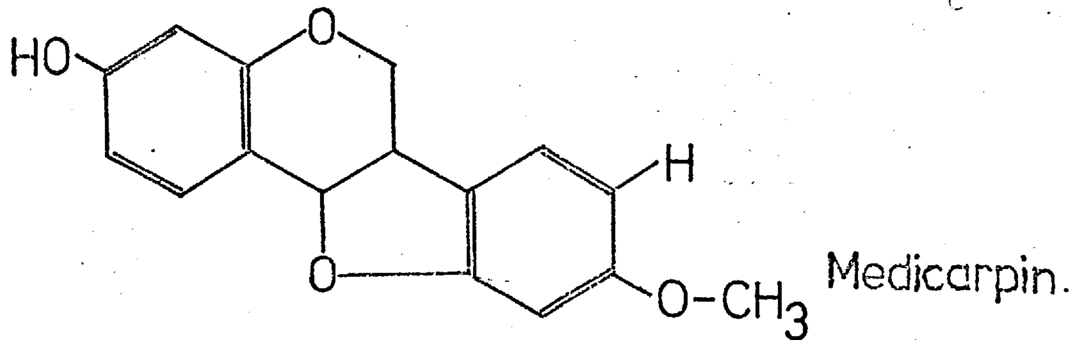
Figure 2

Skeletal Structure.

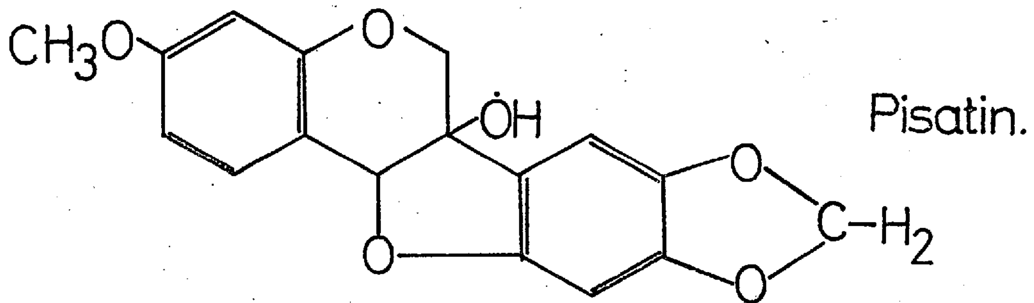




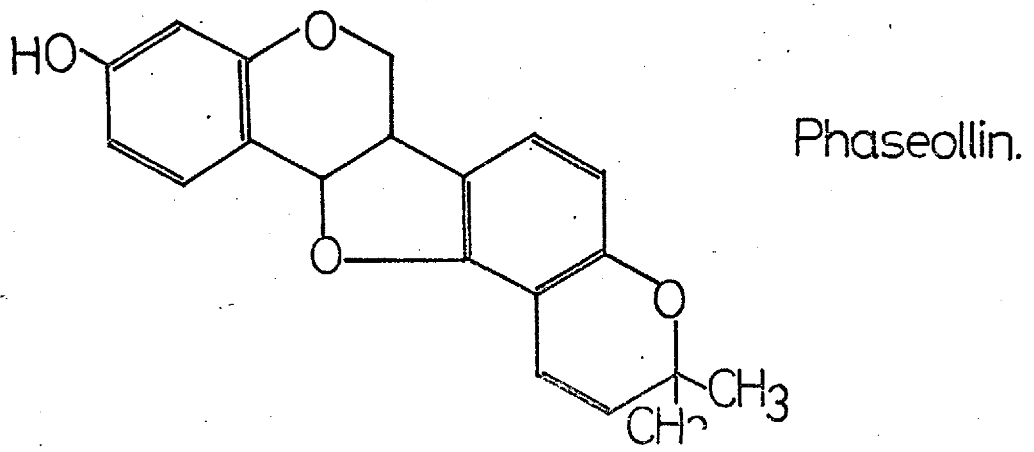
Maackiain.



Medicarpin.



Pisatin.



Phaseollin.

Methods of Controlling Clover Rot

1. Cultural Methods

Red clover is normally included in rotation farming where an autumn sown legume is grown at least once in four years but Lester and Large (1958) found clover rot most serious in 4 or 5 course rotation. Sclerotia can survive for 4 or more years (Halkilahti, 1962; Pape, 1937) and a small population of sclerotia can cause an attack of clover rot. If an 8 year rotation is used, control of clover rot should be achieved. However, ascospores can be windblown from other infected fields and so a long rotation is only effective if no contaminated clover is in the vicinity. If a severe attack has occurred, burying sclerotia to below two inches by deep ploughing of the old ley should reduce apothecial numbers (Loveless, 1951b; Williams and Western, 1965a).

To reduce infection by ascospores, the surface area of the crop can be reduced by grazing or mowing in autumn, at the time of ascospore discharge. However, Loveless (1951b) showed this may have a more deleterious effect than clover rot to the final yield.

2. Use of fungicides

Wadham (1925) used copper and sulphur compounds without conclusive results. Gram (1945) reduced loss due to clover rot by using chloronitrobenzenes, this being confirmed by Houston, Oswald and Peterson (1954), Ylimäki (1955), Zogg (1958) and others. The best results obtained with Brassicol (20% pentachloronitrobenzene plus 80% filler) were using 30 kg/1000 l/ha (Zogg, 1958) or 35 kg/ha (Malmus, 1957). Kupricol suppressed development of S. trifoliorum (Zuara, 1967) and Benlate at 4.5 kg/ha gave good control of clover rot (Glaeser, 1971). At present, however, the cost of using fungicides is excessive compared with the value of the crop and fungicides are therefore little used.

3. Biological Control

Organisms that attack Sclerotia have been studied. For example, Zub (1962) found Coniothyrium minitans could give biological control, but this method has not been used.

4. Resistant Varieties

These are probably the best way to combat clover rot at present. Different resistances of clovers to S. trifoliorum were first noted in 1911 by Hiltner in Bavaria and Zimmerman in Mecklenburg (reported by Baudys, 1923). Early work suggested that clovers adapted to the regions in which they were grown were more resistant (Valleau, Fergus and Henson, 1933). By breeding, more resistant varieties have been obtained, but if grown in a different area this resistance may be lost. Bingefors (1957) found that clover was resistant in Southern Sweden but susceptible in central Sweden. A breakthrough was achieved with the production of induced autotetraploids which Vestad (1960) showed to be generally more resistant to clover rot. In Britain, the Plant Breeding Institute at Cambridge breeds red clover, and their most resistant variety, Maris Leda, is a tetraploid, although work on new diploids e.g. from the Atlas Mountains (Bond and Toynbee-Clarke, 1967) is still continuing. If the mechanisms of resistance to clover rot were known, breeding for resistance may become easier.

Disease Resistance

A plant is resistant to most potential pathogens and susceptible to only a small number. Different varieties of a single species can differ in their resistance or susceptibility to pathogens. The factors that confer resistance are of great interest, because knowledge of them may help in breeding new varieties with greater resistance, thus cutting loss in yield due to disease. Wood (1972) suggests the main categories of resistance to be prevention of infection, retarded growth due to lack of essential substances, absence of factors that react with products of the pathogen, physical barriers that retard growth of the pathogen, substances that inactivate toxins or enzymes of the pathogen, and substances inhibitory to the parasite. Aspects of the latter will be reviewed.

Substances inhibitory to the pathogen

According to Ingham (1973), and references therein, there are four types of inhibitory substances. Prohibitins are substances present in healthy tissue that are unaltered upon pathogen attack, whereas inhibitins are substances present in healthy tissue that increase to inhibitory levels upon infection. Post-inhibitins are substances present in healthy tissue as glycosides but which, upon infection, are hydrolysed to produce a more inhibitory compound. Glycosides are generally less inhibitory than the aglycone. Phytoalexins are substances produced upon infection and are not present in healthy tissue.

These terms are not widely used, and prohibitins and post-inhibitins are generally known as preformed inhibitors. In practice the differences between prohibitins and inhibitins, and between inhibitins and phytoalexins may not be clear. Because of possible confusion, the terms preformed inhibitor and phytoalexin will be used, with further clarification of the terms, if needed, for particular examples.

Of these types, phytoalexins have received the most attention. The concept of phytoalexins was proposed by Müller and Börger (1941) after

their work on the cut surface of a potato tuber inoculated with different races of Phytophthora infestans. Incompatible races apparently induced the production of an inhibitory compound as cross protection resulted. Müller (1956) defined phytoalexins as "antibiotics which are the result of an interaction of two different metabolic systems, the host and the parasite, and which inhibit the growth of microorganisms pathogenic to plants". However, heavy metal ions and antimetabolites will also induce 'phytoalexins' (Perrin and Cruickshank, 1965; Schwochau and Hadwiger, 1968), and the original definition is, therefore, too rigid. As phytoalexins are apparently only important in disease situations, they can be defined simply as compounds inhibitory to the pathogen induced by infection of the host. Phytoalexins have been found in many plants. For further reference see recent reviews e.g. Cruickshank, Biggs and Perrin, 1971; Kuć, 1972; Ingham, 1972; Deverall, 1972.

The majority of work on antifungal compounds have either been carried out on artificial systems using non-pathogens with large amounts of inoculum or with pathogens that cause 'nectrotrophic' diseases. These result in large amounts of infected tissue that can be extracted, so that detection of antifungal compounds is made easier. With obligate parasites, where pathogens cause as little damage as possible to host cells, or where a hypersensitive reaction often results in the death of a single cell, then antifungal compounds may not be present, or present only in trace amounts so that detection is very difficult. Therefore, this review does not really apply to obligate parasites.

Inhibitors in the Leguminosae

Smith (1971a, 1971b) in a survey of various legumes found three types of response to inoculation with a non pathogen, the presence of no inhibitors, the accumulation of many phenolic compounds and one or more phytoalexins associated with necrosis, and very little necrosis with poor germination of the fungus due to preformed inhibitors. Of these, the

first type is unexplainable. The presence of preformed inhibitors is known only in a few species (chickpea and groundnut) but maackiain, biochanin A and formonononetin are present as glycosides in red clover, maackiain as the glycoside, trifolirhizin, (Bredenberg and Hietala, 1961a, 1961b; Schultz, 1967) and release of antifungal phenolic compounds by hydrolysis is known in lima bean (Bell, 1970). The possible conversion of wyerone to wyerone acid may occur in broad bean (Letcher, Widdowson, Deverall and Mansfield, 1970). Therefore, simple conversion of substances present in healthy tissue may be important in several species. The majority of legumes studied, however, accumulate phytoalexins.

Of the identified inhibitors in legumes only wyerone acid is not isoflavanoid, but a furanoacetylene. Although isoflavones, isoflavans and an isoflavanone (see figures 1 and 3) are known as inhibitors, the characteristic legume inhibitor is pterocarpanoid (figure 2), the majority as phytoalexins. Pterocarpans are widely distributed in the Leguminosae and are mainly autonomous in trees (most primitive) especially in heartwood, although induction may take place in leaves and pods, but formation is generally induced in herbs (Hijwegen, 1973). Perrin and Cruickshank (1968) tested the antifungal activity of a number of pterocarpans and related compounds to *Monilinia fructicola* and came to the conclusion that antifungal activity was due to the aromatic rings not lying in the same plane, and the substitution of small oxygen-containing radicals at certain positions on the periphery of the molecule.

Pterocarpan Biosynthesis

Little work has been done on the biosynthesis of autonomous pterocarpans but Hijwegen (1973) found that trifolirhizin in red clover roots was synthesized at the expense of the isoflavones.

In induced pterocarpan biosynthesis there appears to be two types of induction. The first suggests that pterocarpan and isoflavanoid synthesis is not specifically induced but is part of a general change in phenolic

metabolism. This has been shown in lucerne infected with Pseudopeziza medicaginis (Bickoff, Loper, Hanson, Graham, Witt and Spencer, 1967) and Ascochyta imperfecta (Olah and Sherwood, 1971), in French bean pods inoculated with Colletotrichum lindemuthianum (Smith, 1970) and with etiolated seedlings of soybean, snap bean and lima bean inoculated with Helminthosporium carbonum race 1 (Biehn, Kuć and Williams, 1968). The second type is more specific where only isoflavanoid synthesis is accelerated, especially to the production of the pterocarpan, and other phenols remain at concentrations little changed from healthy tissue. This has been found in French bean hypocotyls where phytoalexin was induced by model systems using culture filtrates of Penicillium expansum and mercuric chloride solution (Rathmell and Bendall, 1971) or by a compatible race of Colletotrichum lindemuthianum (Rathmell, 1973) and in disease-resistant soybean hypocotyls inoculated with Phytophthora megasperma var. sojae (Keen, Zaki and Sims, 1972).

Perhaps the amount of necrosis obtained is important. With severe necrosis then phenolic metabolism may be drastically altered. Paxton, Goodchild and Cruickshank (1974) showed phaseollin was formed in bean endocarp upon inoculation with Monilinia fructicola, aqueous solutions of monilicolin A and aqueous solutions of mercuric chloride, in cells that were apparently healthy. An increase in general phenolic metabolism was not noted, but phytoalexin only may have been induced. However, under natural conditions, necrosis may be the normal situation and a general change in phenolic metabolism may occur.

Several pterocarpans are known to be readily biosynthesised from phenylalanine -^{14}C e.g. pisatin (Hadwiger, 1966), phaseollin (Hess, Hadwiger and Schwochau, 1971) and hydroxyphaseollin (Keen et al., 1972), and phenylalanine ammonia lyase is known to increase in early stages of infection before cells become necrotic (Rathmell, 1973). An increase in protein and RNA synthesis has also been noted (Schwochau and Hadwiger, 1968) and Schwochau and Hadwiger (1969) showed pisatin induction in pods of

Pisum sativum was dependent on newly synthesized protein and RNA, and suggest that pisatin production is due to activation of genes.

Keen et al. (1972) proposed a pathway for hydroxyphaseollin in soybean hypocotyls, partly based on work by Dewick, Barz and Grisebach (1970) on coumestrol, and Hijwegen (1973) extended this to a possible general pathway of biosynthesis for pterocarpanoids (see figure 3).

Degradation of pterocarpan phytoalexins by fungi

Despite phytoalexins, some fungi still cause disease. One reason for this is that some pathogens do not induce phytoalexins e.g. Septoria pisi on pea accumulated only 10 μg/ml of pisatin in infected tissue, whereas other fungi accumulated up to 116 μg/ml (Cruickshank and Perrin, 1963). Another reason is that some pathogens have been shown capable of breaking down or converting phytoalexins of the host. Uehara (1964) first showed this with pea pathogens detoxifying pisatin in vitro, and DeWit Elshove (1968, 1969) confirmed this, whilst also showing pea-pathogenic strains of Fusarium solani and Ascochyta pisi could break down pisatin while strains non-pathogenic to pea could not. The breakdown product had a different ultra-violet spectrum from pisatin and was more soluble in water than petroleum ether, unlike pisatin. Later work (De Wit Elshove, 1971) showed that different fungi degraded pisatin differently. F. solani f. pisi and F. oxysporum f. pisi were incapable of degrading pisatin in high glucose or sucrose concentrations, suggesting catabolite repression. When glucose concentrations were below 2750 μg/ml, degradation took place completely to CO_2. Ascochyta pisi, however, degraded pisatin to a compound with similar spectral properties to pisatin, and sugar concentrations had no effect. This compound has now been identified as 6a-hydroxymaackiain (Van't Land, Wiersma-Van Duin and Fuchs, 1975).

Higgins and Millar (1968, 1969a, 1969b, 1970) showed that the lucerne pathogen, Stemphylium botryosum, degraded medicarpin with the production of a new compound. This, on further incubation with the fungus, decreased

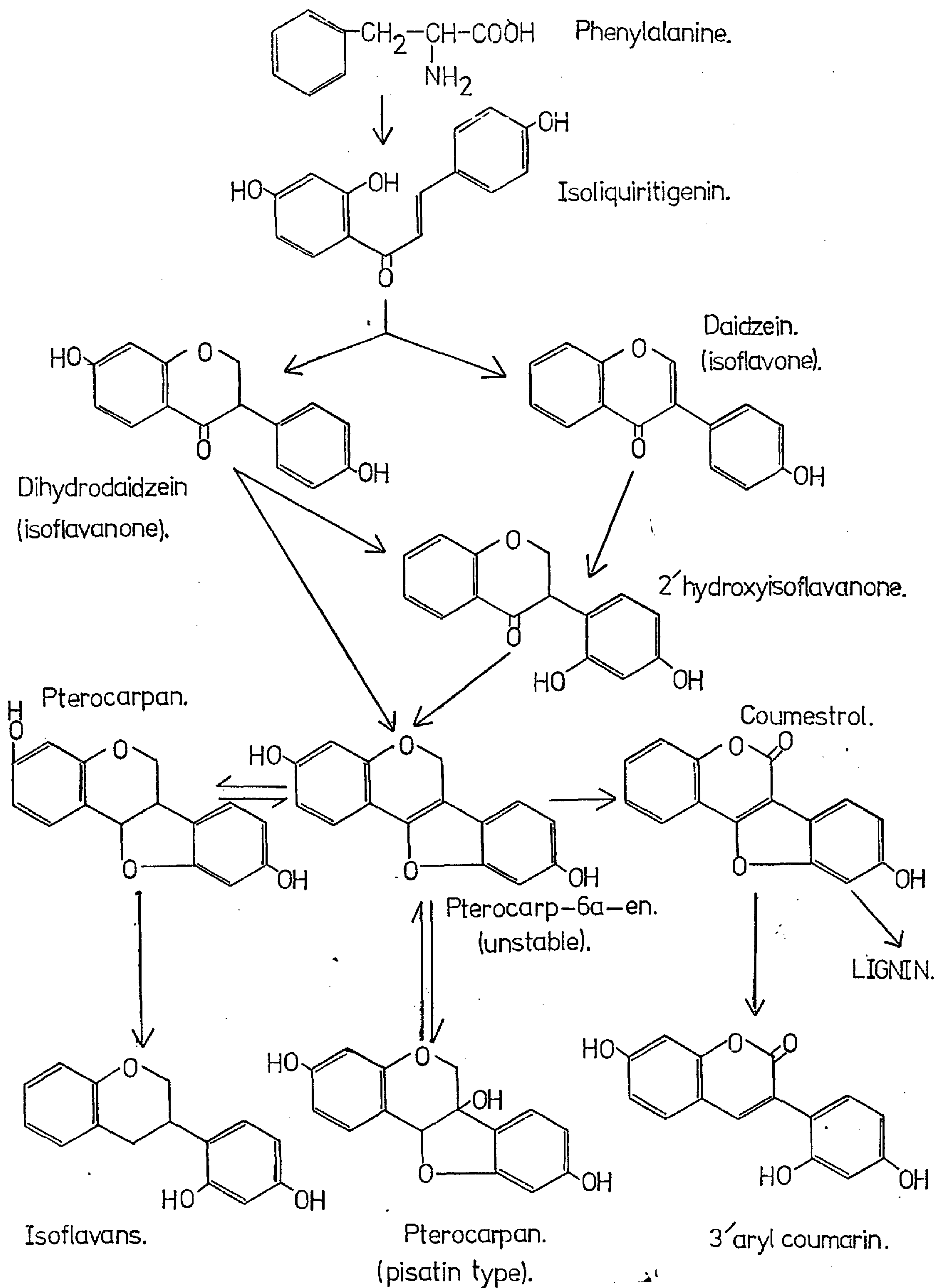


FIGURE 3

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

and a second new compound appeared, which itself slowly disappeared due to degradation or assimilation. The first compound is now known to be vestitol (2-hydroxyisoflavan)(Higgins, Stoessl and Heath, 1974). Stemphylium loti, a weak lucerne pathogen, also degraded medicarpin, but to a compound that was still inhibitory, as did Colletotrichum phomoides, a non-pathogen, but the compounds were different, in the latter case having spectral properties similar to medicarpin. Another non-pathogen of lucerne, Helminthosporium turcicum, was unable to degrade medicarpin at all.

Phaseollin from broad bean is also degraded. Van den Heuvel and Van Etten (1973) found that F. solani f. phaseoli metabolised phaseollin in shake cultures of actively growing mycelium at concentrations non inhibitory to growth. If the fungus was pretreated with low levels of phaseollin, then inhibitory concentrations were also metabolised. Several metabolic products were formed with the disappearance of phaseollin, the most prominent being later identified as la-hydroxyphaseollone (Van den Heuval, Van Etten, Serum, Coffen and Williams, 1974) which was less fungitoxic than phaseollin. Bailey (1974) showed that actively growing Colletotrichum lindemuthianum (race γ) converted phaseollin to a new compound, which itself was converted to a second new compound which disappeared from extracts within 24 hours. Phaseollin and the two new compounds all appeared antifungal to Cladosporium cucumerinum on thin layer chromatography plate bioassays. These two conversion products were later identified by Burden, Bailey and Vincent, 1974. Compound 1 was 6a-hydroxyphaseollin and compound 2 was 6a,7-dihydroxyphaseollin, thus the conversion products were produced by progressive hydroxylation. Washed mycelial fragments of C. lindemuthianum were shown to convert phaseollin and phaseollidin to compounds with almost identical ultra-violet spectra to the phytoalexins (Cruickshank, Biggs, Perrin and Whittle, 1974). Both conversion products were believed to be pterocarpanoid.

Stemphylium botryosum, a lucerne pathogen, has been shown capable of converting phaseollin and pisatin, but at rates far slower than it can

convert medicarpin (Heath and Higgins, 1973). The first conversion product from phaseollin or pisatin was as antifungal as the parent compound, but these were in turn broken down. The first pisatin conversion product had an ultraviolet spectrum very much like that of pisatin. The first phaseollin conversion product had a different spectrum and has been identified as phaseollinisoflavan (Higgins *et al.*, 1974), which is produced as a phytoalexin when French beans are infected with tobacco necrosis virus (Burden, Bailey and Dawson, 1972). *S. botryosum* can readily degrade maackiain from red clover to dihydromaackiain (Duczek and Higgins, 1973; Higgins *et al.*, 1974) but is not pathogenic on red clover. Conversely, *S. sarcinaeforme*, a red clover pathogen, can also degrade both maackiain and medicarpin, but is not pathogenic to lucerne. Clearly, other factors of resistance are operating in these particular cases.

The significance of phytoalexins in disease resistance has recently been challenged by Pueppke and Van Etten (1974) after using three pea pathogens, *Aphanomyces euteiches*, *Fusarium solani* f. sp. *pisi* and *Rhizoctonia solani*, on pea. *A. euteiches* was particularly sensitive to pisatin but eight times the inhibitory levels were found in expanding lesions of this pathogen after 36 hours. Although *R. solani* was not as sensitive to pisatin, after 2 days, concentrations in lesions of *R. solani* were much greater than quantities inhibitory *in vitro*. With *F. solani* f. sp. *pisi*, up to 5 mg/cm^3 of pisatin were found in lesions but this fungus was insensitive to pisatin *in vitro*. Perhaps pisatin is not always in a form that can reach the fungus, and, as suggested by Pueppke and Van Etten (1974), further investivation of the *A. euteiches*-pea combination may explain this contradiction to the phytoalexin theory.

It seems different pathogens can convert phytoalexins in different ways. Non pathogens in some cases can also convert phytoalexins but normally the degradation products are still inhibitory, or degradation is extremely slow. In most cases, where phytoalexins have been implicated, the antifungal affect of the phytoalexin, either directly or indirectly, is enough to explain why a fungus cannot infect a non-host plant.

MATERIALS AND METHODS

1. Biological Material

a. Higher Plants

Seeds of five varieties of Red clover (Trifolium pratense L.) were supplied by the Plant Breeding Institute, Cambridge (P.B.I.). These were :-

Kuhn (2nR)	- a resistant diploid
Red Head (4nR)	- a resistant tetraploid
Dorset Marl (2nS/1)	- a susceptible diploid
Sabtoron (2nS/2)	- a susceptible diploid
4n/RB	- a less resistant tetraploid

These are graded according to results obtained in field trials against clover rot. As no true resistance is known against clover rot, these are really grades of susceptibility, the so called resistant varieties being less susceptible. Tetraploids are less susceptible to clover rot than the diploids from which they were derived, but tetraploids do vary in their resistance, and variety 4n/RB is a more susceptible tetraploid. 4n/RB is not the variety name but a code name given by the P.B.I. Only one susceptible diploid variety was used in any experiment.

Plants were grown initially at the Chelsea Physic Garden, but later at the Botanical Supply Unit of the University of London, Englefield Green, Surrey. Plants were grown in a mixture of John Innes (No. 2) and coarse sand in disposable plastic pots in greenhouses with some additional lighting, until 2 months old. If not used immediately, plants were kept in the Imperial College greenhouse at South Kensington.

Powdery mildew (Erysiphe polygoni DC) was often a problem, but as it was considered unwise to use any fungicide, control was only possible by discarding heavily infected plants or picking off infected leaves. Only leaves with no visible signs of mildew infection were used.

Leaves from field grown plants were collected from trial plots at the P.B.I. The leaves plus 3 - 4 inches of petiole were cut from plants and put into plastic bags with some water and the bags sealed. The leaves were used the same day or kept overnight in the plastic bags at 4°C and used the following day.

Jack beans (Canavalia ensiformis (L.) DC) obtained from Sigma Chemical Company, were planted directly into small pots (3 inch diameter) containing wet vermiculite and standing in trays containing ½ inch of water. Pots were kept in a warm greenhouse compartment (25°C approximate average) with fluorescent tube lighting, and watered every two days. Cotyledons emerged within 5 - 7 days.

b. Fungi

Sclerotinia trifoliorum Eriksson

The isolate of S. trifoliorum was obtained from the P.B.I. as a culture under paraffin, and as sclerotia. It was cultured on Oxoid potato dextrose agar (PDA). Sclerotia were surface sterilised in 10% sodium hypochlorite solution for 5 minutes, washed in sterile distilled water, and cut aseptically with a scalpel into 3 or 4 pieces. Each piece was seeded into the centre of a petri dish of PDA, and incubated at 20 - 25°C. The time varied for mycelium to grow out from the sclerotial segment, but once actively growing, it took about 3 days to reach the edge of the petri dish, and about 8 - 10 days more for small sclerotia to form, usually around the edge of the plate.

3 plugs of agar taken with a No. 3 cork borer (6 mm diameter) from the edge of an actively growing PDA culture were inoculated into 150 mls of sucrose-casamino acids liquid medium per 250 ml Erlenmeyer flask. The medium was prepared as follows :-

4.6 g	Casamino acids (Vitamin free)
1 g	KH_2PO_4
0.5 g	$MgSO_4.7H_2O$
15 g	Sucrose
0.5 g	Glucose
10 ml	Trace element solution
1 l	Distilled water

This will be referred to as SCA. The solution for trace elements was prepared as follows :-

Stock solution	Final concentration in medium (ppm)
0.02g $FeSO_4.7H_2O$	0.2
0.1g $ZnSO_4.7H_2O$	1.0
0.002g $NaMoO_4.2H_2O$	0.02
0.002g $CuSO_4.5H_2O$	0.02
0.002g $MnCl_2.4H_2O$	0.02

Distilled water to one litre

The culture was incubated at 20°C for 10 days, then aseptically homogenised in a 'Sorvall' Omni-Mixer at setting 6 for 20 seconds. This was used as inoculum.

For production of large numbers of sclerotia, bran (obtained from a pet shop) was put into 1 or 2 litre flasks to a depth of $\frac{3}{4}$ inch, and moistened with distilled water, then sterilised. The bran was seeded with 4 to 6 agar plugs from a S. trifoliorum PDA culture, put into a cupboard and left for one month. The bran was then removed from the flasks and dried overnight in the laboratory with the aid of a fan, and sifted through a coarse sieve. Mostly sclerotia remained in the sieve.

In an attempt to produce apothecia, a method after Henson and Valleau (1940) was used. Sclerotia were embedded in 1% water agar slopes in McCartney bottles and incubated at 12 - 14°C in an illuminated incubator

with an 8 hour daylength. However, no apothecia had been formed up to 3 months, and the method was abandoned.

For production of a crude protein extract (including pectinases), S. trifoliorum was inoculated with 2 plugs of PDA culture, into medical flats containing 20 ml of pectin liquid medium. This medium was prepared as SCA but substituting 10 g of pectin for sucrose. The cultures were incubated at 20 - 25°C for 10 days. The fungus was removed from the medium by centrifugation at 3000 rpm. The medium was cooled to 0°C, then added to an equal volume of acetone at -20°C and left for 15 minutes. The white precipitate that formed was removed by centrifugation at 3000 rpm and taken up in the minimum amount of sterile distilled water.

Botrytis cinerea Fr.

This was obtained from Dr. I.M. Smith from the Imperial College culture collection. It was grown on medium X in 250 ml Erlenmeyer flasks containing about 30 - 40 ml of medium. Medium X was prepared as follows :-

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

Flasks were inoculated by swirling around about ½ - 1 ml of spore suspension so that spores were spread all over the medium. Cultures were put into an incubator containing a 'Phillips, Black Light' fluorescent tube (emission spectrum range of 410 - 310 nm, maximum at 360 nm) at 20°C for

8 - 10 days. Within this time, the culture produced a mass of conidia.

Conidial suspensions for inoculation were prepared by putting 10 ml of glucose-water (5%, w/v, glucose) into cultures. The flasks were shaken and the surface gently scraped with the straight end of a sterile spatula. The resulting spore suspension was passed through 4 layers of muslin and the concentration checked using a haemocytometer and adjusted to 5×10^6 by addition of more glucose-water.

Cladosporium sp.

This was obtained from Dr. I.M. Smith who isolated it from a contaminated culture. It was grown on PDA in 250 ml Erlenmeyer flasks at 20 - 25°C. New cultures were inoculated as B. cinerea, and were ready for use within 5 days.

Spore suspensions for spraying on to chromatograms were prepared by putting 10 - 15 ml of SCA medium into culture flasks and shaking the flask and gently scraping the culture surface so as to release spores. The resulting suspension was filtered through 2 layers of muslin.

Most culture work was carried out in a 'Pathfinder' laminar-flow cabinet. Culture media were sterilised by autoclaving at 15 lbs/sq in.

2. Inoculation Methods

a. Fungi

Leaves were cut from Red clover plants, their surface gently rubbed between the thumb and forefinger to disperse the wax bloom, and placed on expanded metal grids so that the petioles were immersed in 75 ppm benziminazole solution in clear plastic boxes. Occasionally leaves were floated on benziminazole solution in petri dishes. The benziminazole solution was prepared by putting 0.075 g of benziminazole in about 50 mls of distilled water and immersing in a water bath at 45 - 50°C until it had dissolved, then making up to 1 litre with distilled water. Benziminazole is a synthetic kinin hormone and slows down the process of senescence.

S. trifoliorum was inoculated as a single 20 µl drop (using an 'Agla' micrometer syringe) of prepared inoculum (see above) onto the centre of each leaflet.

B. cinerea was inoculated as 6 - 8 20 µl drops of conidial suspension (5 x 10^6 concentration), in 5% (w/v) glucose - water, evenly spaced on each leaflet.

The lids were placed on to the plastic boxes (or petri dishes) which were incubated at 20°C in illuminated incubation for 5 - 7 days.

b. Chemicals

Chemicals were vacuum infiltrated into leaves by wholly immersing leaves and weighing them down in a container of a solution of the chemical. The container was placed in a dessicator which was fitted to a 'Speedivac' high vacuum pump via an anhydrous $CaCl_2$ water trap. A vacuum was applied to the leaves for 30 minutes, in which time the leaves became watersoaked. The excess solution was removed from leaves with blotting paper, and the leaves placed on expanded metal grids in clear plastic boxes, with petioles immersed in 75 ppm benziminazole solution. They were left to dry in air for 3 hours, then the lids were placed on the boxes which were incubated at 20°C in illuminated incubators for 2 days.

Where only a small amount of solution was available, inoculation was by using an artists spray gun (2a, Colour spray Ltd.) held 1 cm from the lower leaf surface, thus introducing the solution by pressure infiltration.

3. Chemicals

These were obtained mainly from BDH, Koch Light and Hopkins and Williams, and were of analar grade. Ethanol and Methanol came from James Burrough Ltd., pectin and pyrogallol red from Sigma, Biochanin A from Kodak-Eastman, formononetin and genistein from NIPA Laboratories Ltd., Casamino acids (vitamin free) from Difco, casein hydrolysate and mycological peptone from Oxoid, and agar from Davis.

4. Extraction Methods

a. Isoflavone glycosides

Healthy or infected tissue only was dropped into boiling ethanol, the ethanol cooled to room temperature, the tissue macerated in an Omni-mixer at setting 6 for 20 seconds, and filtered in a Buchner funnel. The filtrate was washed several times in ethanol, the washings being added together, then concentrated at 45°C at reduced pressure with a 'Büchi' rotary evaporator.

b. Aglycones from healthy and infected tissue

See figure 4.

Areas of infected tissue were cut from leaves with scissors or by a cork borer, the latter being made easier by placing the leaves on a pad of paper tissues.

5. Chromatographic techniques

Purification of extracts was by thin-layer chromatography (TLC) on 0.25 mm 'CAMAG' or 'Merck' silica gel thin layers with glass or aluminium backing. Extracts were applied in ethanol, usually as a streak at 1.5 - 2 cm from the lower edge of the plate, using a drawn-out Pasteur pipette, the ethanol being evaporated off quickly using a 'Morphy Richards' hair dryer, blowing a stream of cold air. Plates were developed in Shandon S/P chromatotanks.

For isoflavone glycosides, TLC plates were developed in a solvent of ethyl acetate : methyl-ethyl ketone : formic acid : water (50:30:5:10, v/v/v/v)(Francis, Millington and Bailey, 1967) with filter paper soaked in solvent up the sides of the developing tank to ensure the air was solvent saturated. For isoflavone aglycones and other phenols, plates were developed in methanol : chloroform (2:98, v/v), with no filter paper. Further purification of unknown compounds was by developing in diethyl

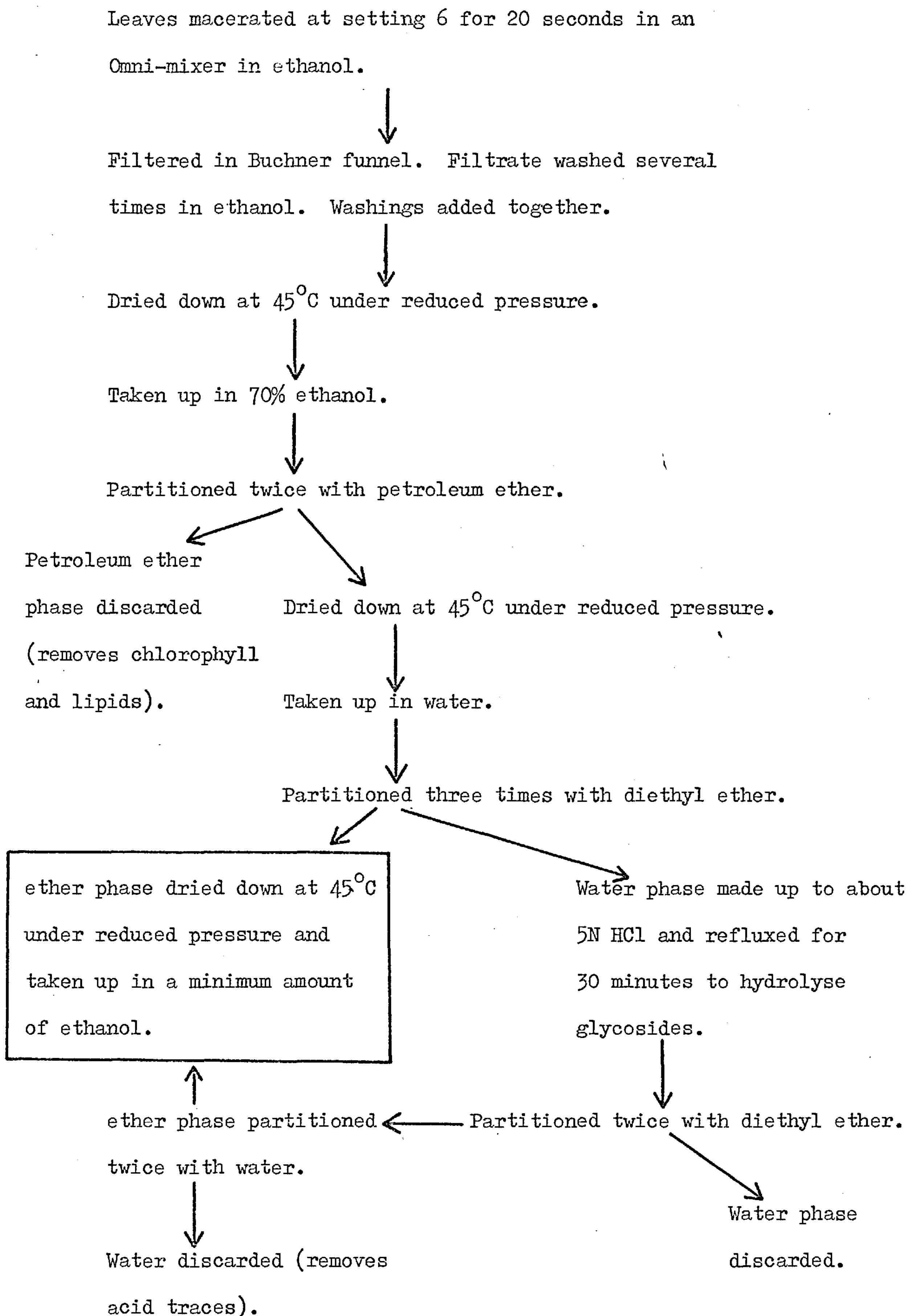


FIGURE 4. Flow chart diagram of extraction method for aglycones from healthy and infected tissue.

ether : petroleum ether (50:50, v/v or 70:30, v/v) or toluene : chloroform : acetone (40:25:35, v/v/v).

Occasionally, 'Merck' polyamide thin layers on aluminium backing were used for comparative TLC using a solvent of water : methanol (15:85, v/v).

Visualisation was usually by viewing TLC plates under ultraviolet (UV) light of 254 or 366 nm. To aid in this, TLC plates incorporating a fluorescence indicator were used. Most phenolic compounds either fluoresce and appear bright blue or purple, or absorb UV light and appear as dark bands.

Some bands did not show under UV light and so the TLC plates were sprayed with various location reagents. These were :-

a. Sulphanilic acid reagent

A. 9g 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 added and it was used immediately. This shows phenols and aromatic amines that will couple.

b. Antimony pentachloride : 2 parts to 8 parts of chloroform. Shows phenols and steroids. If plate is heated to 120°C then colour changes may occur.

c. Antimony trichloride : A saturated solution in chloroform was sprayed. The TLC plate was heated to 100°C for ten minutes. Shows steroids.

d. Ferric chloride : 1% aqueous solution. Shows phenols.

e. Potassium permanganate and acetic acid.

A. 0.1 N $KMnO_4$

B. 2N Acetic acid

Equal volumes mixed. This shows oxidisable materials yellow against a purple background.

f. Vanillin/sulphuric acid : 1 g of vanillin dissolved in 30 ml of methanol plus 0.2 ml concentrated H_2SO_4. Shows certain phenols and steroids.

g. Folin and Ciocalteu's Reagent. Shows phenols.

Elution of appropriate bands was by scraping off the silica gel with a spatula and putting into small sintered glass funnels. Filter paper had been put over the sintered glass to prevent blockage by the silica gel. Ethanol was used as the eluting solvent.

6. Quantitative Determination of Compounds

Concentrations of compounds were calculated by using the molar extinction coefficient (ε)

$\varepsilon = \log\left(\frac{Io}{I}\right)$ for a 1M solution in a cell with a length path of 1 cm, where Io = intensity of incident light, and I = intensity of light after passage through the absorption layer.

From Beer's law :

O.D. = C x ε where C = concentration (g mol/l), O.D. = optical density with a 1 cm path length

$$C = \frac{\text{O.D. x molecular weight (M.W.)}}{\varepsilon} \text{ gm/litre}$$

$$= \frac{\text{O.D. x M.W. x } 10^6}{\varepsilon \times 10^3} \ \mu\text{g/ml}$$

$$\text{Therefore } \mu\text{g compound} = \frac{\text{O.D. x M.W. x } 10^3}{\varepsilon} \text{ x volume ethanol}$$

Published extinction coefficients are :-

a. Biochanin A $\epsilon^{1\ cm}_{1\%} = 1.23 \times 10^3$ (at 263 nm)

Therefore O.D. of 1.00 ≡ 8.1 μg/ml

b. Formononetin $\epsilon^{1\ cm}_{1\%} = 1.01 \times 10^3$ (at 250 nm)

Therefore O.D. of 1.00 ≡ 9.9 μg/ml

c. Genistein $\epsilon^{1\ cm}_{1\%} = 1.32 \times 10^3$ (at 263 nm)

Therefore O.D. of 1.00 ≡ 7.6 μg/ml

Where $\epsilon^{1\ cm}_{1\%}$ is the extinction coefficient of a 1% solution.

All taken from Wong (1962).

d. Maackiain $\log \epsilon = 3.63$ (at 287 nm)

Therefore O.D. of 1.00 ≡ 66 μg/ml (Bredenberg and Hietala, 1961)

e. Medicarpin $\log \epsilon = 3.90$ (at 287 nm)

Therefore O.D. of 1.00 ≡ 34 μg/ml (Smith, McInnes, Higgins and Millar, 1971)

In extracts from infected tissue, medicarpin and maackiain could not be easily separated from each other and therefore the UV spectra obtained were a mixture of the two compounds. To obtain concentrations of the two compounds, simultaneous equations were used as follows :-

$0.0294\ x + 0.0150\ y$ = absorbance at 287 nm

$0.0052\ x + 0.0238\ y$ = absorbance at 310 nm

where x = medicarpin, y = maackiain, and the figures are based on the absorbance of a 1 μg/ml solution at the appropriate wavelength.

7. Methods for obtaining maackiain and medicarpin

A mixture of maackiain and medicarpin, as obtained from infected tissue, was very difficult to separate, and therefore alternative sources were used for these compounds.

a. Maackiain

This was obtained from red clover roots which contain trifolirhizin, a glycoside of maackiain. Soil-free, washed roots were cut up and ground with a pestle and mortar using acid washed sand and a little water. The mixture was left for 2 to 3 days to allow enzymic hydrolysis to take place, which converts trifolirhizin to maackiain, then sieved through 4 layers of muslin, the filtrate being washed several times with ethanol. The washings were added and filtered through a Buchner funnel, then dried down at 45°C at reduced pressure, taken up in water and partitioned three times with diethyl-ether. The ether phases were combined, dried down and taken up in a little alcohol and applied to silica gel TLC plates and run in a solvent of n pentane : diethyl-ether : acetic acid (75:25:1, v/v/v) (Higgins and Smith, 1972). The impure maackiain band was eluted and re-run on TLC in a methanol : chloroform (2:98, v/v) solvent system.

b. Medicarpin

Jack bean cotyledons were thinly sliced and placed onto expanded metal grids over water in plastic boxes, then inoculated with a spore suspension of B. cinerea in 5% glucose-water and incubated for 5 days in the dark at room temperature. Extraction was as for infected clover leaves, and TLC was as for maackiain. Medicarpin is the phytoalexin of Jack beans (Keen, 1972).

8. Bioassay techniques

a. Cladosporium on TLC plates

Cladosporium has been used as an indicator for antifungal compounds (Bailey and Burden, 1973) because it grows quickly, is coloured and is reputed to be particularly sensitive to antifungal compounds. Thick spore suspensions of Cladosporium in SCA were sprayed onto TLC plates until a heavy spore load was deposited, and the medium was dried off in a fume cupboard until the plate was moist but there was no free surface medium. The plates were incubated for 3 - 4 days in a moist chamber at room temperature, after which the fungus would have grown over the whole plate except in bands where inhibitory compounds were present.

b. Spore Germination tests

These were used against B. cinerea and was the method of Purkayastha and Deverall (1965). To obtain slides which had no deleterious effect on spore germination or spreading of test drops, the following technique was used. Soda-glass slides were rubbed with the hand in hot water and detergent and by repeated washing in tap water, then distilled water. An overnight soaking in 5% acetic acid, then overnight in distilled water was followed by a few hours in acetone. The slides were dried in an oven.

Solutions to be bioassayed were made up in 5% ethanol. 20 µl drops were pipetted onto slides using a 0.1 ml graduated pipette so that there were three drops per slide, each of a different dilution. There were at least three replicates per dilution. One µl of spore suspension (5×10^5 spores/ml) was added to the centre of each drop using an 'Agla' micrometer syringe, which gave 500 spores per drop. Only drops shaped as shallow, approximately circular domes were used, other drops being discarded, thus standardising the position of spores relative to the edge and surface of the drop. Slides were placed on bent glass tubing over moist filter paper

in plastic boxes and incubated at room temperature, in the dark, for 22 - 25 hours.

After incubation, spores were killed and stained by adding a small drop of cotton blue in lactophenol to each drop. Percentage germination per drop was measured by examining all spores, up to 100, in several low power microscope fields, germination being considered as the production of a germ-tube at least the length of the spore diameter. If there was 100% or 0% germination, there was no need to count spores.

Germ-tube length was measured by using a calibrated micrometer eyepiece at high power. The first 10 germ-tubes per drop were measured, giving at least 30 replicates. Accurate measurement was often difficult due to curving of the germ tube.

c. Effect on mycelial dry weight

As S. trifoliorum could not be made to produce spores, a method for bioassaying using mycelial dry weights was used, as measuring colonies on agar plates was found not to be satisfactory. 1 ml of homogenised mycelium (as used for plant inoculation) was added to 19 ml of SCA in a 50 ml flask, or 0.25 ml of homogenised mycelium was added to 4.75 ml of SCA in a glass vial. The SCA contained 4.5% sucrose (not 1.5%). This was left for 24 hours to allow the mycelium to recover from homogenisation, then the test compound was added in dimethyl sulphoxide, so that the concentration of dimethyl sulphoxide in the culture was 1%. Flasks, or vials, were incubated at 24 - 27°C in a 'Griffin' orbital incubator at speed 7 for 10 days. After this time, the fungus was removed by centrifugation at 3000 rpm and put into weighed aluminium boats, dried for one day in an oven at 100°C, transferred to a dissicator, cooled, then weighed.

This particular combination was found to be the best after trying several different solvents and comparing agar plug inocula with homogenised mycelium. The following solvents gave no growth of S. trifoliorum after

three days at 2% in SCA : acetone, chloroform and ethanol. Dimethyl formamide gave some growth but with 2% dimethyl sulphoxide growth was almost as much as a control with no solvent. It was found that at 1% dimethyl sulphoxide and 4.5% sucrose in the SCA, that growth was as good as in the control. A solvent was necessary as isoflavones and pterocarpans are not very water soluble.

An agar plug inoculum did not grow as well in shake culture as did homogenised mycelium, and differences between replicates were often large, whereas homogenised mycelium gave good replication. Sterile conditions were maintained throughout.

9. Method to study degradation of phytoalexins by *S. trifoliorum*

A method based loosely on that of Higgins and Millar (1969a) was used. 5 ml of homogenised *S. trifoliorum* mycelium (as used for plant inoculation) was pipetted into 50 ml flasks and left for 18 hours to recover from homogenisation. 150 μg of phytoalexin (30 ppm) dissolved in 50 μl of dimethylsulphoxide was added to each flask. Three replicate flasks were taken at times 0, 1, 3, 6, 9, 12, 18 and 24 hours after inoculation. The fungus was removed by centrifugation at 3000 rpm and the supernatant partitioned twice with equal volumes of carbon tetrachloride, followed by two partitions with ethyl acetate. The fungal pellet was extracted with ethanol. All extracts were dried down at 45°C at reduced pressure and redissolved in either 2 or 4 ml of ethanol. The ethanolic extracts were scanned in a spectrophotometer from 360 to 200 nm, then applied to silica gel TLC plates and run in a solvent system of methanol and chloroform (2:98, v/v).

10. Measurement of electrolyte loss

Electrolyte loss from leaves was measured using a 'Chandros' conductivity bridge, and noting the change in conductivity. 10 ml of distilled water was put into glass vials and the conductivity measured.

5 leaf disks were cut using a cork border (No. 4) and added to the vials, and the conductivity measured at intervals. The water blank was subtracted from the leaf disk readings.

EXPERIMENTAL

Inoculation of fungi onto detached leaves

1. S. trifoliorum

It was hoped that by inoculation of detached leaves that a simple method for detecting differences between the varieties of red clover could be found. It was expected that the rate of spread of the lesion would be the criterion on which to base the varietal differences. As mentioned in Materials and Methods, a method for producing the natural infective agent of the fungus, the ascospores, was unsuccessful. Therefore, different types of artificial inocula were tried, and the varieties compared qualitatively.

Use of agar plugs of mycelium as inoculum

S. trifoliorum was grown on PDA and clover leaves were floated on benziminazole solution in petri-dishes, both as described in Materials and Methods. When the fungal colonies were about 5 cm in diameter, plugs of medium (using a No. 3 cork borer) were cut from the edge of the colony, inverted and placed in the centre of the petioles so that part of the plug was overlapping each leaflet. The inoculated leaves were incubated at four temperatures, 25°, 20°, 16° and 12 - 14°C. With the lower temperature the clover plants were pretreated at this temperature for at least one week before removal and inoculation of leaves, this temperature being approximately that at times of natural infection. At the three higher temperatures there was continuous light but at the lower temperature there was a 6 hour dark period.

Rotting of the leaves usually began within the first day after inoculation and the rot continued to spread until the whole leaf was completely rotted. By visually comparing the varieties, there appeared to be no differences between them in the rate of spread of the lesions. Both tetraploids took longer for the whole of their leaves to become infected, but

this was simply due to their being larger. The different temperatures affected the rate of spread of lesions by slowing down those at the lower temperatures, so that a leaf at 25°C that would have been completely rotted in 5 days, would have taken about 7 days at 12 - 14°C.

Use of mycelial fragments as inoculum

The inoculum was prepared, clover leaves floated on benziminazole solution in petri dishes and the leaves inoculated as described in Materials and Methods. Two temperatures were used, at 12 - 14°C and 20°C, the clover plants being pretreated at these temperatures for at least one week.

Rotting of the leaves took about two days to begin, and there appeared to be no visual differences in the rate of spread in the different varieties, it being slower at the lower temperature.

Two variations of this type of inoculation were also tried. The culture of S. trifoliorum was homogenised, the mycelial fragments removed by centrifugation at 3000 rpm and resuspended in the same amount of sterile distilled water. Using this as an inoculum, no rotting took place after 6 days incubation. This may have been due to the contents of the hyphae being washed out.

The other type of inoculum used was prepared by removing the fungus from the medium by centrifugation at 3000 rpm, putting the mycelium into an equal volume of sterile distilled water and then homogenising. Rotting took place in the majority of leaves, although some leaflets from all the varieties took much longer for the infection to visibly spread from the inoculation drop, up to 5 or 6 days in some cases. However, a visual comparison showed there to be no distinct differences between the varieties. The last two types of inoculum were tried at 12 - 14°C only, the clover plants being pretreated at that temperature for one week.

The final method attempted was to support the leaves on expanded metal grids above the benziminazole solution, with only the petioles in contact with the solution. This was considered a better method as the

disease, being a soft rot, was possibly aided by the leaves being floated on the benziminazole solution. However, the rate of spread of the lesions was the same in all the varieties at both 20°C and 12 - 14°C, but it took 1 to 2 days for the lesion to start spreading from the inoculation drop. The spread was a little slower at the lower temperature. Leaves inoculated in this way are shown in figure 4.

All of the above experiments were carried out on greenhouse grown plants, and also used the variety 2nS/1. When inoculations were carried out on field grown material (that was collected in March) for an experiment described later, there were large differences in the weights of the infected tissue from equal numbers of inoculations between varieties after 5 days incubation, the mean weights being as follows :-

2nR	1.5g	4nR	2.3g
2nS/2	3.3g	4/RB	5.0g

There was more rotted tissue in tetraploids because the size of the leaf was limiting with the diploids, these having smaller leaves that were often completely infected. The extent of the rot was less than that found in greenhouse grown plants after the same incubation time. This could have been due to either the field grown leaves being thicker or to field grown plants having a greater resistance, especially in the more resistant varieties. This natural resistance may be greater at the time the leaves were collected as at that time of year clover rot spreads from the limited ascospore lesions and resistance would be of greatest value.

Because of the failure to obtain any differences between varieties with detached leaves from greenhouse grown plants, such plants or a method using detached leaves from such plants was of little value in testing of varieties in a simple test procedure.

2. B. cinerea

The inoculation method was as described in Materials and Methods. Conidial suspension droplets of 20 µl at concentrations as high as 10^7 spores/ml, but without additional nutrients, failed to cause any necrosis of the leaf below the droplet, and so various concentrations of conidia with added glucose were tried. Necrosis below the inoculation drop was always obtained with a concentration of conidia of 5×10^6/ml with 5% (w/v) of added glucose. Leaves 4 days after inoculation with such drops are shown in figure 5. Occasionally necrosis spread beyond the inoculation drop, but this may have been caused by the leaf beginning to senesce. However, no study was made of the factors that caused spreading in such lesions.

FIGURE 4 Leaves inoculated with a single drop per leaflet of homogenised S. trifoliorum in SCA. Photographed 4 days after inoculation. Note spreading lesions.

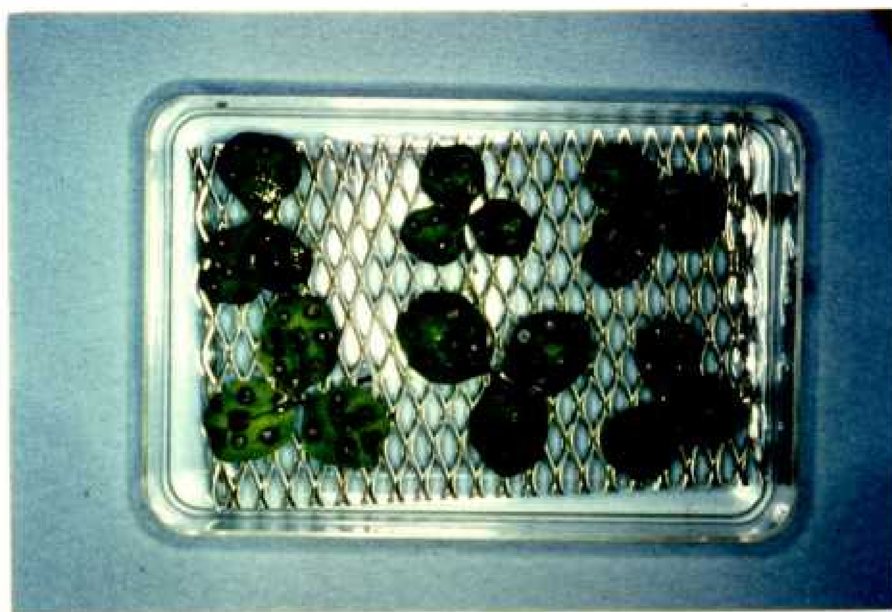


FIGURE 5 Leaves inoculated with 4 or more drops per leaflet of B. cinerea spores (5×10^6) in 5% (w/v) glucose-water. Photographed 4 days after inoculation. Note that lesions are limited to below the inoculation drop.

Extraction and Identification of Reported Antifungal Compounds

a) Isoflavones

As these were normal constituents of clover leaves, healthy leaves were extracted, as described in Materials and Methods for aglycones, and a chromatography system similar to that of Beck (1964) was used for further purification. Thin layer, silica gel chromatography plates were developed in a solvent system of 10% methanol (v/v) in chloroform. The equivalent of 4 to 6g of leaf fresh weight was applied to each plate. After the plates had been developed there were two major bands visible under UV light, one at an R_f of approximately 0.8 which appeared as a dark blue absorbing band under UV light of 254 nm, the other at an R_f of approximately 0.7 which appeared as a blue band under UV light at 254 nm, and as a bright-blue fluorescing band under UV light at 366 nm.

Several visualisation sprays were used for further characterisation of these bands. When sprayed with diazolised sulphanilic acid, the top band gave an orange colour, whilst the lower band gave no colour. With antimony pentachloride, the top band was yellow, and the lower band was pale yellow.

When eluted from the plates, and in 95% ethanolic solutions, these two bands gave UV spectra. The top band had a major peak at 262 nm and is shown in figure 6. The lower band had a major peak at 248 - 250 nm, with shoulders either side of this peak at 240 and 255 nm approximately, as shown in figure 7.

This data compares favourably with that reported for the two isoflavones, biochanin A and formononetin, the upper band being the former and the lower band the latter. Beck (1964) reported the R_f values on silica gel plates with an 11% (v/v) methanol in chloroform solvent system as 0.85 for biochanin A, and 0.75 for formononetin. These are in good agreement with the R_f values reported here, especially as R_f values are not reliable characters because they vary with changes in temperature.

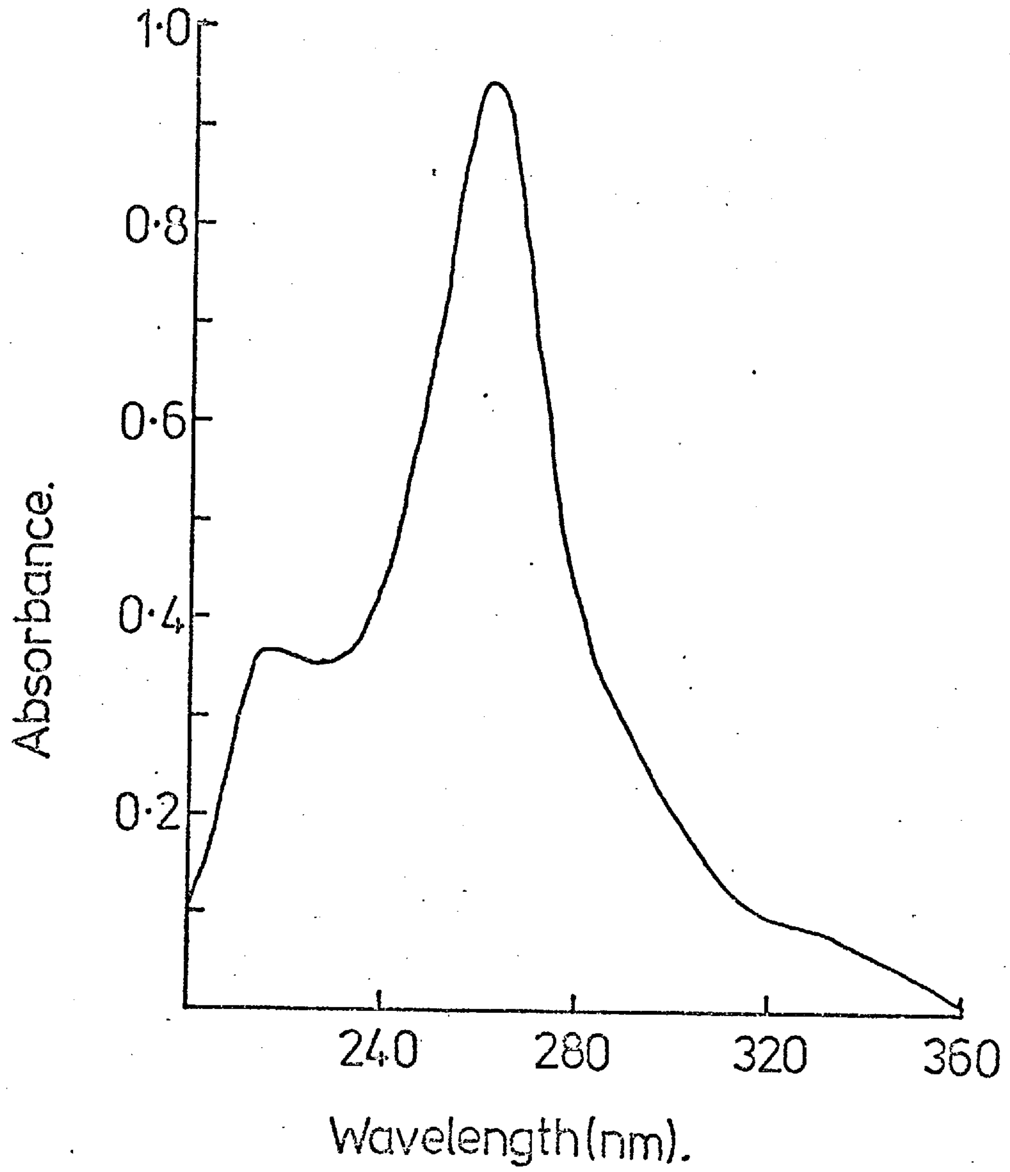


FIGURE 6

UV Absorption Spectrum of biochanin A in ethanol

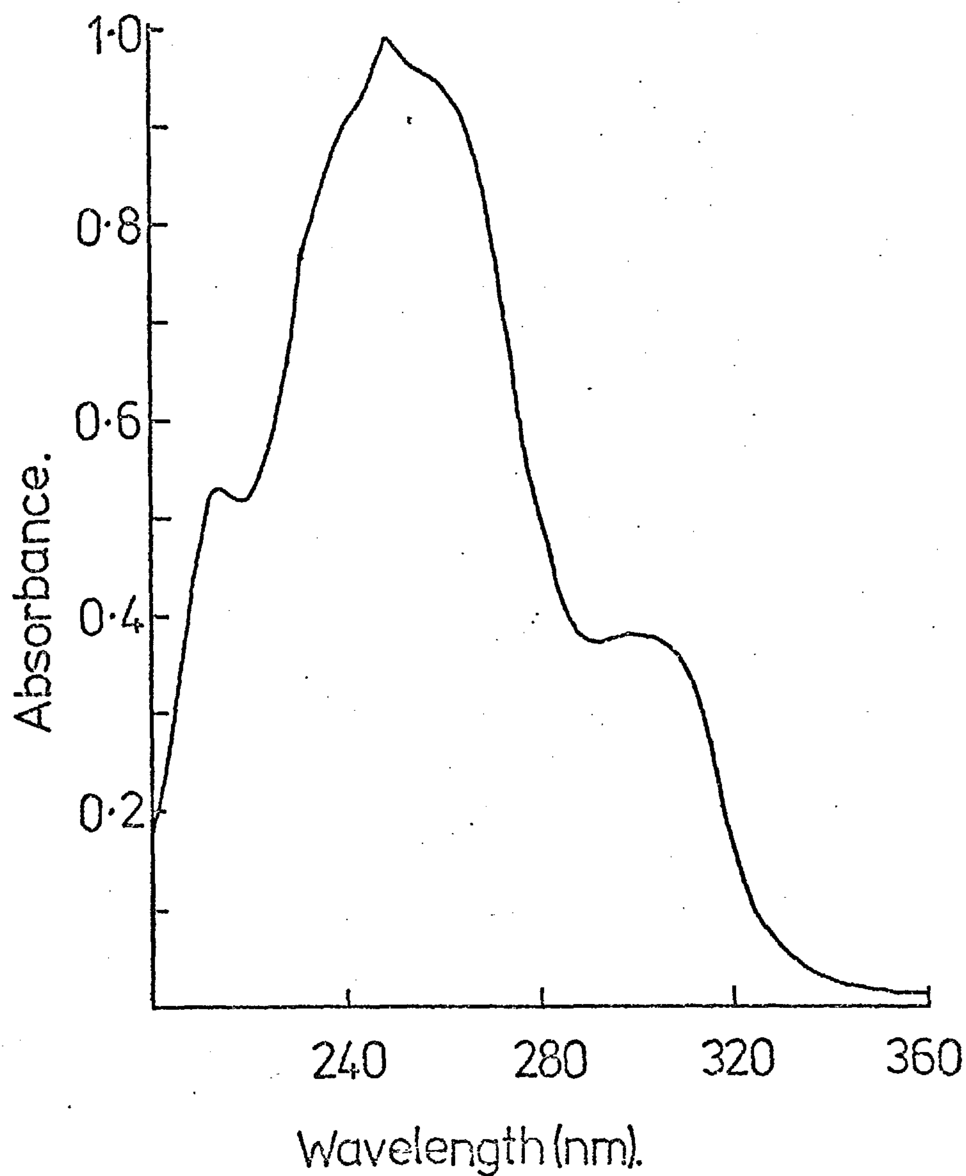


FIGURE 7

UV absorption spectrum of formononetin from red clover leaves, in ethanol

The UV spectra are the same as previously reported.

A few extractions of chickpea (Cicer arietinum) leaves were also carried out, as these too are reported to have large quantities of biochanin A and formononetin. Using the same methods as used with clover, identical results were obtained with regard to R_f values, UV fluorescence, visualisation spray reactions and UV spectra.

As a final check, the two isoflavones were compared by TLC and UV spectrophotometry with samples obtained from Kodak-Eastman (biochanin A) and NIPA Laboratories Ltd. (formononetin) and found to be identical.

If a 2% (v/v) methanol in chloroform solvent system was used, the R_f values were for formononetin 0.3 and for biochanin A 0.4 approximately. This was a better solvent system to use with infected tissue extracts as it gave better separation of the phytoalexins.

b) Pterocarpans

The two pterocarpan phytoalexins, medicarpin and maackiain, had to be induced as they were not detected in healthy tissue. Several methods of induction were tried including fungal and chemical means. Induction by fungi was by inoculation with either S. trifoliorum or B. cinerea. Initial qualitative attempts at using these fungi showed B. cinerea-infected tissue to contain far more of the phytoalexins than did S. trifoliorum-infected tissue.

As Higgins and Smith (1972) were unsuccessful in separating medicarpin and maackiain by TLC except by repeated use of a more complicated method, it was expected that the two compounds would run together. Therefore, alternative sources of the two compound were used to establish TLC and spectral data for the individual compounds, and mixtures of the two compounds, and this data compared to the suspected mixture obtained from clover-leaf extracts.

Medicarpin, from Jack beans, and maackiain, from clover roots, were

obtained as described in Materials and Methods. When run on TLC the following R_f values were obtained with the solvent systems mentioned :

2% (v/v) methanol in chloroform - 0.65 for both

pentane:diethyl ether:acetic acid (75:25:1, v/v/v) - 0.4 for both

When sprayed with antimony pentachloride, medicarpin gave a yellow colour, whilst maackiain was reddish-brown. When the two compounds were added together and run on TLC, the R_f values were the same as above, and the colour of the mixture with antimony pentachloride was brick-red.

Medicarpin in 95% ethanol gave a UV spectrum which consisted of a main peak at 287 nm with a pronounced shoulder at 282 nm (see figure 8). The shape of the peak at 280 to 290 nm is characteristic of many of the pterocarpans. When alkali was added, a shift in the peak to 295 nm was observed, with loss of the shoulder.

Maackiain in 95% ethanol gave a similar UV spectrum to medicarpin, with the same type of peak at 287 nm with the shoulder at 282 nm. However, an additional larger peak was present at 310 nm (see figure 9). When alkali was added, a shift resulting in a single peak at 305 nm took place. The UV spectra for both compounds corresponded to the published data (Bredenberg and Hietala, 1961; Smith _et al_., 1971).

When infected clover leaves were extracted and run on TLC with a 2% (v/v) methanol in chloroform solvent system, many more bands were visible under UV light than were present in extracts of healthy tissue. At the approximate R_f value (0.65) at which medicarpin and maackiain were known to run, a band was visible under UV light at 254 nm as an absorbing dark-blue, which at lower concentrations was shadowy and not very conspicuous. Upon spraying with diazotised sulphanilic acid a yellow to brown colour was obtained, the darker colour being due to higher concentrations whilst with antimony pentachloride, a brick-red colour was formed. When eluted from the TLC plate, the UV spectrum was intermediate between medicaprin and maackiain. The peak at 287 and 282 nm was identical, but a peak at

310 nm was present which was less pronounced than the 287 nm peak (see figure 10). Therefore, the R_f values, UV spectrum and colour reactions were in agreement with this band at R_f 0.65 being a mixture of medicarpin and maackiain.

An attempt was made to try and separate the two compounds from infected clover extracts on TLC by the method described by Higgins and Smith (1972). This useda solvent system of pentane: diethyl ether:acetic acid (75:25:1, v/v/v). The solvent was allowed to run 9cm the first time, the plate removed and dried, then the solvent allowed to run 13 cm before removal of the plate, then finally to 18 cm. It had been reported that the front of the absorbing band seen under UV light contained one compound relatively pure, the middle of the band was a mixture, and the tail of the band contained the other compound relatively pure, as seen by the presence or absence of the 310 nm peak of the UV spectrum. The band was divided into a number of sections running parallel to the solvent front. Each section was eluted separately and the UV spectrum taken, but no separation of the two compounds was found. Obviously the method had to be used very carefully to obtain separation, but as alternative sources of both phytoalexins were available, the method was not needed.

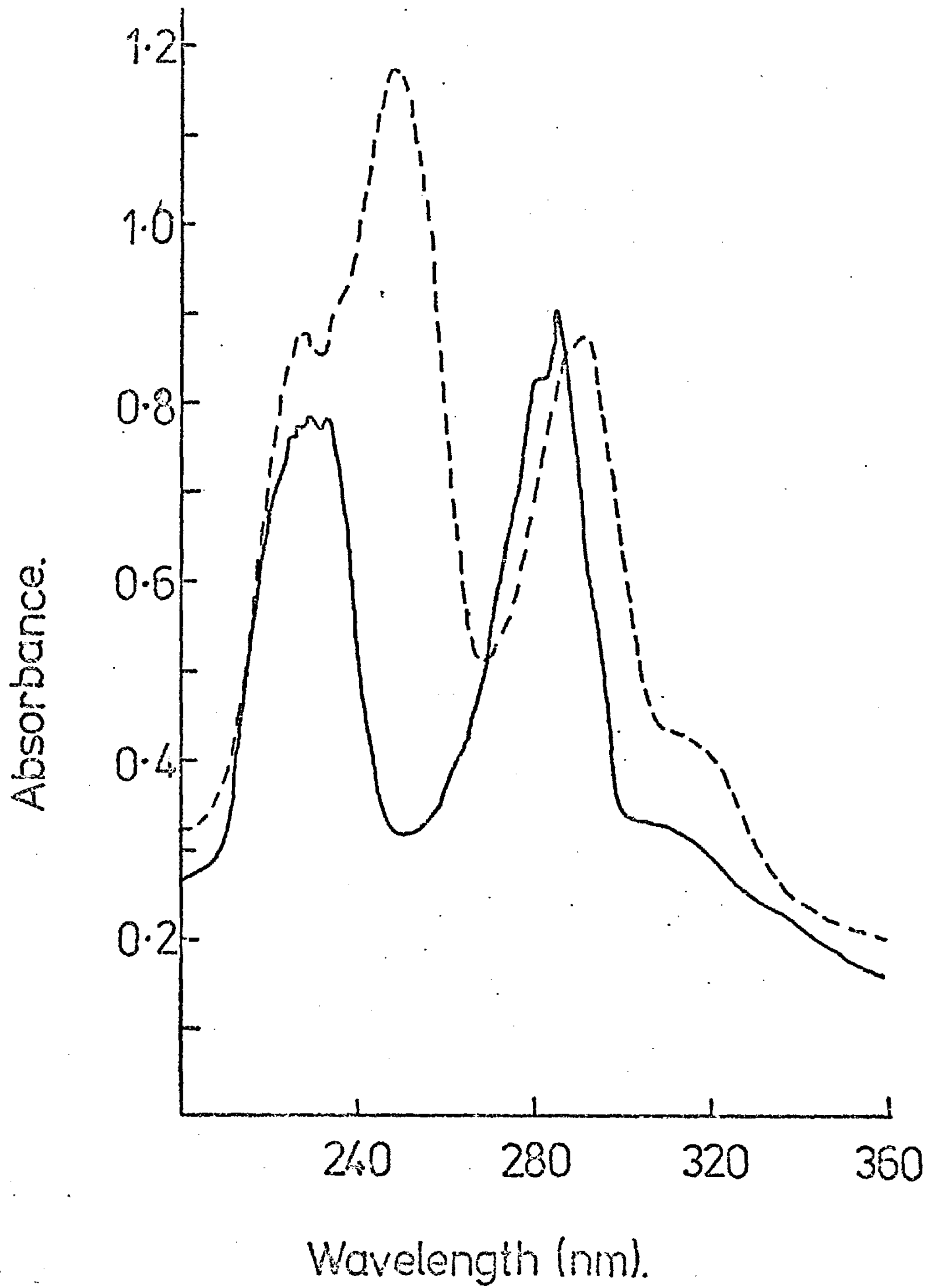


FIGURE 8

UV Absorption Spectra of medicarpin from Jack Bean cotyledons

In ethanol ———

In alkali ------

Concentrations differ

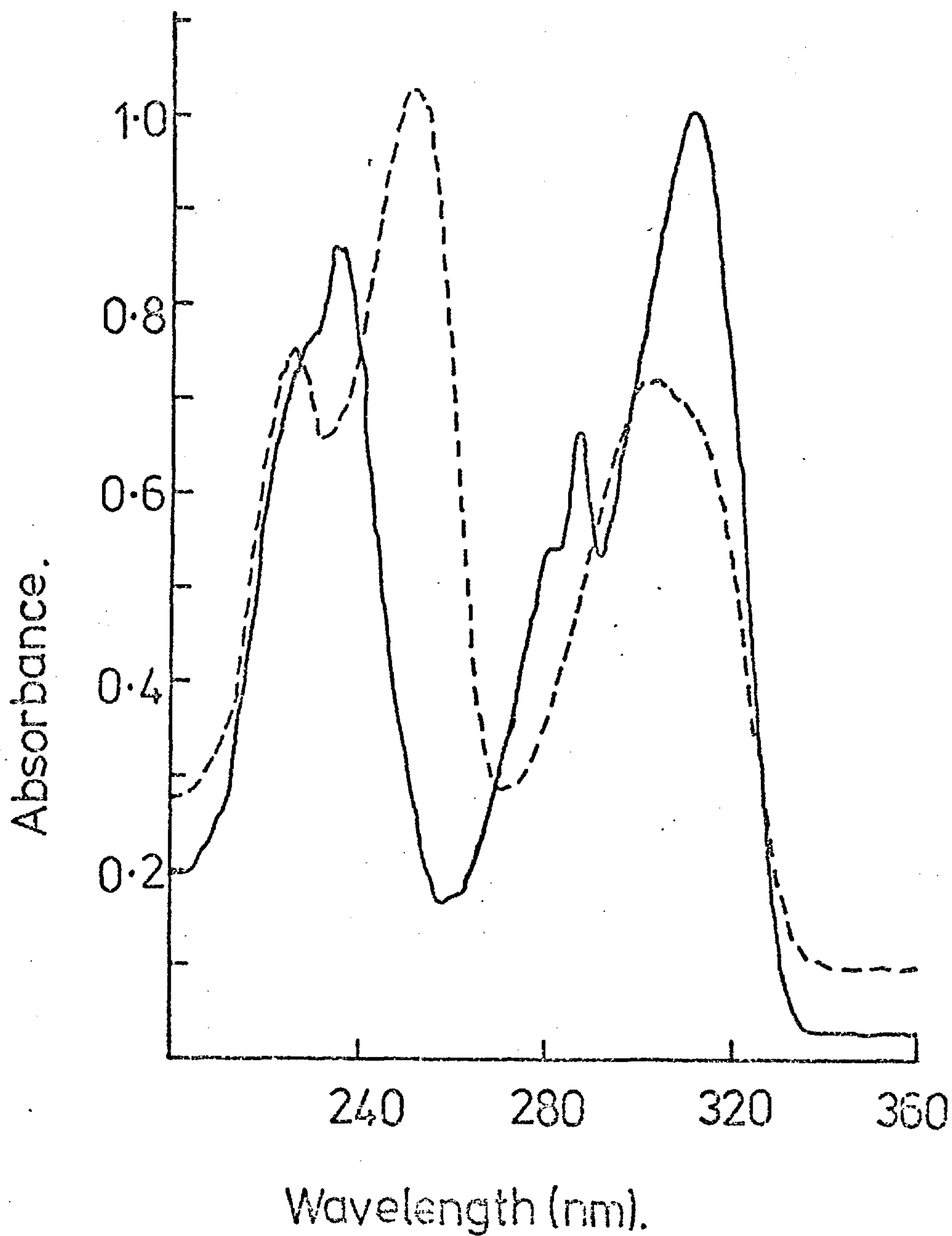


FIGURE 9

UV Absorption spectra of maackiain from red clover roots

In ethanol ——— Note peak at 310 nm

In alkali - - - - Concentrations differ

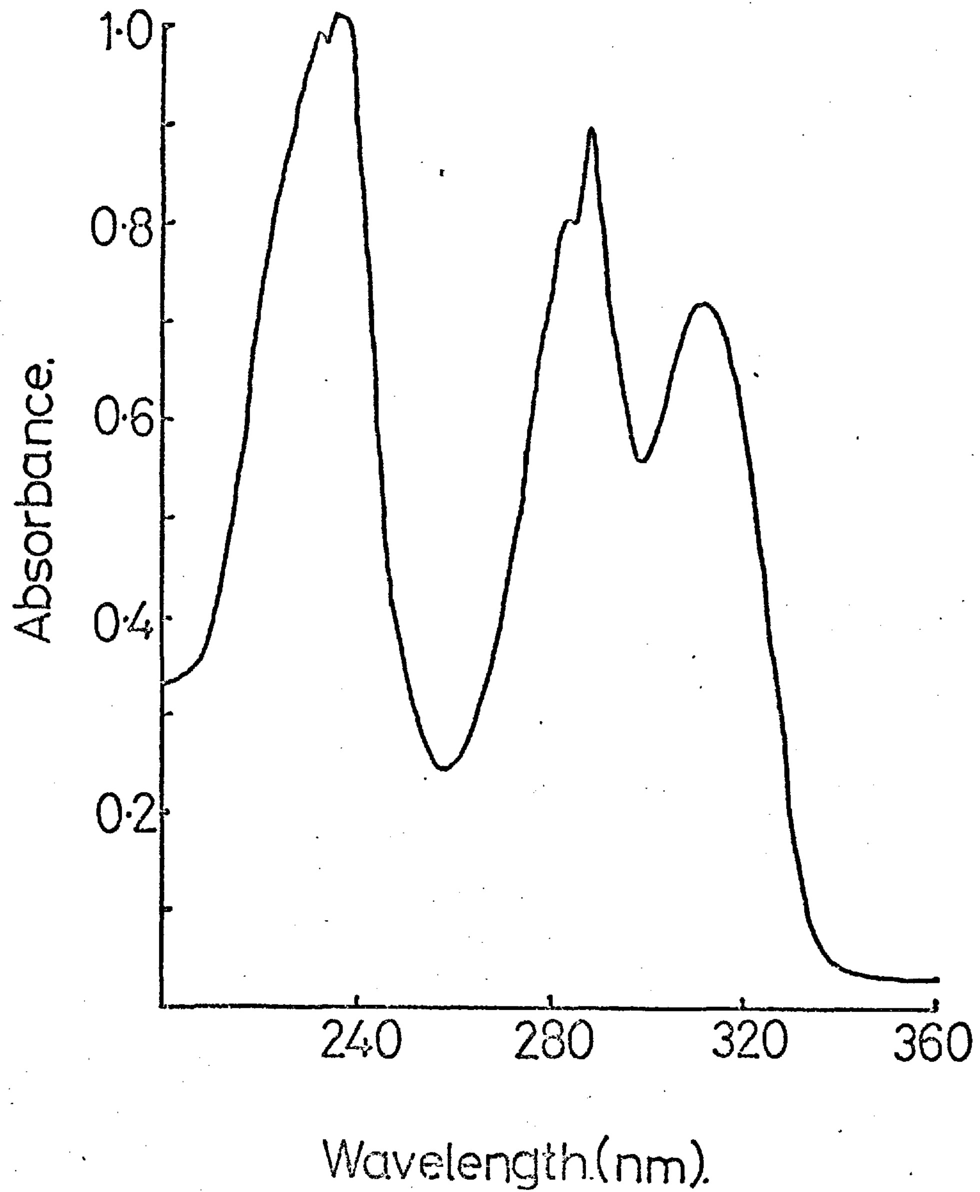


FIGURE 10.

UV absorption spectrum of a mixture of medicarpin and maackiain from extracts of B. cinerea-inoculated clover leaves.

Chemical Methods of Inducing Phytoalexins

Phytoalexins in other plants were known to be induced by chemical methods. Three possible chemical inducers were tried on clover. This was attempted, firstly, to see if clover phytoalexins could be chemical induced; secondly, to confirm that the same compounds were induced by chemicals and fungi; and thirdly, it was hoped that a chemical method could have been used as a standard method of phytoalexin induction. This could be used to compare different clover varieties in their capacity to produce phytoalexins.

a) Protein extract from *S. trifoliorum* culture filtrates

A crude protein extraction of culture filtrates of *S. trifoliorum* grown on liquid pectin medium was prepared, as described in Materials and Methods. It was introduced into detached clover leaves of 2 months of age using the artist's spray gun method. Leaves were incubated for 3 days, and then the whole leaf extracted.

Very little visible damage could be seen on the leaves, and no softening of the tissue was apparent. The latter was unexpected as it was supposed that pectic enzymes were present. Upon extraction, few new bands were seen on the TLC plate compared to the control, but a band was present at the appropriate R_f value for the phytoalexins. This gave, upon elution, the correct UV spectrum for the phytoalexins, but only small amounts were present.

As *S. trifoliorum* causes a soft rot of red clover, pectic enzymes must be present in infected tissue and probably diffuse into areas ahead of the fungus. As it seems that pectic enzymes can possibly induce phytoalexins, then *in vivo*, phytoalexins are possibly induced in advance of the fungal mycelium. Therefore, it would seem unlikely that *S. trifoliorum* was pathogenic to red clover because it failed to induce phytoalexins.

b) Copper chloride $CuCl_2$

Heavy metals, including copper, are known to induce phytoalexins in several plants. The vacuum infiltration method (as described in Materials and Methods) was used for infiltration of copper chloride into leaves. The method was tried initially in a qualitative way using 10^{-3}M $CuCl_2$. After three days incubation at 20°C, the leaves showed purple-brown blotches on them. Therefore there had been some effect on the leaves. The whole leaves were extracted and run on TLC with a 2% (v/v) methanol in chloroform solvent system. Some phytoalexin at the correct R_f value was found to have been induced which gave the expected UV spectrum after elution.

It was hoped that this method could have been used to compare the four different varieties in their capacity to produce phytoalexins. Therefore the above procedure was repeated quantitatively by measuring the absorbance (at 287 nm) of the phytoalexin mixture from 1g of tissue, as shown in Table 1. However, $CuCl_2$ did not seem to be a very potent phytoalexin inducer as the results were very low. There were no significant differences between the varieties when the results were subjected to analysis of variance (see Table 1). Because of the very low concentrations of phytoalexin induced this method was abandoned.

c) Pyrogallol Red (Pyrogallol Sulphonphthalein)

This is a DNA intercalating compound that was reported by Hadwiger and Schwochau (1971) to be a good inducer of pisatin in pea pods. It was therefore hoped that pyrogallol red could have been used to compare phytoalexin production in the different clover varieties instead of copper chloride.

A 1 mg/ml solution of pyrogallol red was vacuum infiltrated into two month old clover leaves, as described in Materials and Methods. The leaves were incubated at 20°C for three days, then the whole leaves extracted

Table 1. Induction of phytoalexins by $CuCl_2$ vacuum infiltration of leaves

Variety	Replicate (absorbance units/g fresh weight*)					
	1	2	3	4	5	Mean
2nR	0.176	0.208	0.258	0.252	0.354	0.249
4nR	0.183	0.288	0.330	0.242	0.228	0.254
2nS	0.191	0.309	0.263	0.239	0.306	0.261
4nS	0.130	0.219	0.265	0.236	0.214	0.213

* From mixed UV spectra of medicarpin and maackiain measured at 287 nm

Analysis of variance of above results

	SS	df	Variance	F
Varieties	0.0070	3	0.0023	0.676
residual	0.0538	16	0.0034	
total	0.0608	19		

and run on TLC in a 2% (v/v) methanol in chloroform solvent system. However, no band was observed under UV light on the developed TLC plates at the appropriate R_f value for the phytoalexins despite heavier loading of plates (the extract from 10g of leaf tissue being applied to a single plate). The area at the R_f value for the phytoalexins was eluted but no UV spectrum was obtained. Therefore pyrogallol red does not induce phytoalexins with this method.

State of the isoflavones in healthy and diseased tissue

As the isoflavones had been reported to be present in the healthy plant as glucosides (mono-, di-, and tri-) with only minute quantities, if any, of the aglycones, the first consideration was whether the glycosides persisted in infected tissue or were hydrolysed to the aglycones. If the latter was the case, then bioassays could be carried out with only the aglycones.

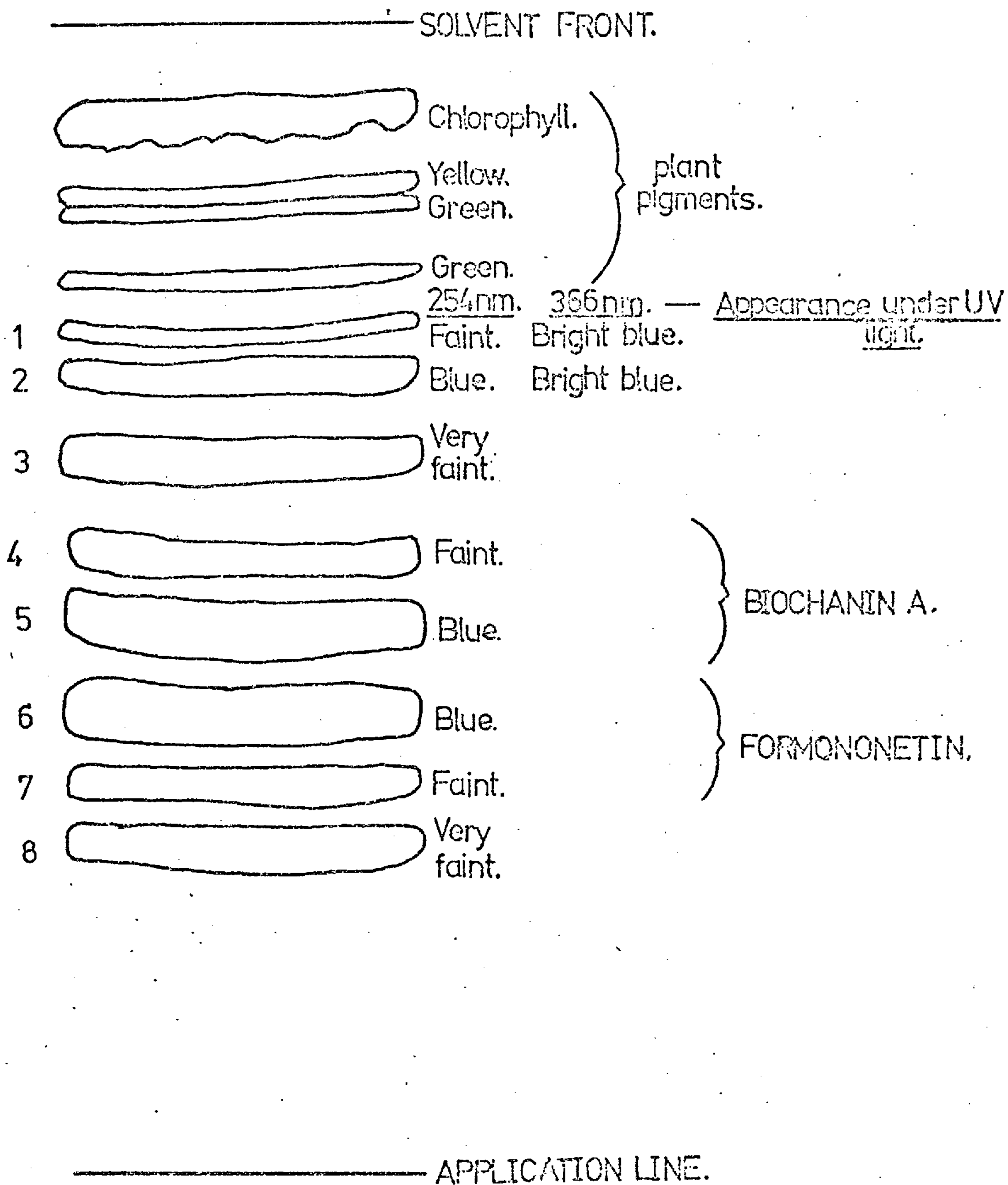
a) Healthy leaf tissue

The extraction and TLC was as described for glycosides in Materials and Methods. Extracts from about 8g of leaf fresh weight were applied to each TLC plate. The bands were eluted and the UV spectra taken in ethanol. Each sample was then dried down in a boiling tube at reduced pressure at 45°C, redissolved in 10 ml of 5N HCl, a glass bubble put on the tube, and the sample boiled for 30 minutes. This hydrolysed the glycosides to the aglycones. After cooling, the sample in HCl was partitioned twice with equal volumes of diethyl ether, the ether phase being further partitioned with an equal volume of water to remove traces of HCl. The ether phase was dried down at reduced pressure, redissolved in ethanol and a new UV spectrum taken. These latter spectra were of the aglycones.

A diagram of a TLC plate of healthy tissue is shown in figure 11, showing the appearance of the bands under UV light, and indicating which bands gave isoflavones upon hydrolysis. Two glycosides of both biochanin

FIGURE 11

Diagram of TLC plate of healthy tissue developed in a solvent system of ethyl acetate - methyl ethyl ketone - water - formic acid (50:30:10:5, v/v/v/v) to show glycosides of isoflavones



A (bands 4 and 5) and formononetin (bands 6 and 7) were found, the biochanin A glycosides coming at approximate R_fs of 0.53 and 0.47, and those for formononetin at approximate R_fs of 0.4 and 0.34. The UV spectra of the glycosides of both isoflavones were very similar, having a peak at about 260 nm, and resembling quite closely the UV spectrum of biochanin A aglycone (figure 6). The other bands present were not investigated further. An attempt to bioassay TLC plates was not successful as the *Cladosporium* sprayed onto the plates did not grow, presumably due to traces of acetic acid from the solvent system that had not evaporated off.

b) *S. trifoliorum* infected leaf tissue

When tissue infected with *S. trifoliorum* was investigated in the same way, the TLC plate showed many more bands under UV, with a large number of overlapping bands near the solvent front. Upon elution none of the bands at the R_fs found for glycosides in healthy tissue yielded a UV spectrum like those of the isoflavone glycosides, and upon hydrolysis, no UV spectrum resembling an isoflavone aglycone was found. The whole top region of the plate with many overlapping bands was eluted with ethanol and rerun on TLC in a 10% (v/v) methanol in chloroform solvent system. It had been found that both the isoflavone aglycones ran to an R_f of approximately 0.95 in the glycoside solvent and therefore it was expected that only aglycone would be found on the rerun. As predicted, both biochanin A and formononetin aglycones were found at their appropriate R_fs and gave the correct UV spectra, before and after treatment with HCl.

This qualitative experiment suggested that very little, if any, of the glycosides were present in *S. trifoliorum*-infected tissue. A further experiment was carried out in an attempt to quantify this. Infected tissue was put into boiling ethanol for 5 minutes, so that if glycosides were present they would not be hydrolysed enzymatically after homogenisation. The ethanol was allowed to cool, the leaves blended in an Omni-mixer, then filtered through a Buchner funnel. The filtrate was washed several times in

ethanol, the washings added together, then dried down at 45°C under reduced pressure. This was taken up in 70% ethanol and partitioned twice with petroleum ether, the ethanol phase being retained, then dried at 45°C under reduced pressure. This was taken up in water and partitioned three times with diethyl ether. Into the ether phase went the aglycones, whilst the water phase retained the glycosides. The water phase was made up to 5N HCl and refluxed for 30 minutes to hydrolyse the glycosides. This was partitioned twice with diethyl ether, the ether being washed twice with water to remove acid traces. This ether phase contained aglycones derived from glycosides in the original extract. The two ether extracts were analysed separately so as to compare the proportion of aglycone to glycoside in *S. trifoliorum*-infected tissue. Both extracts were dried down at 45°C under reduced pressure, taken up in the minimum amount of ethanol and purified on TLC using a 2% (v/v) methanol in chloroform solvent system. The biochanin A and formononetin bands were eluted in ethanol and the absorbance measured for each. The tissue weight was not taken and the absolute amount of isoflavones was not calculated as the results were only comparisons of proportions. The results are given in Table 2.

Although the results give a very high percentage of aglycones, these figures are still probably overestimating the amounts of the glycosides. Partitioning into ether is not highly efficient and some aglycone does remain in the water phase. After hydrolysis of the water phase it is possible that this aglycone plus the aglycone from glycosides is taken up into the ether upon partitioning again, thus exaggerating the amount of glycoside. However it is clear that most of both the isoflavones in *S. trifoliorum*-infected tissue were present as aglycones.

Table 2 Percentage of isoflavone aglycone in *S. trifoliorum*-infected tissue

	Wavelength at which absorbance measured	Absorbance[+] of aglycone	Absorbance[x] of glycoside	Percentage of total as aglycone
Biochanin A	262 nm	78.0	12.75	86
Formononetin	250 nm	66.5	22.0	75

\+ From ether phase of extraction.

x From ether phase of ether/hydrolysed water phase partition.
Figures corrected for dilution.

Bioassaying of TLC plates for antifungal compounds

Higgins and Smith (1972) reported that substances, other than medicarpin and maackiain, were inhibitory to growth of Helminthosporium turcicum in extracts from red clover. No further work has been published on these substances. Therefore, simple bioassays of TLC plates of extracts from healthy, S. trifoliorum-infected and B. cinerea inoculated leaf tissue were carried out in order to locate and possibly identify unknown antifungal compounds in red clover. To begin with, agar replicas of TLC plates were made and sprayed with homogenised S. trifoliorum mycelium in liquid medium, or heavy spore suspensions of B. cinerea or Verticillium albo-atrum (supplied by E.C. Tjamos). The agar replicas were made by placing a thin layer of PDA or X medium agar (1 - 1.2 mm in thickness), with glass backing, onto TLC plates for 1 to 2 hours to form a replica by diffusion of the compounds into the agar, as described by Tjamos and Smith (1974). After spraying with fungus, the replicas were incubated at room temperature in a moist, dark chamber for 2 to 4 days. However, S. trifoliorum would not grow with such a method, and as there was no other method of obtaining an even distribution of S. trifoliorum on the replica, use of this fungus was abandoned. When B. cinerea or V. albo-atrum were sprayed onto the replicas, bands of inhibition were occasionally found but these were not consistent from one replica to another.

An even simpler method of bioassaying was to spray fungus directly onto TLC plates in liquid medium. Bailey and Burden (1973) had found Cladosporium cucumerinum to work very well, and so an available Cladosporium sp. (species unknown) was used. This worked well and gave consistent results. Spores of B. cinerea were also sprayed onto TLC plates in the same way and found to grow well. However it was very difficult to locate antifungal zones because of the whitish mycelium against the white background of the silica gel on the TLC plate. Viewing under UV light did not show up the fungus but only the compounds present on the TLC plate. Therefore the method using Cladosporium was used exclusively.

Cladosporium bioassays on TLC plates

1. Healthy tissue

Uninfected leaves were macerated, extracted and purified on TLC in a 2% (v/v) methanol in chloroform solvent system as described in Materials and Methods. Extracts of about 3 to 4g of tissue (fresh weight) were applied to each TLC plate. A spore suspension of Cladosporium was sprayed onto plates which were incubated as described in Materials and Methods. An example of such a plate is shown in figure 12. Three inhibitory bands were seen, the lower of which was found, upon elution and taking the UV spectrum, to be biochanin A. Formononetin, which has also been reported to be antifungal to several species of fungi, although found to be present on the TLC plates, was not inhibitory to Cladosporium in this bioassay. The other two inhibitory zones were unknowns and these were investigated further.

A slightly different extraction procedure was used, namely putting leaves into boiling ethanol for 5 minutes, followed by the aglycone extraction procedure used previously until the ether-water partition stage. At this point, the water phase was discarded. The object of this method was to omit all glycosides from the final extract, these being left in the water phase. As isoflavones are present in healthy tissue as glycosides, these would therefore not be in the final extract and if the unknown inhibitory zones were also glycosides, these would also not be present. The loading of the TLC plates and the solvent system used were the same as before. The plates were sprayed with Cladosporium, and after incubation were found to show two antifungal zones as seen in figure 13. Both of these zones corresponded to the unknown bands found previously, being at the same R_f values. No other zones of inhibition were found.

The approximate R_fs of these two bands were 0.9 and 0.65 in the above solvent system. The band at R_f 0.9 was not always easy to distinguish due to some growth of Cladosporium upon it. The band at R_f 0.65 was always

very clear with very little or no fungal growth over it. When a plate unsprayed with Cladosporium was viewed under UV light no bands could be seen at the appropriate R_fs, with or without fuming with ammonia. The following sprays were used in an attempt to help visualisation : ferric chloride, diazotised sulphanilic acid, potassium permanganate in acetic acid, vanillin, antimony trichloride and antimony pentachloride. However, none of these showed up anything at the appropriate R_fs.

When the bands of inhibition on Cladosporium bioassays were eluted, or the areas at the same R_fs were eluted from unsprayed plates, and the UV spectrum taken, no peaks could be observed between 380 and 200 nm (see figure 14 - data for 4g loading).

One possibility for the inability to locate these compounds could have been that they were present in low concentrations, too low to show up with any of the methods used, and so a larger bulk of tissue, 20g, was extracted into boiling ethanol following the same method as used previously. Six TLC plates were used to further purify the extract, and the approximate areas of inhibition were eluted from each plate, bulked together and run on two TLC plates, one for the 0.9 inhibitor and the other for the 0.65 inhibitor, using the same solvent system. A strip was cut from each plate and sprayed with Cladosporium, the remainder of the plate being stored at -20°C. The bioassays of the 0.9 band did not show a very distinct inhibitory zone, but the equivalent position on the unsprayed portion of the plate did show a brown band when viewed under UV light at 366 nm. When eluted in ethanol a UV spectrum was taken which had a peak at about 270 nm (see figure 14).

The Cladosporium bioassay of the 0.65 band showed a large clear inhibitory zone. When the unsprayed part of the plate was viewed under UV light there were two bands visible, one running just above the other. The higher one at UV light of 254 nm was very pale blue, and at 366 nm was bright blue-violet. Upon elution the UV spectrum was found to be almost identical to the 0.9 band, with a peak at about 270 nm (see figure 14).

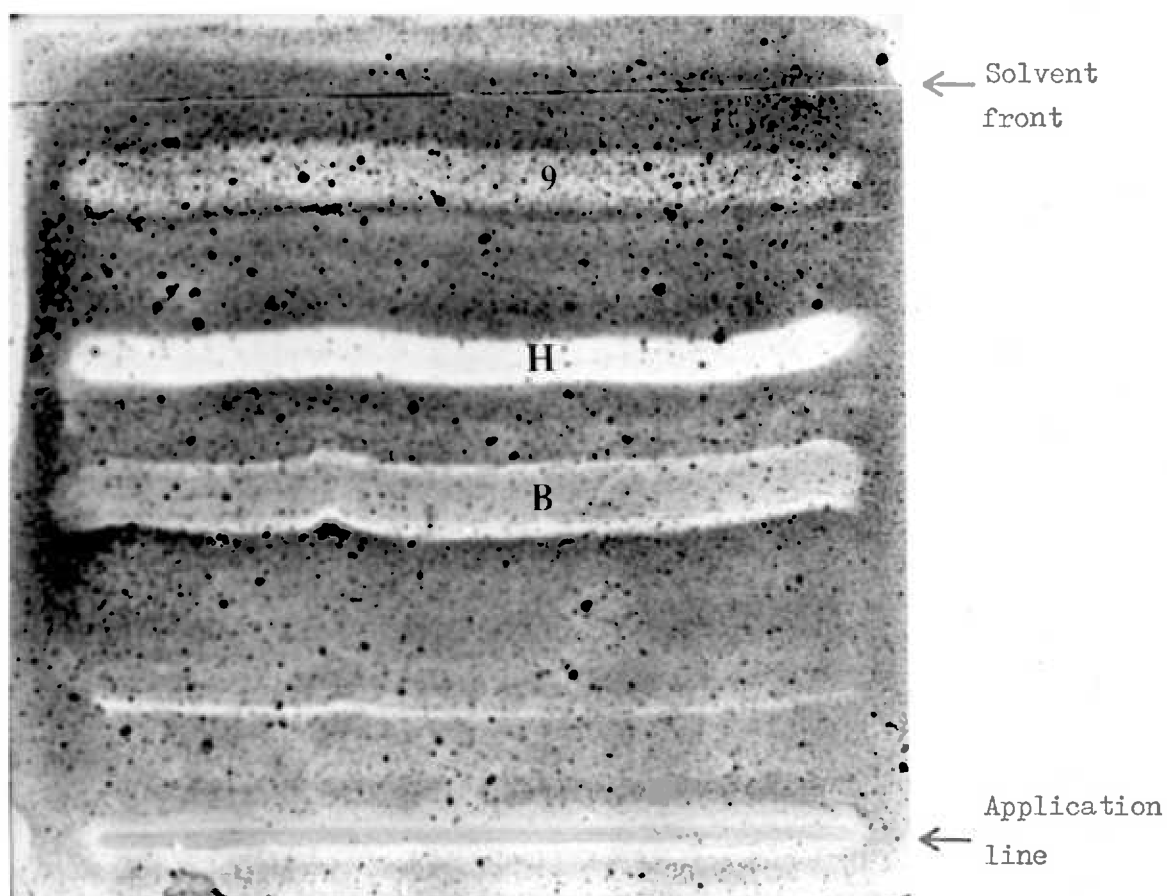


FIGURE 12.

Cladosporium TLC plate bioassay of healthy leaf extract following normal extraction procedure.

The plate was run in a solvent system of 2% (v/v) methanol in chloroform. Note the three antifungal zones (lighter bands). Formononetin was not antifungal in this bioassay.

9 = Band at R_f 0.9

B = Biochanin A

H = Band at R_f 0.65

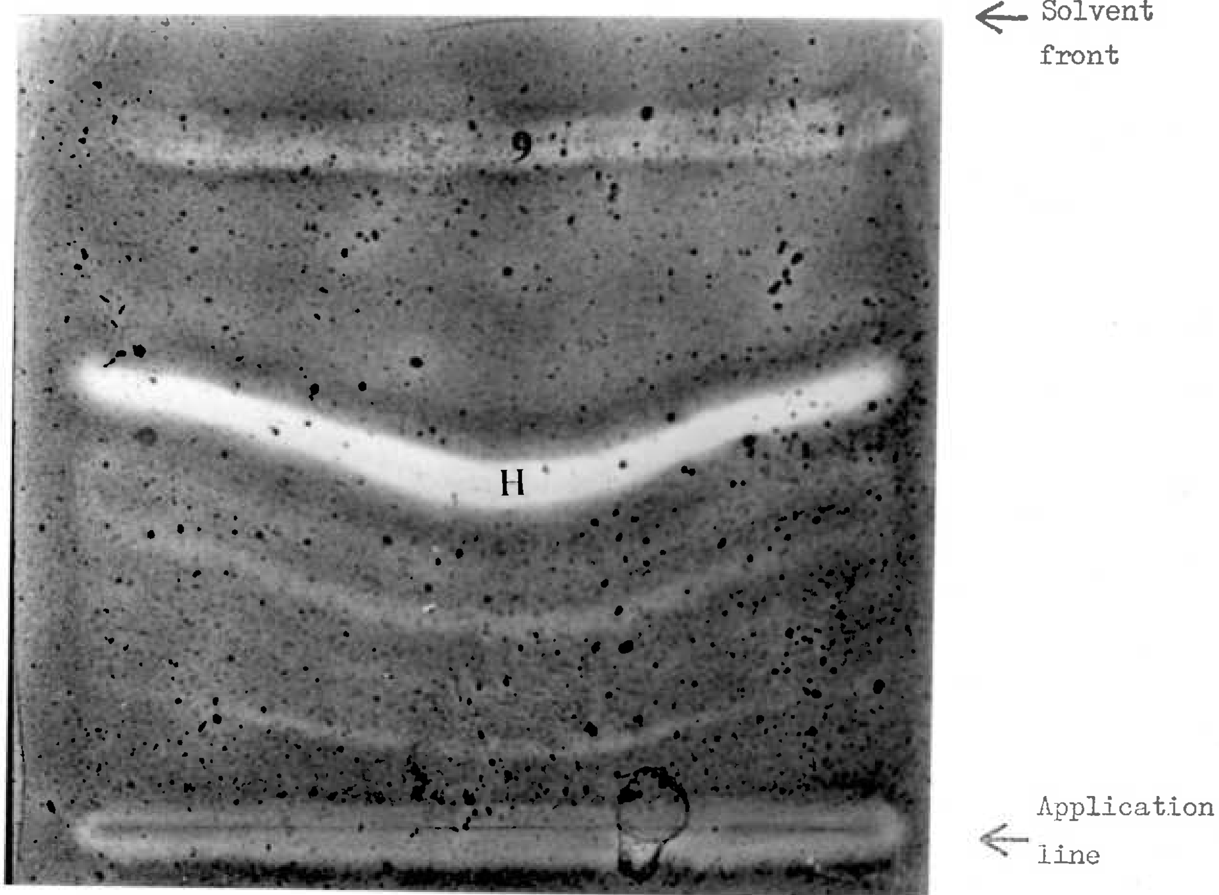


FIGURE 13.

Cladosporium TLC plate bioassay of healthy leaf extract excluding glycosides.

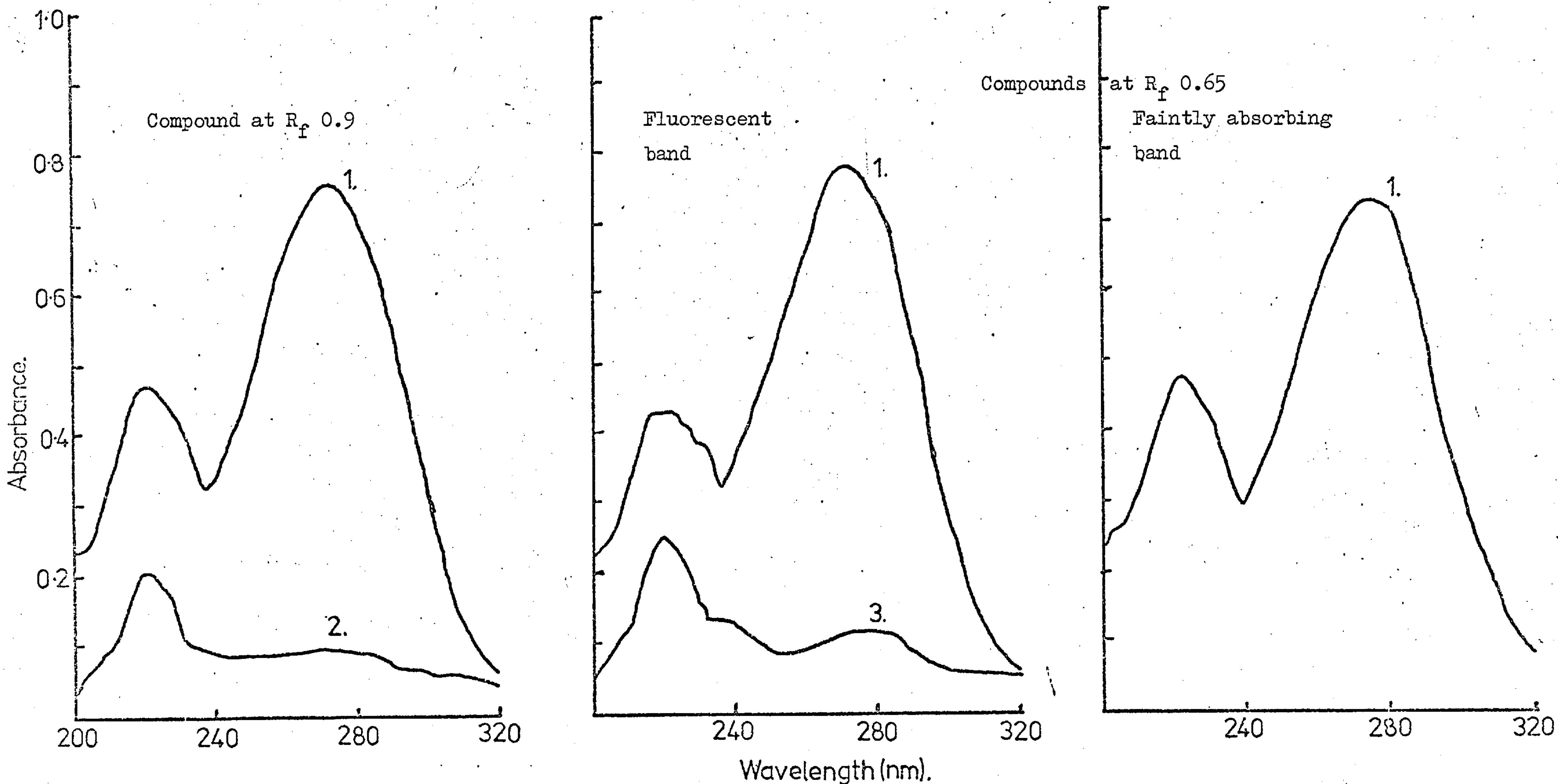
The plate was run in a solvent system of 2% (v/v) methanol in chloroform.

Note the obvious antifungal zone at R_f 0.65 and the less clear zone at R_f 0.9. Biochanin A was not present.

9 = Band at R_f 0.9

H = Band at R_f 0.65

FIGURE 14. UV spectra of unknown compounds causing inhibitory zones on Cladosporium TLC bioassays of healthy tissue extracts



1 = From extract of 20g of tissue. 2 = From antifungal zone on TLC plate (4g per plate)

3 - As 2 but combination of both bands at R_f 0.65

The lower band could be seen on the TLC plates under UV light of 254 nm as a faintly absorbing band. Upon elution with ethanol, it too gave a very similar UV spectrum, with the peak slightly shifted to 275 nm (see figure 14).

2. S. trifoliorum infected leaf tissue

Leaves were inoculated with S. trifoliorum as described in Materials and Methods. The infected areas of the leaves were cut out after 5 days incubation, extracted and further purified on TLC using a 2% (v/v) methanol in chloroform solvent system. The developed TLC plates were sprayed with Cladosporium sp. The extract from about 3 - 4g of leaf tissue was applied to each chromatogram and an example of such a plate is shown in figure 15. Six bands of inhibition could usually be seen but the antifungal band from healthy tissue at R_f value 0.65 was never present. The other two antifungal bands found in healthy tissue extracts were, however, present i.e. biochanin A and the band at R_f value 0.9, although the latter was not always very clear and sometimes consisted of two separate bands. The antifungal band just above biochanin A was composed of the two phytoalexins, medicarpin and maackiain, this being confirmed by the joint UV spectrum and R_f value. The identities of the three remaining bands were unknown and these were investigated further.

The most conspicuous of the three unknown antifungal bands was the one that was usually of lowest R_f value, this being approximately 0.2. When viewed under UV light at 254 nm, the band absorbed strongly giving a dark blue colour, similar to that obtained with biochanin A. With diazotised sulphanilic acid an orange colour was formed and with antimony pentachloride a yellow colour. Upon elution, the UV spectrum in ethanol was found to be almost identical to that of biochanin A with a peak at 262 nm, and when alkali was added the bathochromatic shift of the peak was 10 nm to 272 nm. When developed on TLC with a 10% (v/v) methanol in chloroform solvent system,

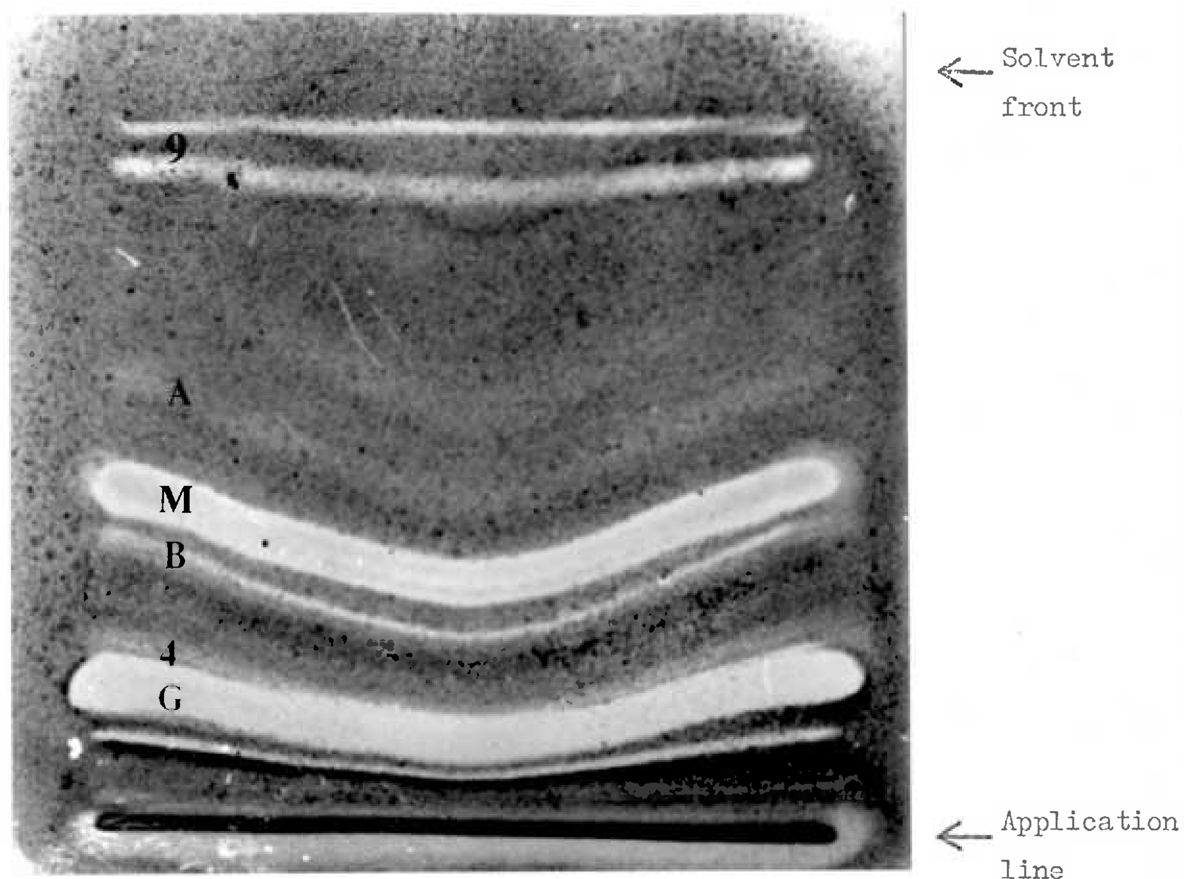


FIGURE 15.

Cladosporium TLC plate bioassay of S. trifoliorum-infected leaf tissue.

9 = Bands at R_f 0.9 (two present on this plate)

A = Fraction A (not very clear on this plate)

M = Medicarpin/maackiain

B = Biochanin A

4 = Fraction 4 (merges into top of genistein)

G = Genistein

The narrow antifungal zone of lowest R_f was only present on a few bioassays.

the R_f value was 0.5 approximately. The UV spectra, with bathochromatic shift, and R_f values corresponded to those of genistein (Wong, 1962; Harborne, 1967; Beck, 1964). To confirm this compound to be genistein, its UV spectra and TLC on several solvent systems were compared with a sample of authentic genistein ('genistin' obtained from NIPA Laboratories Ltd.) and found to be identical. This is another isoflavone (see figure 1) that, although known in red clover (Pope and Wright, 1954), was found in far smaller concentrations than either formononetin or biochanin A in healthy tissue. However, in subterranean clover (T. subterraneum L.) it was found as one of the major isoflavones (Beck, 1964). There had obviously been a great increase in genistein concentration from healthy tissue to that found in S. trifoliorum infected tissue. Due to the extraction method it was not known whether genistein was present in vivo in infected tissue as predominently the aglycone or whether some was present in glycosidic form.

The antifungal band that was present just above genistein and often confluent with it (as in figure 15) at an R_f of approximately 0.25 was faintly absorbing under UV light of 254 nm. When eluted with ethanol, the UV spectrum was often not very clear, due in part to contamination from the genistein band. Therefore, the compound usually had to be further purified (and bioassayed with Cladosporium) on TLC using a diethyl ether: petroleum ether (50:50, v/v) solvent system, followed by a further run with a similar system of diethyl-ether:petroleum ether (70:30, v/v). The R_f values in these latter two solvent systems were very variable. The UV spectrum of the purified compound was very much like that of the medicarpin/ maackiain spectrum, with peaks at 310 and 287 nm and a shoulder at 282 nm. When alkali was added, there was a shift in the peaks, so that the 287 and 310 nm peaks merged together to form a single peak at approximately 300 nm, as shown in figure 16. The alkali shift was almost identical to that found with maackiain. Samples of this compound were given to the Chemistry Department of Imperial College where Mr. J. Bilton carried out analysis by mass spectrometry and other methods, and the compound became known as fraction 4.

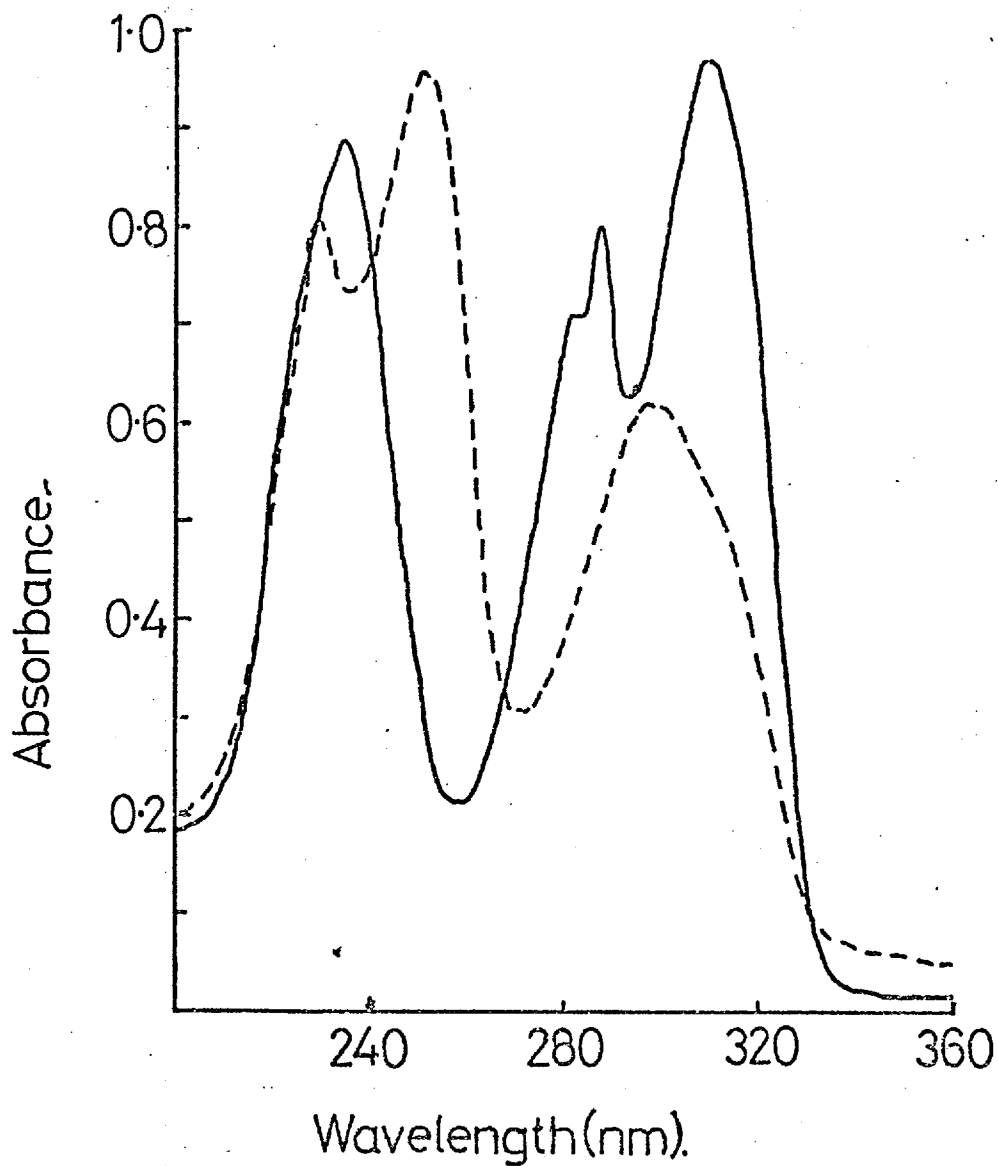


FIGURE 16.

UV spectra of compound 4 from <u>S. trifoliorum</u> infected clover leaves

——— in 95% ethanol

------- with added alkali

When a concentrated spot of 4 on a TLC plate was exposed to UV light for a short while, the spot turned red and when the coloured spot was eluted and rerun on TLC with a diethyl ether: petroleum ether (50:50, v/v) solvent system, the red spot ran in advance of the absorbing spot. This shows that 4 was light sensitive, the red compound being the product. This colour reaction was often useful as a reference.

From the characteristic UV spectrum, it was supposed that 4 was a pterocarpan, and therefore most interesting because of the supposed function of pterocarpans as antifungal compounds in resistance mechanisms.

The third antifungal zone, which became known as fraction A, was found at approximately R_f 0.8. It was not very conspicuous but was of interest because it occurred more prominently with bioassays of extracts from tissue inoculated with B. cinerea, in which section it is described.

B. cinerea inoculated leaf tissue

Leaves were inoculated with B. cinerea, incubated, extracted, further purified on TLC, and the TLC plates bioassayed with Cladosporium all as described in Materials and Methods. Extracts from about 3 - 4g of tissue were applied to each TLC plate and an example of such a plate bioassayed with Cladosporium is shown in figure 17. Six antifungal zones could usually be seen, some of these being the same compounds as found in S. trifoliorum infected tissue, including biochanin A, genistein, fraction A, the band at R_f 0.9 and the combined medicarpin/maackiain band. The last three of these were usually more extensive than the equivalent zones with S. trifoliorum infected tissue extracts. Once again, the antifungal band from healthy tissue at R_f value 0.65 was absent, as was fraction 4 that was found in S. trifoliorum infected tissue. One new antifungal band was present at an R_f value of 0.4 approximately.

Fraction A, at R_f 0.8, appeared on unsprayed TLC plates as a faintly absorbing band when viewed under UV light of 254 nm. When this

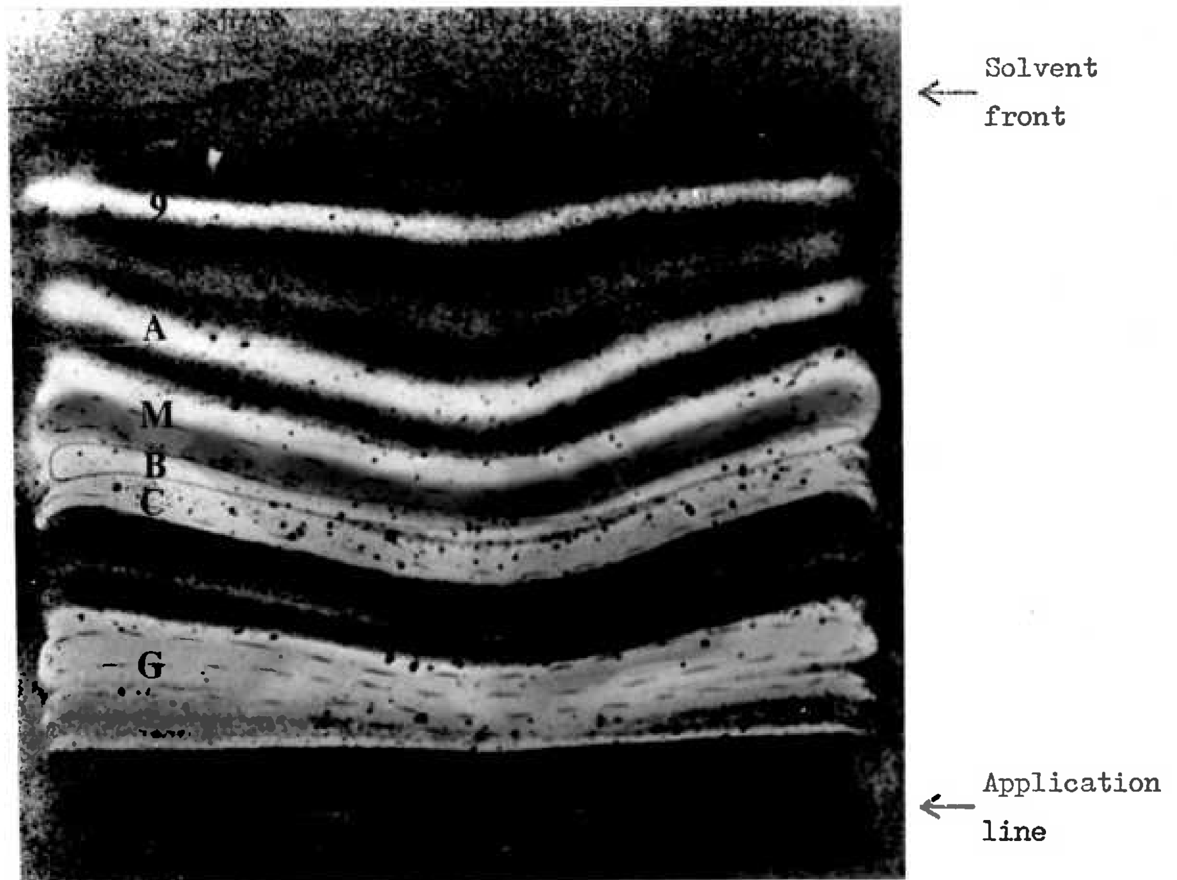


FIGURE 17.

<u>Cladosporium</u> TLC plate bioassay of <u>B. cinerea</u>-infected leaf tissue.

9 = Band at R_f 0.9

A = Fraction A

M = Medicarpin/maackiain

C = Band between biochanin A and formononetin

G = Genistein

B = Biochanin A

This particular plate does not show clearly the distinct antifungal zones commonly obtained with such bioassays.

band was eluted from TLC plates and the UV spectrum taken in ethanol, the spectrum obtained was not very distinct and so the extract had to be further purified using TLC with a solvent system of diethyl ether: petroleum ether (70:30, v/v or 50:50, v/v). The main components were found to be two absorbing bands and when bioassayed with Cladosporium, the one of higher R_f value caused an antifungal zone. The UV spectrum of the purified antifungal fraction (as shown in figure 18) was very similar to that of the medicarpin/maackiain combined UV spectrum, or that of fraction 4, with a major peak at 287 nm with a shoulder at 282 nm, but the peak at 310 nm was less pronounced than that of 4, being smaller than the 287 nm peak. The characteristic shape of the peak suggested this compound to be another pterocarpan. When alkali was added, there was very little shift in the spectrum. Samples at this purification were given for analysis to J. Bilton of the Chemistry Department.

The new antifungal zone at R_f 0.4 ran between biochanin A and formononetin on TLC plates, but when viewed under UV light, this area was found to consist of a number of overlapping absorbing and fluorescing bands. Therefore, this whole area was eluted with ethanol and rerun on TLC with a solvent system of toluene:chloroform:acetone (40:25:35, v/v/v). A quarter of the plate cut off and bioassayed with Cladosporium, the remainder being stored at -20°C. An antifungal zone occurred at an R_f value of approximately 0.5, but when the equivalent portion was viewed under UV light on the unsprayed plate, no bands were observed. A number of visualization sprays were used, including antimony pentachloride, Folin-Ciacalteu reagent, diazotised sulphanilic acid and antimony trichloride, the latter only giving any reaction, forming a very pale, almost indistinguishable yellow colour. When an unsprayed region of the TLC plate was eluted, equivalent to the antifungal zone of the bioassay, a UV spectrum was obtained (as shown in figure 19) which consisted of a single peak, the optimum of which was approximately 272 nm. This spectrum was very similar to those of the antifungal zones in healthy tissue (see figure 14)

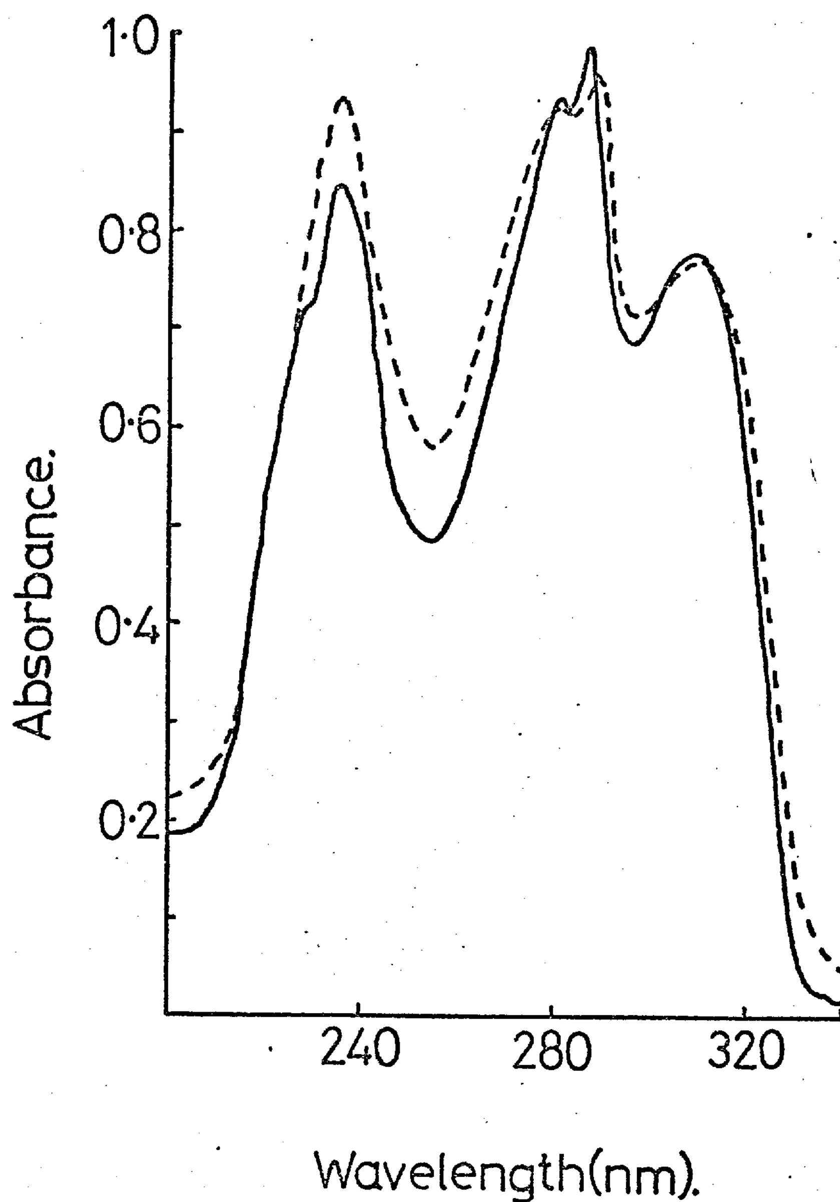


FIGURE 18.

UV spectra of compound A from B. cinerea inoculated clover leaves.

——— in 95% ethanol

- - - - with added alkali

Note that there is no significant shift in alkali.

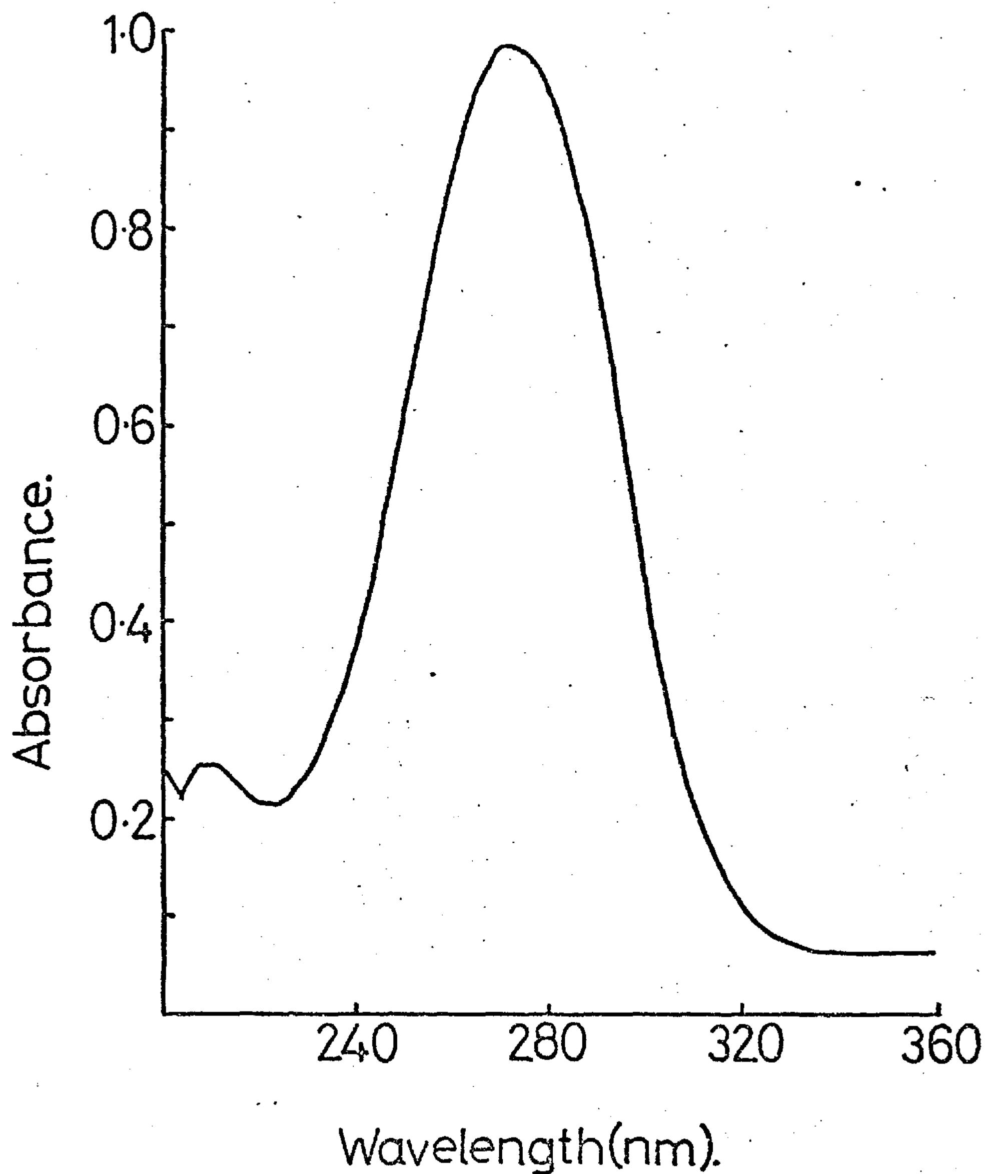


FIGURE 19.

UV spectrum in 95% ethanol of band at R_f value 0.4 on TLC with a 2% (v/v) methanol in chloroform solvent system, which had been purified on TLC with a toluene:chloroform:acetone (40:25:35, v/v/v) solvent system, in which it occurred at R_f 0.5.

and therefore could be of a similar type of compound.

Because of the increase in size of the antifungal zone at R_f 0.9, and also that there were two bands often visible instead of the one band always found in healthy tissue extracts, some further work was carried out on this area. The top area of the chromatogram (between approximately R_f 0.8 and 1.0) was eluted with ethanol and rerun in a solvent system of toluene:chloroform:acetone (40:25:35, v/v/v). Part of the TLC plate was cut off and bioassayed, the remainder of the plate being stored at -20°C. A single inhibitory zone was found at an R_f of approximately 0.7 to 0.75. When the unsprayed portion of the plate was viewed under UV light, a faintly absorbing band was seen at an R_f of approximately 0.7, this covering the lower portion of the antifungal zone. Folin-Ciocalteu spray reagent followed by fuming with ammonia showed two distinct blue bands equivalent to the antifungal zone, one at R_f 0.7, the other at R_f 0.75. With antimony pentachloride spray, the same two bands were present, the one at 0.7 being brick-red and the one at 0.75 being brown in colour. Therefore the antifungal zone was caused by two different compounds, which show up easily with spraying, unlike the band of the same R_f value in healthy tissue.

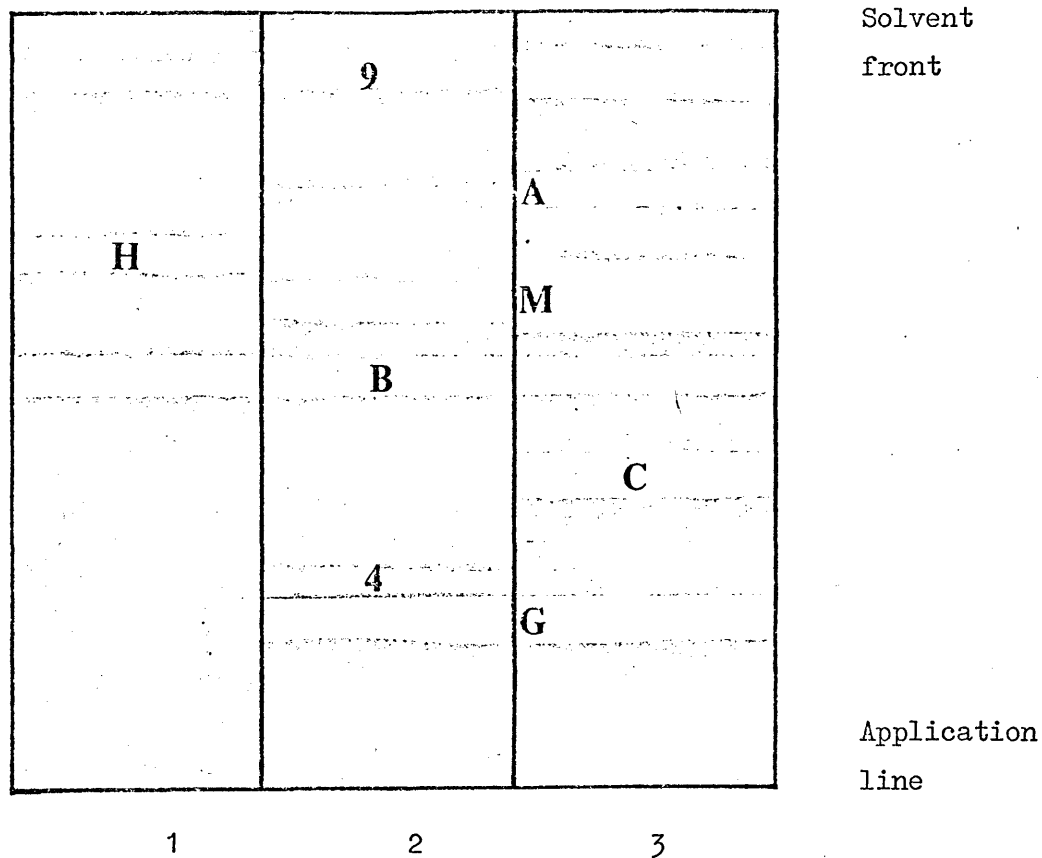
Comparison of *Cladosporium* bioassays

Figure 20 gives a diagrammtic representation of *Cladosporium* bioassays of the three types of tissue, i.e. healthy, *S. trifoliorum*-infected, and *B. cinerea* inoculated leaf tissue. The most obvious difference was the greater number of antifungal bands present in infected tissue extracts than in healthy tissue extracts but this was expected. It is, however, unusual to find so many antifungal compounds in healthy tissue.

The band found in healthy tissue at R_f 0.65 was puzzling in that it did not occur in either of the infected tissue extracts. The R_f value reported for it was the same as that for the phytoalexins medicarpin and maackiain, but this may be slightly misleading especially as R_f values are

FIGURE 20.

Diagrammatic representation of Cladosporium bioassays on extracts from leaves of red clover.



1 = Uninoculated

2 = S. trifoliorum-infected

3 = B. cinerea inoculated

Developed in solvent system of 2% (v/v) methanol in chloroform.

9 = Bands at R_f 0.9

A = Fraction A

H = Band at R_f 0.65 in healthy tissue extracts

M = Medicarpin/Maackiain

B = Biochanin A

C = Band between biochanin A and formononetin

4 = Fraction 4

G = Genistein

not constant but vary with changes in temperature. When compared the two bands did run differently, the band from healthy tissue extracts running ahead of the medicarpin/maackiain band. Although the spectra also differed, this was probably not absolute evidence of the two being different as the spectra for the healthy band were obtained from a large extract where background contamination would be correspondingly high. But it was likely that the healthy extract band was not trace amounts of the phytoalexins because the R_f value was slightly different and the visualization sprays showed no colour reactions that would be expected with the phytoalexins. Why this band was not present in infected tissue is not easy to explain. If the compounds were phenolic, then upon infection the increase and release of many enzymes, especially ones such as polyphenol oxidase, could cause such compounds to be converted to other substances e.g. polyphenols. These would then have different R_f values.

The appearance of fraction 4 only in S. trifoliorum-infected tissue was very interesting. Its characteristically pterocarpanoid UV spectrum shows it to be closely related to medicarpin and maackiain and therefore the immediate reaction is that it is another phytoalexin. But this seems unlikely because phytoalexins are usually more concentrated in tissues infected with weak or non-pathogens, the reverse of the case here. In other diseases of legumes, the appearance of pterocarpanoid compounds, other than the phytoalexins, in pathogen infected plants has been shown to be due to the phytoalexins being converted by the pathogen to these new compounds. This could be the situation occurring here.

An observation that may well support the above view was the lower concentrations of medicarpin/maackiain and fraction A in S. trifoliorum-infected tissue extracts than in B. cinerea inoculated tissue extracts. This could clearly be seen by the extent of the antifungal zone, and by the intensity of absorbance under UV light of 254 nm. The two possible reasons for this are, firstly, that degradation by the pathogen takes place

(supporting the theory on the appearance of 4), or secondly, that less induction takes place with the pathogen than with the non-pathogen.

The appearance of a new antifungal band with B. cinerea infected tissue extracts (at R_f 0.4) was a little unusual as there was never any trace of such a band in S. trifoliorum infected tissue extracts. As it had a similar UV spectrum to those of the unknown bands in healthy tissue, it was possibly a conversion product. Alternatively it could have been a completely new compound, but why it was absent from S. trifoliorum infected tissue extracts was not known.

The isoflavones were interesting in that biochanin A caused an inhibitory zone, but formononetin did not. The appearance of genistein in infected tissue also raised several questions e.g. was genistein being synthesised at the expense of existing isoflavones or was there de novo synthesis ? Also, if genistein was present could daidzein be present ?

Because of the large number of antifungal bands it was decided to concentrate on the isoflavones and pterocarpanoid compounds only. There were two reasons for this, firstly the importance that pterocarpans were believed to play in resistance, and the uncertain position of isoflavones in resistance, and secondly because these compounds were easier to locate on TLC plates with UV light which made working with them simpler.

The state of Genistein in S. trifoliorum infected tissue

The two isoflavones biochanin A and formononetin, although present in uninfected leaf tissue as glycosides, are present in S. trifoliorum infected leaf tissue as predominantly the glycosides. It was expected that genistein would also be present as the aglycone in S. trifoliorum infected leaf tissue. An experiment was conducted in precisely the same way as with biochanin A and formononetin in infected tissue to determine if this assumption was correct. The results are given as the optical densities at 262 nm from an unknown weight of tissue to give a proportion of aglycone to glycoside. They were as follows :-

Absorbance of aglycone	-	300	(Figures
Absorbance of glycoside	-	45	corrected for
% as aglycone of total isoflavone	-	87	dilution)

Clearly, most of the genistein was present as the aglycone.

Occurrence of daidzein in infected tissue

When a comparison was made between the structures of genistein and biochanin A, then genistein only differed in not having the $-CH_2$ group (see figure 1). A similar loss of $-CH_2$ from formononetin would produce daidzein. It was not known if daidzein was present on TLC plates, and as formononetin did not appear antifungal on Cladosporium TLC plate bioassays, then equally daidzein also may not appear antifungal in such a system. Therefore a cursory look for daidzein was carried out on S. trifoliorum infected leaf tissue.

Daidzein, like formononetin, is brightly fluorescent on TLC plates viewed under UV light of 366 nm, but it has a lower R_f value than genistein. With developed TLC plates some brightly fluorescent compounds were observed at low R_f values when a 2% (v/v) methanol in chloroform solvent system was used. When eluted and rerun in a 10% (v/v) methanol in chloroform solvent system, two fluorescent bands were found, the higher of which (at R_f 0.5 approximately) was daidzein as shown by its UV spectrum, which is

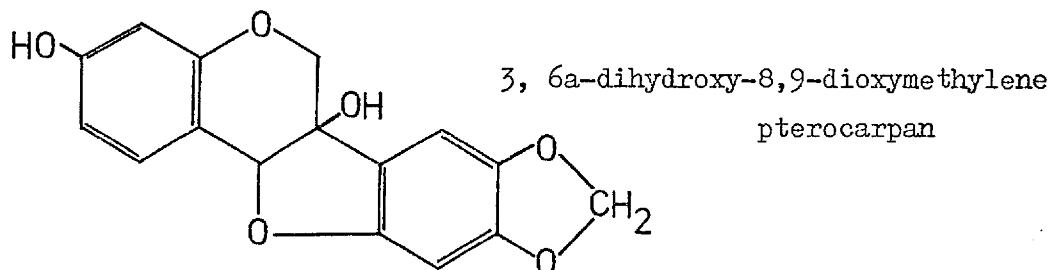
almost identical to that of formononetin (shown in figure 7). Therefore daidzein was present and included as one of the compounds to be looked at in a quantitative analysis.

Identifications of unknown pterocarpans from infected tissue extracts

The data on which these identifications were based was obtained by Mr. J. Bilton of the Imperial College Department of Chemistry.

Fraction 4

This compound was very similar to maackiain but differed in mass by 16 units, i.e. one oxygen atom. The structure was elucidated as :-

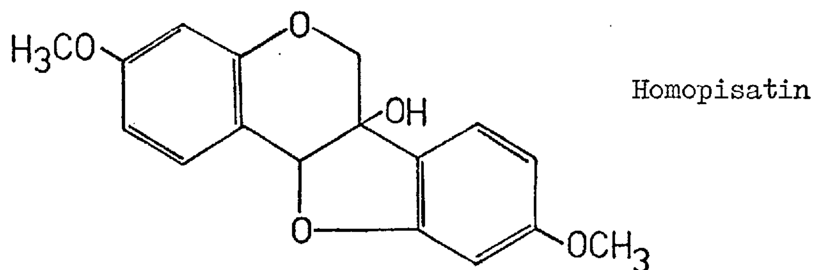


This can also be called 6a-hydroxy maackiain or 6a-hydroxy inermin. It had been found previously as a conversion product of pisatin by Fusarium solani f. sp. pisi (Van Etten, Pueppke and Kelsey, 1975) and Ascochyta pisi (van't Land, Wiersma-Van Duin and Fuchs, 1975). The extinction coefficient was known, log ε being 3.64 at 286.5 nm (Van Etten et al., 1975), therefore an absorbance of one was equivalent to 69 μg/ml.

Fraction A

In the mass spectrometer this was found to be composed of two compounds, one of which was certainly pisatin (see figure 2). Maackiain and pisatin are very similar, both having the 8, 9-dioxymethylene group, but pisatin is methylated at the 3 position and has an hydroxyl at the 6a position.

The second compound was tentatively identified as :-



Homopisatin

This compound has been synthesised and found to be strongly antifungal (Perrin and Cruickshank, 1969). It is not known as a naturally occurring compound and no chemical details have apparently been published about it.

The extinction coefficient for pisatin is known, log ε being 3.68 at 286 (Perrin and Bottomley, 1962) therefore an absorbance of one is equivalent to 66 µg/ml. Concentrations calculated for fraction A were based on the extinction coefficient of pisatin alone. Being a mixture of pisatin and homopisatin without a method of separating them, the name fraction A is used for this mixture.

Quantitative analysis of antifungal compounds in field grown red clover

An experiment was conducted on 4 varieties of red clover to find the concentrations of some of the antifungal compounds. Leaves from red clover plants were collected from field trials at the P.B.I. on 25th March 1974, as described in Materials and Methods. At that time of year clover rot was in its spreading lesion phase. Therefore it was considered the best time to carry out the analysis of the antifungal compounds, especially as it was known that isoflavone concentrations varied throughout the year (Dedio and Clarke, 1968). There were three replicates of each treatment for each variety, each replicate consisting of 10 inoculated leaves with the S. trifoliorum and B. cinerea inoculated treatments, and of 5 uninoculated leaves for the healthy treatments. The plants were inoculated, incubated, extracted, purified on TLC, appropriate bands eluted and concentrations of compounds from known weights of tissue calculated by using the extinction coefficient, all as described in Materials and Methods.

The compounds analysed were the isoflavones formononetin, biochanin A, genistein and daidzein, and the pterocarpans medicarpin, maackiain, fraction A and 6a-hydroxy maackiain. In the cases of daidzein, fraction A and 6a-hydroxy maackiain, a second TLC was necessary to obtain a fairly clean sample. The first TLC was carried out with a solvent system of 2% (v/v) methanol in chloroform. The second TLC for daidzein and 6a-hydroxy maackiain used a solvent system of 10% (v/v) methanol in chloroform, and for fraction A the TLC solvent system was diethyl ether:petroleum ether (50:50, v/v).

a) Isoflavones

Only the total amount of hydrolysed aglycone was measured. Because the isoflavones biochanin A and formononetin were reported as being anti-Sclerotinia and were preformed, the first consideration was whether the resistant varieties had more than the susceptible varieties. The second consideration was whether the increased concentrations of some isoflavones induced upon infection were produced from existing isoflavones or if de novo

synthesis took place. The results are given in Tables 3, 6 and 9. These results were treated to statistical analysis using analysis of variance (Tables 4, 6 and 10) and Duncan's multiple range test (Tables 5, 6 and 10) using the $\log_{10}$ of the concentrations.

Biochanin A

There were highly significant differences between the varieties and between the treatments.

In healthy tissue the 2nR variety had less than the other three varieties, the latter not being significantly different from each other.

In S. trifoliorum-infected tissue there were two groups, one composed of 2nR and 4nR, the other of 2nS/2 and 4n/RB. The first group had significantly more than the other group, but in all four varieties there was a very large and significant drop from concentrations in healthy tissue.

In B. cinerea—inoculated tissue there was a significant rise in the 2nR and 4nR varieties but a significant drop in the 2nS/2 and 4n/RB varieties when compared to healthy tissue. The only difference between varieties was between the 2nS/2 (lowest concentration) and 4nR (highest concentration).

There was no correlation in healthy tissue between resistant varieties and higher concentrations of biochanin A. With S. trifoliorum-infected tissue the resistant varieties did have more than the 2nS/2 and 4n/RB varieties, though less than in healthy tissue. The concentrations with B. cinerea were variable, but there was a rise from healthy concentrations in resistant varieties but generally no differences between the concentrations in all the varieties.

Formononetin

There were significant differences between varieties and between treatments.

In healthy tissue, the 2nR had significantly less than the other three varieties which were not significantly different from each other.

In S. trifoliorum-infected tissue, the 4nR variety had significantly more than the other three varieties. The 2nS/2 and 4n/RB varieties were not significantly different from each other and the 2nR was not different from the 4n/RB. All concentrations were very much lower than in healthy tissue.

In B. cinerea-inoculated tissue, the diploid varieties had less than the tetraploid varieties. However all varieties had less than healthy tissue although more than in S. trifoliorum-infected tissue.

Once again there was no correlation between resistance and higher concentrations in healthy tissue. With S. trifoliorum-infected tissue there were no clear differences but there was a trend towards the resistant varieties. In B. cinerea-inoculated tissue there was less of a drop in concentration from healthy levels, and the tetraploids had more than the diploid varieties.

Genistein

There were significant differences between treatments.

In healthy tissue, the 2nR and 4n/RB varieties had significantly lower concentrations than the other two varieties.

In S. trifoliorum-infected tissue, there was no significant differences between varieties but a highly significant rise from healthy concentrations.

In B. cinerea-infected tissue the only difference was between the 2nS/2 and 4n/RB varieties. There was significantly more than in healthy tissue but significantly less than in S. trifoliorum-infected tissue.

There was no correlation between resistance and concentrations in different varieties, but there was generally much more in infected tissue compared to healthy tissue.

Daidzein

This was only detected in tissue infected with S. trifoliorum. The concentrations did not differ significantly from each other in the four varieties (Tables 9 and 10).

Table 3 Concentrations (μg) of main isoflavones in lg fresh weight of leaf tissue

Variety	BIOCHANIN A			FORMONONETIN			GENISTEIN		
	H	S.t	B.c	H	S.t	B.c	H	S.t	B.c
2nR	226	110	385	334	35	207	56	333	198
4nR	389	118	623	528	72	320	92	296	218
2nS/2	524	68	344	502	24	189	102	452	136
4n/RB	540	67	470	515	29	327	44	471	278

Each figure is the mean of 3 replicates

H = uninoculated tissue, S.t = S. trifoliorum-infected tissue, B.c = B. cinerea-inoculated tissue

Table 4 Analysis of variance of isoflavone concentrations ($\mu g/gm$) as $\log_{10}$ numbers

	S.S.	df	Variance	F	P
<u>Biochanin A</u>					
Varieties	0.1343	3	0.0447	6.21	0.1%
Treatments	3.7006	2	1.8503	257	0.1%
Interaction	0.4006	6	0.0667	9.3	0.1%
Residual	0.1740	24	0.0072		
Total	4.4095	35			
<u>Formononetin</u>					
Varieties	0.346	3	0.1153	21.35	0.1%
Treatments	7.9573	2	3.9786	736.8	0.1%
Interaction	0.2535	6	0.0422	7.8	0.1%
Residual	0.1297	24	0.0054		
Total	8.6865	35			
<u>Genistein</u>					
Varieties	0.0752	3	0.025	1.786	-
Treatments	3.4917	2	1.7458	124.7	0.1%
Interaction	0.4962	6	0.0819	5.85	0.1%
Residual	0.2516	24	0.014		
Total	4.2247	35			

(Note on presentation of Duncan's Multiple Range Test - Table 5 etc. The means of each treatment are given. Any two means underscored by the same line are not significantly different)

Table 5 Use of Duncan's multiple range test to compare varieties within a certain treatment of isoflavone concentrations

Biochanin A

	2nR	4nR	2nS/2	4n/RB
Healthy	7.03	7.77	8.09	8.19

	4n/RB	2nS/2	2nR	4nR
S. trifoliorum-inoculated	5.47	5.49	6.08	6.20

	2nS/2	2nR	4n/RB	4nR
B. cinerea inoculated	7.60	7.75	7.99	8.36

Formononetin

	2nR	2nS/2	4n/RB	4nR
Healthy	7.56	8.09	8.10	8.16

	2nS/2	4n/RB	2nR	4nR
S. trifoliorum-inoculated	4.14	4.39	4.60	5.56

	2nS/2	2nR	4nR	4n/RB
B. cinerea inoculated	6.82	6.95	7.51	7.52

Genistein

	4n/RB	2nR	4nR	2nS/2
Healthy	4.93	5.00	5.89	6.02

	4nR	2nR	2nS/2	4n/RB
S. trifoliorum-inoculated	7.41	7.51	7.96	8.02

	2nS/2	2nR	4nR	4n/RB
B. cinerea inoculated	6.41	6.87	7.01	7.32

Total Isoflavones

The isoflavone totals are shown in Table 6. Analysis of variance showed there to be significant differences between varieties and treatments with also a significant interaction. Using the means of the treatments and of the varieties, Duncan's Multiple Range test showed that the S. trifoliorum-inoculated tissue had lower concentrations than the other two treatments, and the 2nR variety had generally less isoflavone than the other varieties. A study of the interaction showed that there was no general trend in the differences.

b) Pterocarpans

The concentrations of medicarpin and maackiain are given in Table 7. The data was treated to statistical analysis.

Medicarpin

This was not detected in healthy tissue. There was significantly more in B. cinerea-inoculated tissue than in S. trifoliorum-infected tissue. In the latter there was only a significant difference between the highest (2nR) and lowest (2nS/2) concentrations. In B. cinerea-inoculated tissue the 2nR and 4nR varieties were not significantly different from each other, but the 4nR variety had higher concentrations than the other two varieties.

The general trend was for the resistant varieties to have higher concentrations of medicarpin, and there was much less in pathogen-infected than in non-pathogen-infected tissue.

Maackiain

Again, this was not detected in healthy tissue, and there were higher concentrations in B. cinerea-inoculated tissue than in S. trifoliorum-infected tissue. In the latter the 2nR and 4nR varieties were not significantly different from each other, but both had higher concentrations

Table 6 Total isoflavones in µg/g fresh weight

Variety	H	S.t	B.c
2nR	616	547	790
4nR	1009	537	1161
2nS/2	1128	599	669
4n/RB	1099	626	1075

H = healthy, S.t = *S. trifoliorum*-infected, B.c = *B. cinerea* inoculated.

All weights expressed as the mean of three replicates.

Each replicate isoflavone total was derived by adding together the weights of biochanin A, formononetin, genistein and, when it occurred, daidzein.

Analysis of variance of total isoflavone data.

	SS	df	Variance	F	P
Varieties	441081	3	147027	8.3	0.1%
Treatments	1081419	2	540709	30.56	0.1%
Interaction	569765	6	94961	5.4	1.0%
Residual	424624	24	17692		
Total	2516889	35			

Duncan's Multiple Range Test

Treatments

S.t	B.c	H
577	923	963

Varieties

2nR	2nS	4nR	4n/RB
651	799	902	932

than the 2nS/2 and 4n/RB varieties. The 4n/RB variety had more than the 2nS/2 variety. With B. cinerea-inoculated tissue, all the varieties had significantly different amounts, but the more resistant varieties had higher concentrations.

As with medicarpin, the resistant varieties had higher concentrations and there was far less maackiain in tissue infected with the pathogen than with the non-pathogen.

6a-hydroxy maackiain

This compound was only detected in S. trifoliorum-infected tissue. The 2nR variety had more than the other varieties. The two tetraploid varieties and the 4n/RB and 2nS/2 varieties were not different but the 4nR variety contained more than the 2nS/2 variety (Tables 9 and 10).

Fraction A

This band was only detected in B. cinerea-inoculated tissue in this experiment although it had been found in S. trifoliorum-infected tissue in qualitative extractions and Cladosporium bioassays. Only the 2nS/2 variety differed from the other varieties having significantly less (Tables 9 and 10).

Summary of quantitative analysis

With the isoflavones there was generally no correlation between resistance and higher concentrations. With S. trifoliorum-infected tissue, both biochanin A and formononetin concentrations dropped whereas genistein concentrations rose from the concentrations in healthy tissue. With B. cinerea inoculated tissue, the concentrations of formononetin again dropped and genistein again rose, but less so than with S. trifoliorum. Biochanin A concentrations varied, with the 2nR and 4nR varieties having higher and the 2nS and 4n/RB varieties having lower concentrations than found in healthy tissue.

There was a correlation with resistance and higher concentrations of medicarpin and maackiain, but with S. trifoliorum-infected tissue, the concentrations were far lower than in B. cinerea-inoculated tissue. There was none detected in healthy tissue. Fraction A, found only in B. cinerea inoculated tissue, was also in higher concentrations in the more resistant varieties, as was 6a-hydroxy maackiain in S. trifoliorum-infected tissue, but not all of these differences were statistically significant.

Table 7 Weight (μg) of medicarpin and maackiain in 1g fresh weight of leaf tissue

(Mean of 3 replicates)

	MEDICARPIN			MAACKIAIN		
Variety	H	S.t	B.c	H	S.t	B.c
2nR	0	18	220	0	87	1421
4nR	0	12	401	0	82	2550
2nS/2	0	9	154	0	31	896
4n/RB	0	12	177	0	65	1153

H = healthy tissue, S.t = *S. trifoliorum*-infected tissue, B.c = *B. cinerea*-inoculated tissue

Table 8 Statistical analysis of $\log_{10}$ concentrations per gm of a medicarpin and maackiain

A) Main effects and interactions - analysis of variance

	SS	df	Variance	F	P
<u>Medicarpin</u>					
Varieties	0.3668	3	0.1222	4.364	1%
Treatments	9.7741	1	9.7741	349	0.1%
Interaction	0.2687	3	0.0895	3.196	-
Residual	0.4490	16	0.028		
Total	10.7586	23			
<u>Maackiain</u>					
Varieties	0.6305	3	0.2101	70.033	0.1%
Treatments	11.0637	1	11.0637	3687.9	0.1%
Interaction	0.0836	3	0.0278	9.267	
Residual	0.0485	16	0.0030		
Total	11.8263	23			

B) Duncan's multiple range test

<u>Medicarpin</u>

	2nS/2	4n/RB	4nR	2nR
<u>S. trifoliorum-</u> inoculated	2.575	3.199	3.207	3.734

	2nS/2	4nS/RB	2nR	4nR
<u>B. cinerea</u> inoculated	6.517	6.728	6.995	7.791

<u>Maackiain</u>

	2nS/2	4n/RB	4nR	2nR
<u>S. trifoliorum-</u> inoculated	4.44	5.42	5.72	5.77

	2nS/2	4n/RB	2nR	4nR
<u>B. cinerea</u> inoculated	8.27	9.18	9.45	10.19

all different

Table 9 Concentrations (μg/g fresh weight) of daidzein, fraction A and 6a-hydroxy maackiain in infected clover leaves

Variety	Daidzein S.t	Fraction A B.c	6a-hydroxy maackiain S.t
2nR	69	153	407
4nR	51	166	283
2nS/2	55	81	173
4n/RB	59	126	214

S.t = *S. trifoliorum*-infected tissue, B.c = *B. cinerea*-inoculated tissue

Although a mixture of 2 compounds, the concentrations of fraction A are calculated using the extinction coefficient of pisatin alone.

The results are expressed as a mean of 3 replicates.

Table 10 Statistical analysis of data in Table 9

	SS	df	Variance	F	P
Daidzein					
Varieties	496	3	165.3	0.3758	-
Residual	3519	8	439.9		
Total	4015	11			

No differences with Duncan's multiple range test

	SS	df	Variance	F	P
Fraction A					
Varieties	2.929	3	0.976	9.385	0.01
Residual	0.834	8	0.104		
Total	3.763	11			

2nS	4nS	2nR	4nR
1.23	1.91	2.32	2.52

	SS	df	Variance	F	P
6a-hydroxymaackiain					
Varieties	19.87	3	6.62	12.73	0.01
Residual	4.14	8	0.52		
Total	24.01	11			

2nS	4nS	4nR	2nR
2.5	3.1	4.1	5.9

Bioassays against S. trifoliorum

The method used is described in Materials and Methods. It was decided to use a liquid medium after attempting bioassays with biochanin A in agar medium of several types. The problem with using solid medium was the uneven distribution of the biochanin A. This accumulated at the bottom of the medium and the fungus did not come into contact with it but grew mostly near and on the surface of the medium. When concentrations above 50 µg/ml were used, these were higher than the solubility in water, and the undissolved compound settled out when put into molten agar medium. To try and overcome this, the biochanin A was autoclaved with the medium, but this made no difference. It was also found very difficult to evenly distribute a small amount of solvent, containing the biochanin A, into molten agar medium.

In liquid medium a small amount of solvent, containing isoflavone, was easily distributed. However, even in this system the isoflavones came out of solution as soon as they were added to the medium at concentrations above 50 µg/ml, but this was always in a very fine form which could be maintained at an even distribution by incubating the flasks in a rotary shaker.

Because this method required quite large amounts of the compounds for testing, it was only possible to bioassay biochanin A, formononetin, genistein, medicarpin and maackiain against S. trifoliorum.

1. Isoflavones

The results of bioassays using the isoflavones (from commercially available sources) are shown in Table 11. The statistical analysis of this data (Table 12) showed that none of the isoflavone concentration significantly reduced the dry weight of S. trifoliorum from the controls. Therefore the isoflavone do not have any effect against mycelial growth of S. trifoliorum in liquid medium.

2. Pterocarpans

The results of bioassays using medicarpin and maackiain are given in

Table 13. It is clear from this table that medicarpin reduces the dry weight of S. trifoliorum with increase in concentration. The weight shown for the 100 μg/ml concentration was no more than that of the initial inoculum, therefore there was complete inhibition at this concentration. The $E.D._{50}$ for mycelial dry weight was approximately 50 - 60 μg/ml of medicarpin, but there was no effect on the dry weight with concentrations up to 30 μg/ml. However, all of these concentrations were higher than those found in leaf tissue infected with S. trifoliorum, the highest concentration for a replicate being 20 μg/gm. Therefore, in infected tissue, medicarpin would have had no effect on S. trifoliorum.

The maackiain data, when subjected to statistical analysis showed there to be no deviation from the controls (F value 0.86). Therefore maackiain had no effect against S. trifoliorum.

Of the isoflavones and pterocarpans tested, only medicarpin had any effect on the growth of S. trifoliorum, but this was at concentrations higher than those found in S. trifoliorum-infected tissue.

Table 11 Bioassays of S. trifoliorum against isoflavones

Concentration (μg/ml)	Mycelial dry weights* (g)		
	BIOCHANIN A	FORMONONETIN	GENISTEIN
0	0.09592	0.09149	0.13300
50	0.08244	0.06700	0.12366
100	0.08355	0.10113	0.12916
200	0.09478	0.06942	0.10863
350	0.07799	0.10693	0.14090
500	0.05507	0.10121	0.10515
650	0.06756	0.12543	0.10354

* Means of 4 replicates. Weights taken after ten days

Table 12 Analyses of variance of bioassays of isoflavones against *S. trifoliorum*

	SS	df	Variance	F
Formononetin				
Treatments	0.010457	6	0.001742	2.07
Residual	0.01764	21	0.000840	
Total	0.028097	27		
Biochanin A				
Treatments	0.005108	6	0.000851	1.3
Residual	0.013769	21	0.000655	
Total	0.018877	27		
Genistein				
Treatments	0.005285	6	0.0008808	1.8
Residual	0.01035	21	0.0004928	
Total	0.01564	27		

Table 13 Bioassays of S. trifoliorum against medicarpin and maackiain

Concentration (μg/ml)	Mycelial dry weights* (g)	
	MAACKIAIN	MEDICARPIN
0	0.06182	0.06785
10	0.07103	0.07890
20	0.08179	0.08735
30	0.06684	0.08606
50	0.06896	0.03798
80	0.06686	0.00282
100	0.07515	0.00062

* Means of 4 replicates. Weights taken after 10 days.

$E.D._{50}$ for mycelial dry weight of S. trifoliorum against medicarpin is somewhere between 50 and 60 μg/ml of medicarpin.

Bioassays against B. cinerea

For testing compounds against B. cinerea the spore germination and germ-tube growth bioassay method was used, as described in Materials and Methods. The advantages of this method are that it takes only small quantities of the test compound to carry out a full range of concentrations to ascertain the $E.D._{50}$, and as it takes only 24 hours incubation, no special care is needed to avoid contamination.

a) Isoflavones

The results of the bioassays of the isoflavones against B. cinerea are given in Table 14. Spore germination was not significantly decreased by any of the isoflavones. There was, however, a significant reduction of germ-tube length by all three isoflavones, biochanin A reducing the growth to 70% of the control, formononetin reducing growth to 40% of the control and genistein reducing growth to 50% of the control. Low concentrations (10 - 20 μg/ml) of all three isoflavones reduced growth of the germ-tube, but higher concentrations appeared to have little or no further effect (confirmed by using Duncan's multiple range test). Therefore maximum inhibition was reached at concentrations far lower than found in clover leaf tissue.

b) Pterocarpans

The results are shown in Tables 15 and 16. The most active of the pterocarpans was medicarpin which had an $E.D._{50}$ for spore germination of 30 μg/ml and for germ-tube growth of 20 μg/ml, and completely prevented germination at 100 μg/ml. Maackiain did not significantly decrease spore germination. It did significantly affect germ-tube growth but, as with the isoflavones, low concentrations caused large reductions in length and higher concentrations caused little or no further decrease. Fraction A (a mixture of pisatin and homopisatin) was shown to significantly reduce germination but by only 7% to 93% of the control. Germ-tube length was

reduced far more, the $E.D._{50}$ being slightly less than 1.0 absorbance unit/ml (approximately 60 µg/ml if calculated using the extinction coefficient of pisatin). 6a-hydroxy maackiain did not significantly decrease spore germination, but germ-tube length was almost reduced to 50% at 6 absorbance units/ml (414 µg/ml). This concentration was slightly more than that found in S. trifoliorum infected tissue, but this compound was not detected in B. cinerea inoculated tissue.

Table 14 *B. cinerea* spore germination tests against the isoflavones

	BIOCHANIN A		FORMONONETIN		GENISTEIN	
Concentration µg/ml	% Spore Germination	Germ-tube Length (µm)	% Spore Germination	Germ-tube Length (µm)	% Spore Germination	Germ-tube Length (µm)
0	98.3	215.5	99.5	251.3	96.0	127.8
10	-	-	97.5	136.1	89.7	92.2
20	-	-	97.2	139.4	79.7	36.3
40	96.3	195.7	98.0	136.8	89.3	67.1
80	96.8	156.6	-	-	88.8	69.3
100	96.7	154.0	97.6	118.9	88.7	67.8
250	96.0	147.1	96.5	103.3	93.7	66.7
500	97.0	148.4	96.0	104.5	91.7	75.2

Each spore germination figure is the mean of at least three replicate drops of 100 counted spores

Each germ-tube length figure is the mean of at least 30 replicates

F values for germ-tube length were - biochanin A 5.24, formononetin 4.1 and genistein 15.7

Table 15 *B. cinerea* spore germination tests against medicarpin and maackiain

Concentration µg/ml	MEDICARPIN		MAACKIAIN	
	Spore Germination %	Germ-tube length (µm)	Spore Germination %	Germ-tube length (µm)
0	99.8	131.4	99.6	165.9
10	97.2	117.6	94.4	48.4
20	92.2	65.6	88.4	31.3
30	48.0	23.6	93.2	43.5
40	22.6	15.6	91.2	33.9
50	8.0	-	93.5	41.1
80	4.8	-	91.9	39.7
100	0	-	92.3	36.3

Each spore germination figure is the mean of at least 3 replicate drops of 100 counted spores

Each germ-tube length is the mean of at least 30 replicates

Table 16 *B. cinerea* spore germination tests against fraction A and 6a-hydroxy maackiain

Absorbance/ ml	FRACTION A		6a-HYDROXY MAACKIAIN	
	Spore germination %	Germ-tube length (µm)	Spore germination %	Germ-tube length (µm)
0	100	361.5	100	408.0
0.5	100	256.8	-	-
1.0	97.3	195.4	99.3	330.3
2.0	93	101.4	99.7	233.8
4.0	93	89.4	-	-
6.0	-	-	97.7	212.4

Each spore germination figure is the mean of three replicate drops of 100 counted spores

Each germ-tube length figure is the mean of 30 replicates

Studies on degradation of phytoalexins by S. trifoliorum

As shown in the quantitative analysis of compound in clover leaf extracts, the concentrations of phytoalexins in S. trifoliorum-infected tissue extracts were far lower than the concentrations in extracts of tissue inoculated with B. cinerea. There are two possible reasons why this was so. Firstly, production of the phytoalexins was less, due either to failure of S. trifoliorum to induce them, or to inhibition by S. trifoliorum of the biosynthesis. Secondly, production of the phytoalexins did take place but the fungus was able to convert them to less inhibitory compounds. The second possibility was the one favoured, especially as it was known that crude protein from S. trifoliorum culture filtrates were able to induce phytoalexins, albeit in small concentrations in that particular experiment.

Experiments were carried out to study the possible degradation of the phytoalexins by S. trifoliorum. The method used was similar to that of Higgins and Millar (1969a) except that spores could not be used, homogenised mycelium was therefore used instead. The method is fully described in Materials and Methods, but briefly, small amounts of phytoalexin were added to 5 ml of homogenised mycelium for various incubation times. The fungus was then centrifuged off and the supernatant partitioned twice with carbon tetrachloride, followed by twice with ethyl acetate. These two solvents differ in their polarity, more polar compounds (i.e. those more soluble in water) being more soluble in ethyl acetate than carbon tetrachloride. Both maackiain and medicarpin were soluble in carbon tetrachloride. The fungal pellet was washed in ethanol to take up any phytoalexin that may have absorbed onto the mycelium. All these extracts were dried down, taken up in ethanol and scanned in a UV spectrophotometer.

Degradation of maackiain

Figures 21 and 22 show the UV spectra of the carbon tetrachloride and ethyl acetate extracts in ethanol, corresponding to various incubation

times. The carbon tetrachloride extracts show the disappearance of maackiain with time from the medium, which is almost complete by 9 hours. No new peaks appear on the UV scans and therefore no new compounds appear in this extract.

With the ethyl acetate extract at time 0, the UV spectrum had a peak at 265 nm but with subsequent times this peak completely disappears. It is not known what caused this peak, although it may have been in part due to dimethyl sulphoxide which gives a peak at 245 nm. After time 0, new peaks appeared which increased in absorbance with time. These new peaks had similar characteristics to those of maackiain, being optimal at 287 and 310 nm with a shoulder at 282 nm. The 310 nm peak was less pronounced than the corresponding peak of maackiain.

These crude extracts were purified on TLC using a 2% (v/v) methanol in chloroform solvent system, then eluted and the absorbance measured at 287 nm for both maackiain and its degradation product, the results of which are given in Table 17. The recovery of both compounds from the medium was not particularly good, being only 60% recovery for maackiain. When a control was carried out with maackiain in water, 100% recovery was obtained on partitioning with carbon tetrachloride. What causes this apparent disappearance of maackiain is not known, but it may be due to absorption into the fungal mycelium where it becomes tightly bound so that washing the mycelium with ethanol does not recover it. This loss was also observed in similar experiments by Lyon (1971) using phaseollin. 70% of the maackiain applied to TLC plates was recovered, so that the data in Table 17 at time 0 is effectively only 40% of the maackiain originally used.

From Table 17, one can see that most of the maackiain was gone, if not by 6 hours, then certainly by 9 hours. Conversely, the degradation product was first detected after 3 hours, reached a peak at 12 hours, and then began to decline. No new bands could be detected on TLC plates after 12 hours, therefore the degradation product must have been absorbed by the fungus and possibly metabolised.

A control, with maackiain in dimethylsulphoxide dissolved in 5 ml of water for 24 hours, yielded 90% of the maackiain after the incubation time upon partitioning the water with carbon tetrachloride. Therefore the loss of maackiain was due to the fungus.

The ethanol extract of the centrifuged mycelium showed no changes in its UV spectra throughout the entire experiment.

Identification of maackiain degradation product

When it was run on TLC with a 2% (v/v) methanol in chloroform solvent system, the R_f value of the degradation product was approximately 0.25. This was the same R_f value as that of 6a-hydroxy maackiain found in *S. trifoliorum*-infected leaf tissue and because the UV spectra were very similar, these two compounds were compared to confirm if they were the same. The R_f values of the two were compared in two completely different TLC systems, firstly on silica gel with a solvent of 2% (v/v) methanol in chloroform, and secondly on polyamide with a solvent of 15% (v/v) water in methanol. In both systems the R_f values were identical for the two compounds, being 0.25 on silica gel and 0.4 on polyamide. The appearance under UV light at 254 nm was the same for both, being an absorbing dark-blue colour. After exposure to UV light for a short while, both spots turned red and when eluted and run on TLC on silica gel with a solvent of diethyl ether: petroleum ether (50:50, v/v), the red spot ran ahead of the absorbing spot, the R_f values for both spots being identical for both 6a-hydroxy maackiain and the degradation product. With a *Cladosporium* bioassay the degradation product caused an inhibitory zone, as did 6a-hydroxy maackiain. Both UV spectra were identical (see figure 16) and the mass spectra (obtained by J. Bilton) finally confirmed that 6a-hydroxy maackiain and the degradation product were the same compound. Therefore the degradation product of maackiain had been found both *in vitro* and *in vivo*.

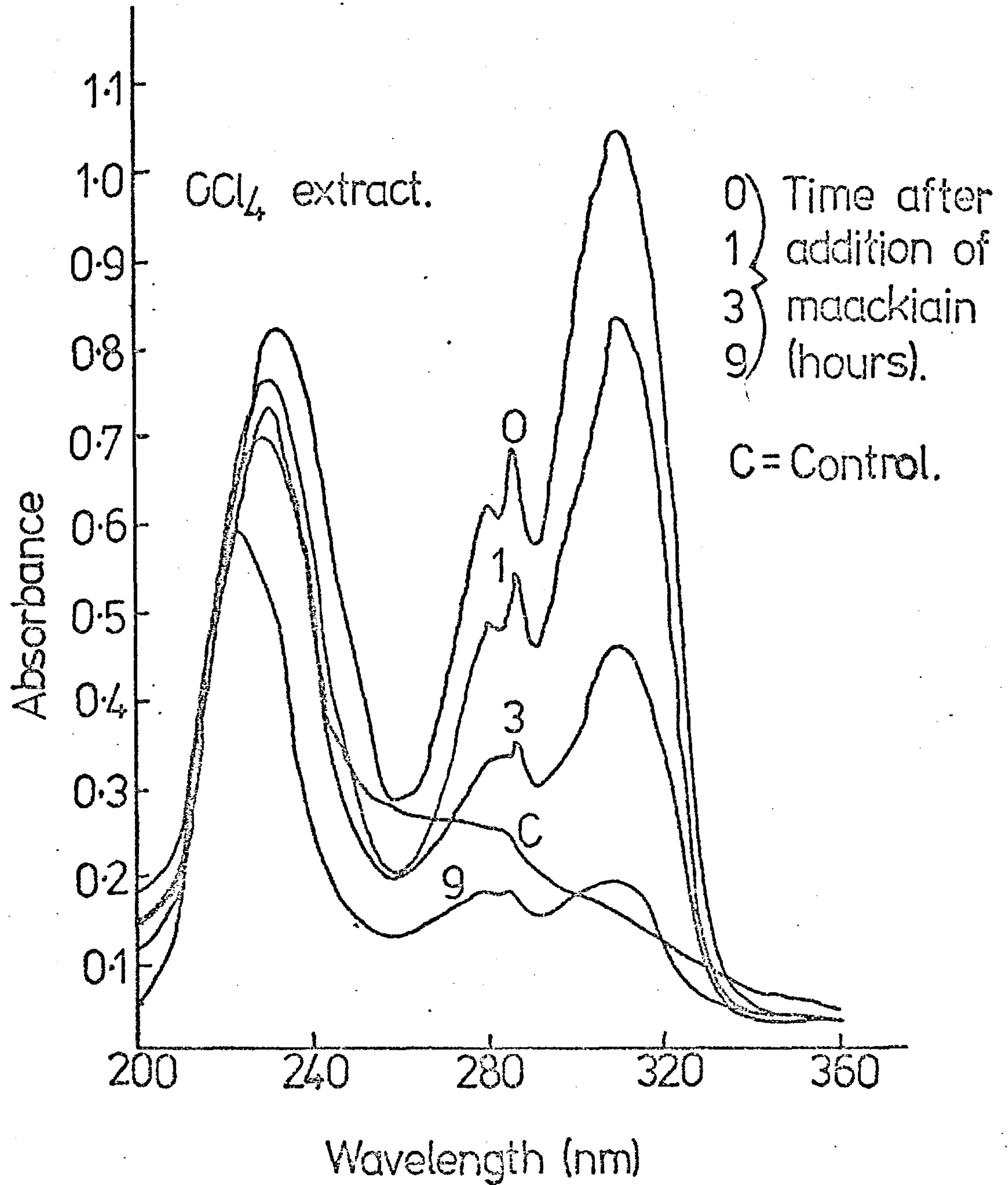
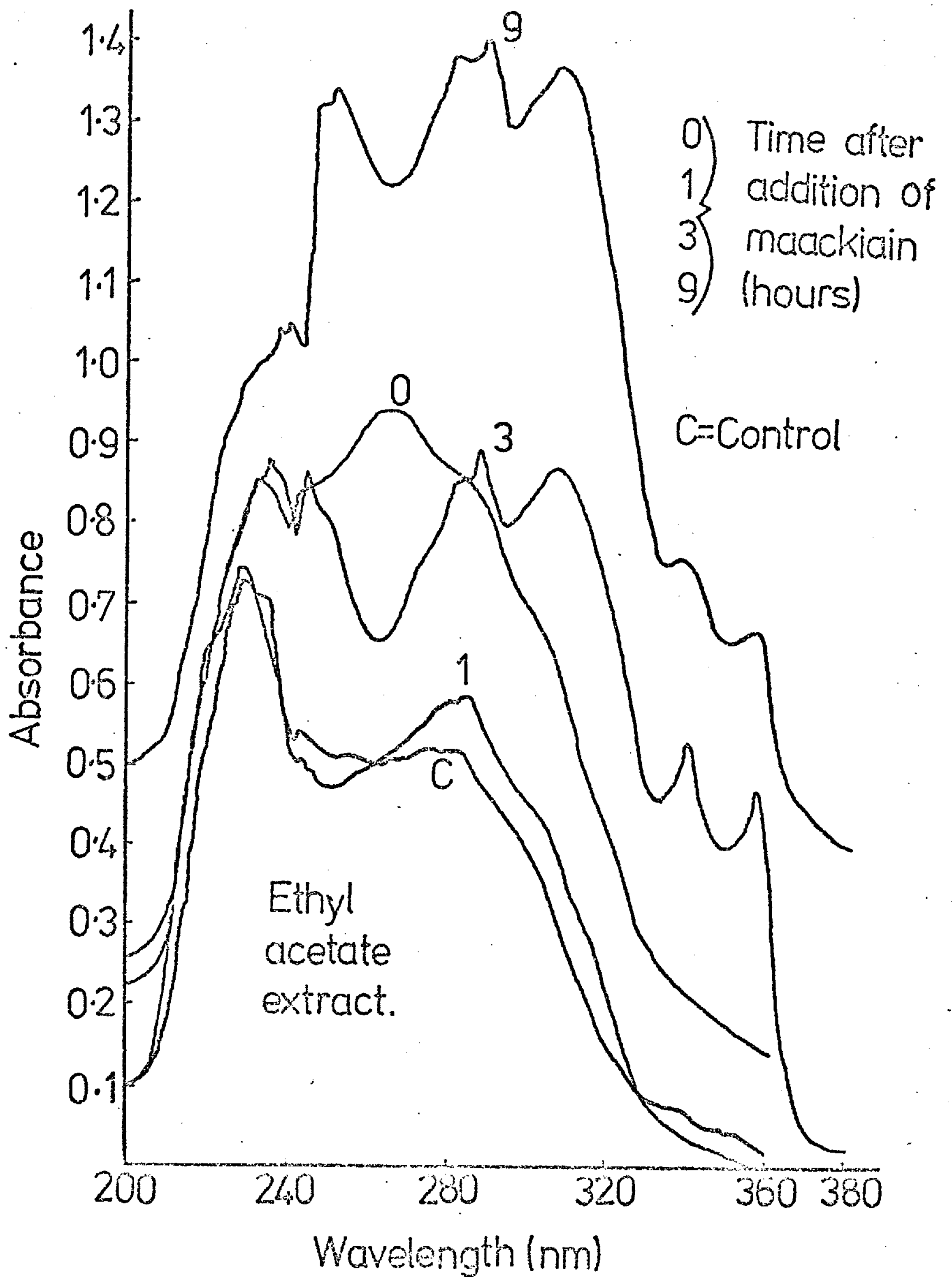


FIGURE 21

METABOLISM OF MAACKIAIN BY S.TRIFOLIORUM (1)

UV spectra of the dried down extract which had been redissolved in 2 ml of ethanol



METABOLISM OF MAACKIAIN BY S.TRIFOLIORUM (2).

FIGURE 22

UV spectra of the dried down extract which had been redissolved in 2 ml of ethanol

Table 17 Studies on degradation of phytoalexins by S. trifoliorum

a) Amount of maackiain and its degradation product after purification on TLC (µg per replicate flask, mean of three)

Time (hours)	Maackiain (µg)	Degradation Product (O.D. at 287 nm)
0	56.76	-
1	55.44	-
3	38.28	0.44 (30.4)*
6	11.88	0.52 (35.9)
9	-	0.48 (33.1)
12	-	0.58 (40.0)
18	-	0.42 (29.0)
24	-	0.32 (22.1)

* Amounts per flask in µg, calculated by using the extinction coefficient of 6a-hydroxy maackiain.

b) Amount of medicarpin and its degradation product after purification on TLC (µg per replicate flask, mean of three)

Time (hours)	Medicarpin (µg)	Degradation Product (O.D. at 287 nm)
0	52.36	-
1	44.20	0.30
3	21.06	0.70
6	7.480	0.46
9	10.20	0.74
12	-	0.70
18	-	0.74
24	-	0.68

Degradation of medicarpin

Figures 23 and 24 show the UV spectra of the carbon tetrachloride and ethyl acetate fractions in ethanol. As happens with maackiain, the medicarpin peak in the carbon tetrachloride fraction became smaller with time, showing its disappearance from the medium. The peak reached its lowest absorbance after 12 hours, at which level it stayed for the remainder of the experiment. This peak present after 12 hours was probably not due to medicarpin but due to the conversion product, some of which was taken up into the carbon tetrachloride.

The UV spectra of ethyl acetate extracts at time 0 were again composed of large peaks from 240 to 280 nm (see figure 24), but with subsequent extracts this disappeared. After time 0, a different peak was present which became more pronounced up to about 12 hours. This new spectrum was very similar to that of medicarpin, with a peak at 287 nm, a shoulder at 282 nm and no peak at 310 nm. These crude extracts were purified on TLC using a 2% (v/v) methanol in chloroform solvent system, eluted and the absorbance measured at 287 nm for both medicarpin and its conversion product, the results being given in Table 17.

The UV spectra of ethanol extracts of the spun down mycelial pellet of controls showed no peaks except at very low wavelengths. However, throughout the whole experiment, a peak with a maximum of 272 nm was present in other ethanol extracts, where medicarpin had been added. As this remained constant throughout the experiment it was not investigated further, but may have been due to small amounts of medicarpin.

Comparison of medicarpin with its conversion product

Purified medicarpin conversion product (from TLC with 2% (v/v) methanol in chloroform) was compared with medicarpin by UV spectra and TLC properties. The UV spectra of both compounds were identical, the same as the spectrum of medicarpin shown in figure 8. When NaOH was added, the bathochromatic shift was identical for both compounds, with peaks at 290 and

250 nm (see figure 8). In different TLC systems, the following R_f values were obtained :-

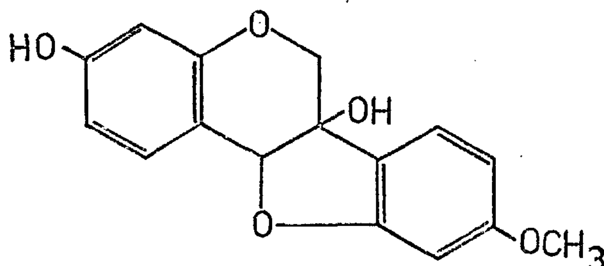
15% (v/v) Water in methanol on polyamide	0.43 for both
2% (v/v) methanol in chloroform on silica gel	0.21 for both
Diethylether:petroleum ether (50:50, v/v) on silica gel	0.27 for both

A Cladosporium TLC plate bioassay was carried out on the second of the above TLC plates and the conversion product was found to cause an antifungal zone. Because of lack of the conversion product, it was impossible to carry out any further bioassays.

From the data known about the conversion product, the only difference between it and medicarpin was the different solubilities in carbon tetrachloride and ethyl acetate. This suggests that the conversion product was more polar than medicarpin, but this difference in polarity was not enough to cause differences of R_f values on TLC plates.

Tentative Identification of the medicarpin conversion product

A sample of the medicarpin conversion product was given to Mr. J. Bilton for chemical analysis. When mass was measured in a mass spectrometer, it was found to be 286. This was 16 units more than medicarpin, that is the addition of one oxygen atom. If the same process occurs with both medicarpin and maackiain, as seems likely, then the additional oxygen was part of a hydroxyl group present at the 6a position, giving the following structure :-



6a-hydroxy medicarpin

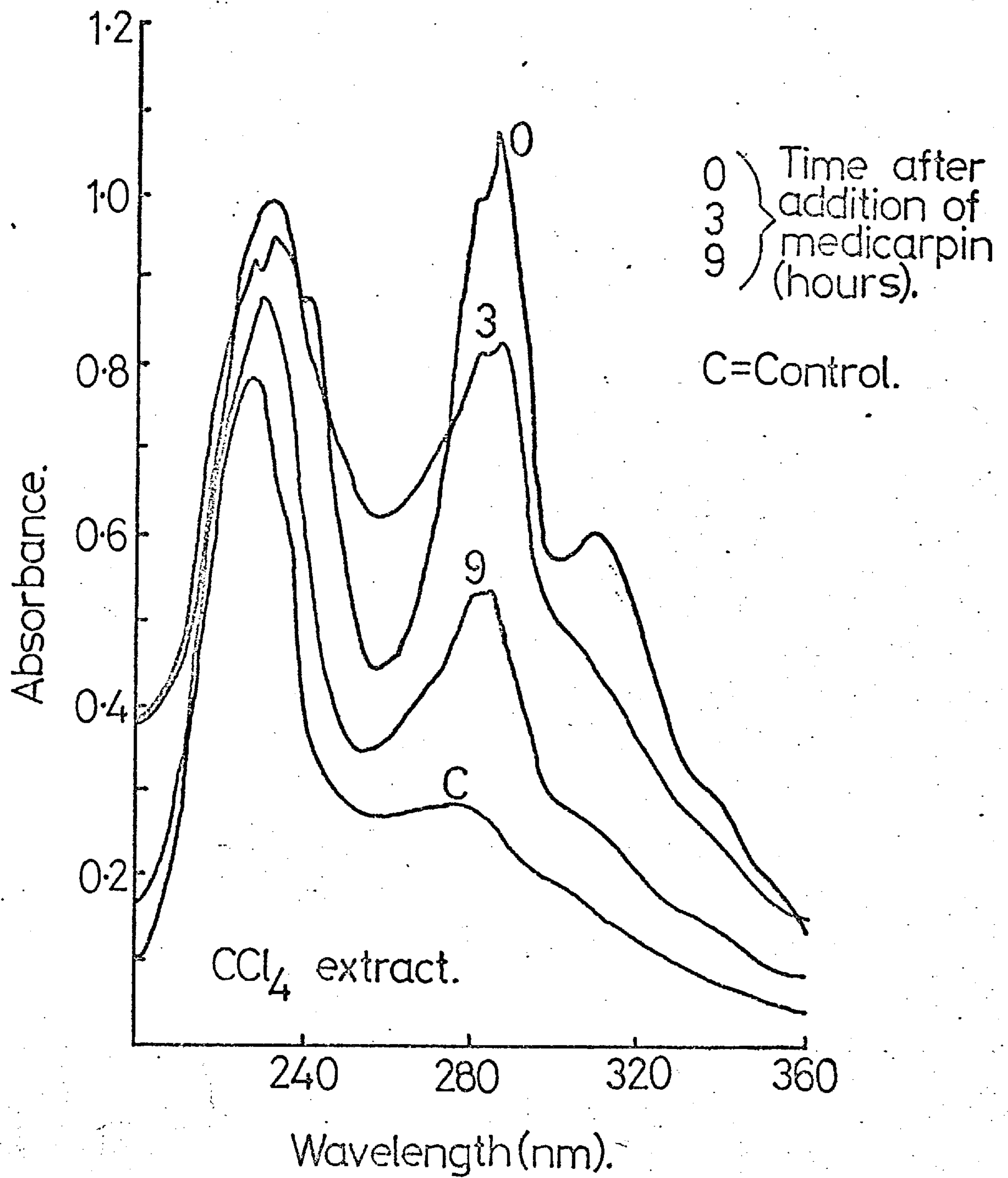


FIGURE 23 Metabolism of medicarpin by S. trifoliorum (1)

UV spectra of the dried down extract, redissolved in 2 ml of ethanol

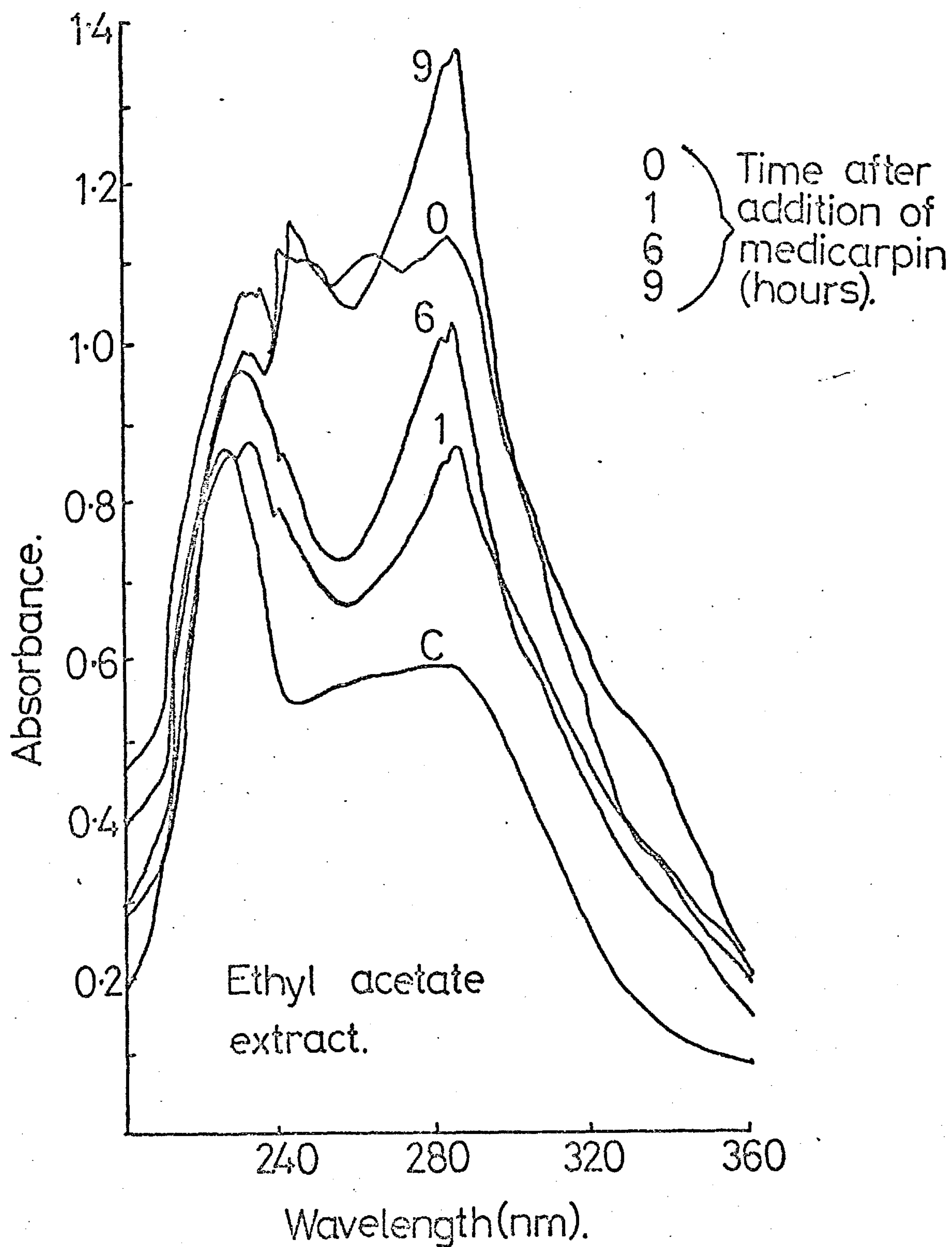


FIGURE 24 Metabolism of medicarpin by S. trifoliorum (2)

UV spectra of the dried down extract, redissolved in 2 ml of ethanol

Isoflavones and S. trifoliorum in vitro

Upon infection of leaves with S. trifoliorum, the concentrations of the isoflavones, biochanin A and especially formononetin, dropped. There were several possible explanations for this reduction; firstly the isoflavones were being metabolised by the plant, or secondly that they were being metabolised by the fungus and either being converted to some other compound, as with the phytoalexins, or used as a foodsource. The second possibility was studied further using a method similar to that used in phytoalexin degradation studies. 10 ml of homogenised S. trifoliorum mycelium was put into 100 ml flasks and left for 24 hours to recover from homogenisation. The isoflavones were then added at a concentration of 100 μg/ml, therefore 1000 μg/flask, this being dissolved in 100 μl of dimethyl sulphoxide. At this concentration, some of the isoflavone came out of solution forming a fine suspension. The flasks were initially shaken to ensure even distribution. After the various incubation times the fungus was removed by centrifugation at 3000 rpm. This also brings down the isoflavone that was in suspension and therefore two extracts were carried out to recover the isoflavones. Firstly, the supernatant was partitioned three times with diethyl ether to obtain the 'ether extract', which was composed of the dissolved isoflavone. Secondly, the pellet was washed with ethanol to obtain the 'ethanol extract' which consisted of the undissolved isoflavone, and also any absorbed onto the mycelium. The absorbance was measured directly for each extract against an equivalent blank obtained by extracting controls (with no isoflavone) in the same way as above.

The results are given in Tables 18 and 19. It is clear that the ether extracts, i.e. the amounts of isoflavones dissolved in the medium, were very low, and most of the isoflavones were either absorbed onto the mycelium or in suspension in the medium. With formononetin, the total concentrations do not differ very much, and analysis of variance showed there to be no significant difference (F value 2.94). However, with

biochanin A there was an initial drop in concentration but by 96 hours the concentration was almost as high as at time 0. Analysis of variance gave a significant F value of 4.55. This initial drop could not have been due to degradation as the levels would not have risen again, but may have been because of some solubility phenomenon.

This shows that little, if any, biochanin A or formononetin was metabolised by S. trifoliorum. The lower concentrations of these two compounds in S. trifoliorum-infected tissue was therefore more likely due to the plant's metabolism, perhaps drawing the isoflavones into the pterocarpan biosynthetic pathway.

Table 18 Formononetin in *S. trifoliorum* liquid cultures

		Time (hours)			
		0	9	24	48
Absorbance	Ether Extract	0.26	0.69	1.62	0.86
	Ethanol Extract	8.7	7.9	8.1	8.1
Total concentration (μg/ml)		88.7	85.2	96.2	89.0

Each figure is the mean of 3 replicates

Table 19 Biochanin A in *S. trifoliorum* liquid cultures

		Time (hours)				
		0	9	24	48	96
Absorbance	Ether Extract	1.36	0.79	1.22	3.45	0.99
	Ethanol Extract	10.2	8.5	8.27	6.77	9.75
Total concentration (μg/ml)		93.6	75.2	76.9	82.8	87.1

Each figure is the mean of 3 replicates

Degradation of maackiain by B. cinerea

Although B. cinerea is not pathogenic to healthy red clover, and high concentrations of phytoalexins were found in tissue inoculated with B. cinerea, it was still possible that B. cinerea could degrade low levels of the phytoalexins. Non-pathogens of other plants had been shown capable of degrading the phytoalexins of those plants. Also, B. cinerea, being the imperfect stage of Sclerotinia fuckeliana, is closely related to S. trifoliorum. Therefore the enzymes necessary for S. trifoliorum to degrade the phytoalexins could also be present in B. cinerea. To study degradation, a similar method to that used for degradation of phytoalexins by S. trifoliorum was used. 5 ml of SCA medium was put into 50 ml Erlenmeyer flasks and autoclaved. After cooling, 0.5 ml of B. cinerea spores at 10^6 concentration was added and the flasks left for 24 hours to allow spore germination. 100 μg of maackiain dissolved in 50 μl of dimethyl/ sulphoxide were added to each flask, and the flasks harvested at 0, 5, 10 and 24 hours. The fungus was removed by centrifugation at 3000 rpm, and the supernatant partitioned twice with carbon tetrachloride, then twice with ethyl acetate. The fungal pellet was washed in ethanol, then the fungus removed by centrifugation at 3000 rpm. All extracts were dried down at 45°C at reduced pressure, taken up in 2 ml of ethanol and scanned in the UV spectrophotometer from 360 to 200 nm.

Figures 25 and 26 show the carbon tetrachloride and ethyl acetate extracts in ethanol corresponding to various incubation times. The carbon tetrachloride extract shows the disappearance from the medium of the maackiain, which by 24 hours had completely gone. The ethyl acetate extract shows the appearance of new peaks, which at 5 and 10 hours were very similar to the parent maackiain, with peaks at 310 and 287 nm with a shoulder at 282 nm. However, at 24 hours, the absorbance of the peaks had more than doubled and there was a slight shift so that the 282 nm shoulder was gone and the main peaks were at 286 and 302 nm.

TLC was carried out on the following fractions, carbon tetrachloride at time 0, and ethyl acetate at times 5, 10 and 24 hours, using a solvent system of 4% (v/v) methanol in chloroform. In the carbon tetrachloride fraction, maackiain was the main component at an R_f value of 0.7. In the ethyl acetate fraction there were two bands, the R_f values being 0.3 and 0.1. The 0.3 band was obvious at all three times, and its R_f value and UV spectrum were the same as fraction 4 (6a, hydroxy maackiain).

The second band, at R_f 0.1, was more conspicuous with the 24 hours extract and was visible under UV light at 254 nm, being faintly absorbing. However, a UV spectrum was not obtained when this band was eluted. Therefore a new compound was appearing after the longer incubation times.

Although not confirmed, it was probable that the band at R_f 0.3 was indeed fraction 4, thus showing _B. cinerea_ to have the same enzymes as _S. trifoliorum_ for converting maackiain. The band at R_f 0.1 was therefore a further conversion product of fraction 4. As _B. cinerea_ can convert maackiain, then why were there such large concentrations in leaf tissue inoculated with _B. cinerea_ ? The speed at which maackiain can be degraded may be important. _S. trifoliorum_ can probably convert maackiain fast enough to keep the concentrations in the tissue very low, whereas _B. cinerea_ cannot. Thus maackiain builds up to concentrations quite inhibitory to _B. cinerea_. Probably more important than this, however, is the presence of other antifungal compounds, and in particular medicarpin. In spore germination bioassays, medicarpin was far more active against _B. cinerea_ than was maackiain. Unfortunately, it was impossible to carry out degradation studies on medicarpin with _B. cinerea_ because of the unavailability of medicarpin at the time.

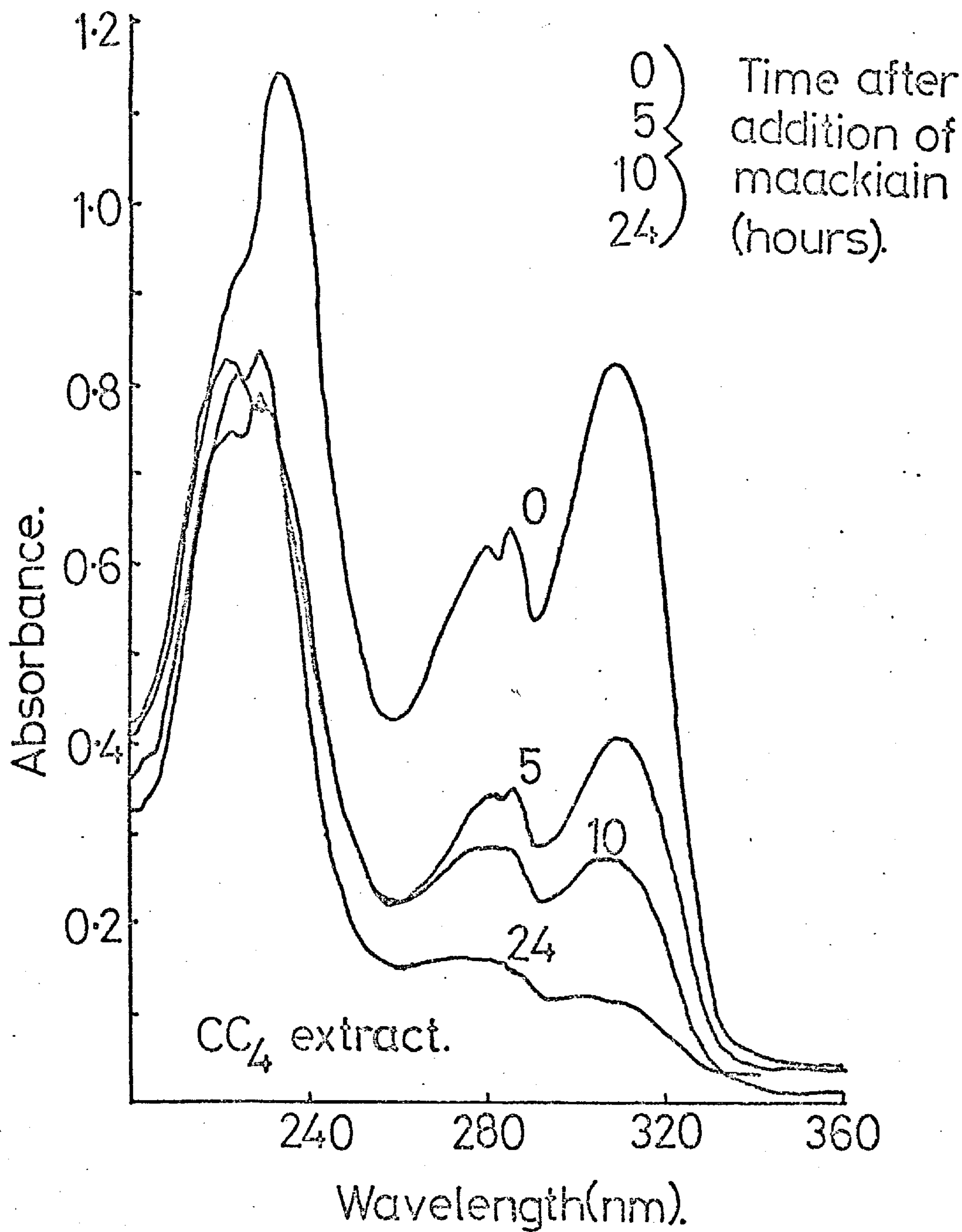


FIGURE 25

Degradation of maackiain by B. cinerea (1)

UV spectra of the dried down extract, redissolved in 2 ml of ethanol.

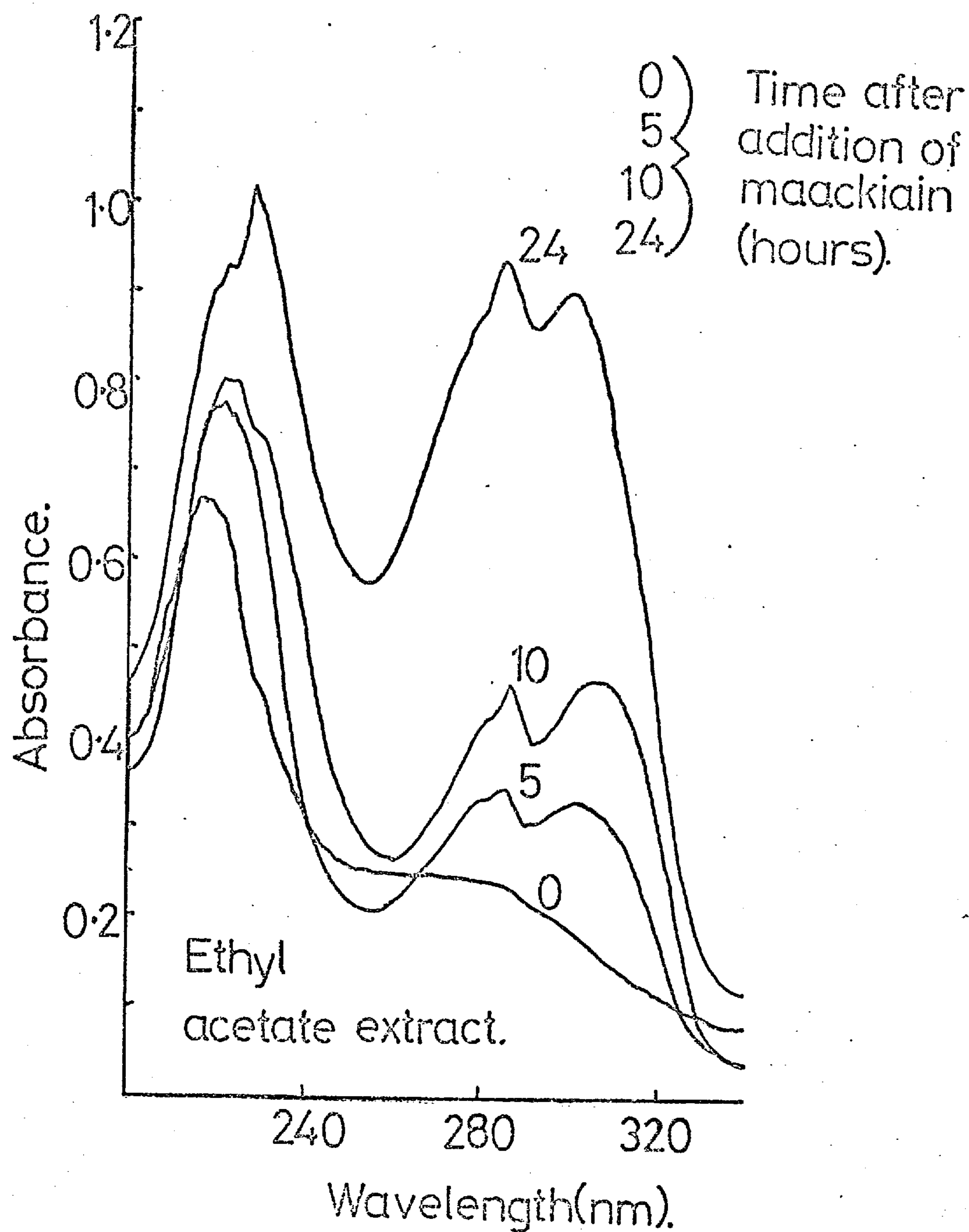


FIGURE 26

Degradation of maackiain by B. cinerea (2)

UV spectra of the dried down extract, redissolved in 2 ml of ethanol.

Development of a method for producing artificially limited lesions of S. trifoliorum on leaves of red clover

When leaves were inoculated with S. trifoliorum and incubated in conditions favouring lesion spread, no differences between greenhouse grown plants of different varieties could be detected. In natural infection, the spreading of lesions usually follows a latent period when only limited ascospore lesions are present. As it had been shown that varieties do not differ in the number of ascospore lesions produced upon them, but only on the spread of infection from the existing ascospore lesions (Dijkstra, 1966), the obvious way to detect varietal differences was to measure the spread of infection from limited lesions. However, ascospores can only be produceed in the autumn, as no reliable method for ascospore production in culture exists. Therefore a method for artificially producing limited lesions using an alternative type of inoculum was attempted. It was hoped that this method could be used at any time of the year.

Toynbee-Clarke (personal communication) had found that seed-tray testing of red clover plants using a bran inoculum of S. trifoliorum was greatly effected by a fall in humidity. When the seed-tray cover was removed so that the humidity dropped from 100%, the infection stopped spreading. Quite badly infected plants were able to produce new leaves and grow out from the disease. Loveless (1951b) had also found that ascospore lesions spread only in very high humidities. No one had reported, however, what happened to a lesion which had stopped spreading due to being removed from 100% relative humidity, when replaced back into a 100% relative humidity. It was not known if the lesion would again start to spread, or whether the drop in relative humidity caused an irreversible cessation of lesion spread. If the lesion started to spread again, then manipulation of the relative humidity could produce an artificially limited lesion.

Inoculation at the centre of the leaflet

The inoculum used was macerated S. trifoliorum mycelium in SCA medium, as used in previous inoculations. Whole plants were taken, their larger leaves were gently rubbed between the thumb and forefinger to disperse the wax bloom, and 20 µl drops of inoculum were placed onto the centre of the leaflets. The plants were placed underneath a glass cover so that the humidity would rise to 100%, and left for 24 hours. The cover was then removed so that the humidity dropped. Development of lesions was quite variable, some inoculation points not showing any browning, whilst others were completely brown underneath the inoculation drop. About an hour after removal of the cover, about half of the inoculated leaflets developed a curling of their distal end which was associated with browning and dessication of the tissue. This was a very similar reaction to that of leaves treated with toxin produced by S. trifoliorum in SCA medium. Some studies on this toxin are described later. Therefore, the leaf curling was probably due to this toxin which was possibly in high concentrations in the SCA medium of the inoculation drop.

To try and overcome the effect of the toxin, the experiment was repeated, but the mycelium before maceration was removed from the old medium by centrifugation at 3000 rpm, then added to an equal volume of fresh medium. This was then macerated and 20 µl drops put onto the leaves. After the 24 hours incubation at 100% humidity, the cover was removed so that the humidity dropped. An hour later, many of the inoculated leaflets had curled up distally, were brown and dessicated in the same manner as before. Apparently, the fungus was therefore producing enough toxin upon infection to cause this effect.

Inoculation at the distal end of the leaflet

The effect of the toxin from the point of inoculation was always towards the distal end of the leaflet. In order to minimise the effect of the toxin, the inoculation drop was placed onto the distal end of the

leaflet. The experiment was otherwise carried out in an identical way to above, using macerated mycelium in its original medium. An hour after the humidity dropped from 100%, only 2 out of a total of 18 inoculated leaflets showed any sign of damage by the toxin. None of the other leaflets showed any signs of toxin damage for two days after the drop in humidity, the period over which they were observed. All inoculation drops were thereafter placed at the distal end of the leaflets.

Standardization of the size of limited lesions

So far limited lesions could be obtained, but the size of them was very variable. It was more desirable, however, to have limited lesions of more or less the same size. There were two possible ways in which to improve the method, firstly to ensure that all inoculation drops would start to infect at the same time, and secondly, to ensure that all plants were in 100% humidity during all the time under the cover.

The only possible way to obtain infection simultaneously in all inoculation drops was by having a more concentrated inoculum. Therefore, the fungus was grown as before until 10 days old, but before maceration, 100 ml of the medium was removed so that the fungus was in 50 ml of medium only. This was macerated and used as inoculum.

After inoculation of the leaflets but before the seed box cover was placed over the plants, the inside of the cover was sprayed with a fine mist of water. This was to ensure that there was 100% humidity.

Using these two refinements, leaflets of several plants were inoculated and left at 100% humidity for 24 hours. After this time the cover was removed and the lesions were found to be all more or less the same size, the majority being slightly larger than the original inoculation drop. None of the leaves showed any signs of toxin damage.

Spread of infection from limited lesions

Leaves with limited lesions were cut from plants and put onto expanded metal grids in small, clear sandwich boxes, so that the petioles passed through the grids and were immersed in 75 ppm benziminazole solution. A fine mist of water was sprayed over the leaves and the sandwich box lid to obtain 100% humidity. The lid was then replaced onto the box. After 24 hours, the lesions had started to spread and continued to spread until the whole leaf was infected. Therefore, limited lesions did spread when returned to 100% humidity. The next step was to see if different varieties showed differences with artificially limited lesions.

Summary of method for production of artificially limited lesions

1. Whole plants used. The larger leaflets were gently rubbed at the distal end between the thumb and forefinger to disperse the wax bloom.

2. 10 day old *S. trifoliorum* culture in SCA medium, from which 100 mls of medium had been removed, was macerated.

3. 20 µl drops of macerated culture were placed onto the distal ends of leaflets.

4. The cover was placed over the plants after the inside of it had been sprayed with a fine mist of water to ensure 100% humidity.

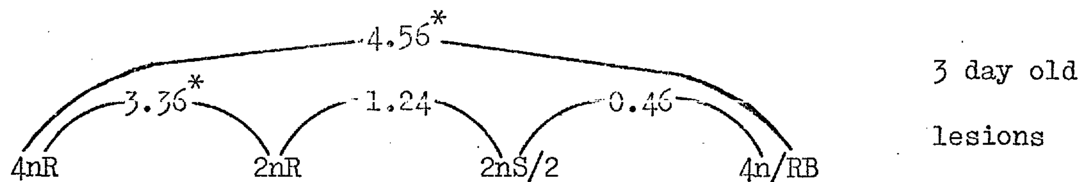
5. Plants were left for 24 hours under the cover, which was then removed so that the humidity dropped and the lesions became limited.

All inoculations were carried out in a temperature range of 20 - 25°C.

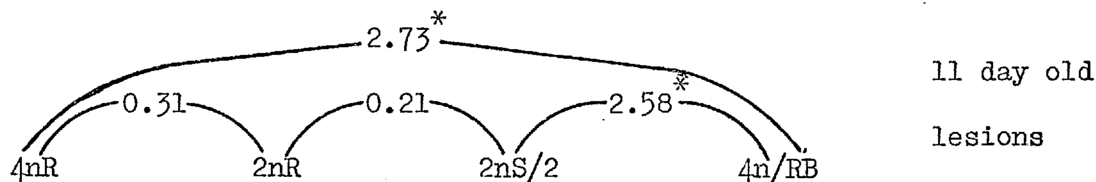
Experiment to test the resistance of different red clover varieties to *S. trifoliorum* by using artificially limited lesions

Two month old red clover plants of four varieties, 2nS/2, 2nR, 4nR and 4n/RB, were inoculated to obtain limited lesions. The inoculations were carried out in a greenhouse at about 25°C. Because the plants were too large to be put under seed tray covers, a wooden frame covered in clear plastic was used to cover them. After inoculation the frame was placed over the plants, then a fine mist of water was sprayed into the frame through slits in the plastic in order to obtain 100% humidity. After 24 hours the frame was removed so that the humidity dropped and the lesions were artificially limited. Leaves with limited lesions were cut from plants 3 days and 11 days after removal of the frame and put onto expanded metal grids in small clear plastic boxes so that the petioles passed through the grid and into 75 ppm benziminazole solution. The leaves and box lid were sprayed with a fine mist of water so that there was 100% humidity. The boxes were put into an incubator at 17°C with a 12 hour daylength. The lesions were measured at time intervals, the mean of two diameters being taken. The results are shown in Table 18.

It was encouraging to find that the limited lesions were still viable after 11 days. The size of the limited lesions was generally larger at 11 days than at 3 days but this may have been due to either growth of the fungus, or reaction of the host. The sizes of the spreading lesions of the different varieties at the last readings were compared statistically by using the t-test. The results were :-



3 day old lesions



11 day old lesions

* 0.1% probability

The order of the sizes of the spreading lesions of the different varieties was the same both times, 4nR having the smallest, then 2nR, 2nS/2 and finally 4n/RB which had the largest lesions. The differences were not always significant, but with 3 day old lesions, 4nR had significantly less spread of infection, whereas with 11 day old lesions, 4n/RB had significantly more spread of infection. As the tetraploids had larger leaves, their spreading lesions were less limited by the edge of the leaves, which may partly account for the 4n/RB variety having the largest lesions. However, the 4nR variety had the smallest lesions.

These results were quite encouraging. However, the use of only four varieties whose resistance to S. trifoliorum is known to differ greatly in the field, does not prove the worth of this test in a general screening for S. trifoliorum resistance. To do this, a large number of varieties would have to be tested. Because the lesions were viable for at least 11 days, the method may be useful for studying the factors that cause lesion spread to occur.

Table 18 Spread of infection (cm) from artificially limited lesions on detached leaves in 100% humidity

a) 3 day old limited lesions

	Time (hours)			
Variety	17	41	66	93
2nR	0.52	0.90	1.36	1.85
4nR	0.51	0.76	1.06	1.46
2nS/2	0.49	0.98	1.35	1.98
4n/RB	0.56	1.06	1.52	2.03

b) 11 day old limited lesions

	Time (hours)			
Variety	18	42	66	90
2nR	0.61	0.78	1.09	1.27
4nR	0.58	0.78	1.06	1.22
2nS/2	0.54	0.82	1.13	1.29
4n/RB	0.73	1.00	1.34	1.57

Each measurement is expressed as the mean of at least 18 replicates.
Each replicate measurement was the mean of two diameters of each lesion.

<u>The effect of frosting on red clover leaves</u>

The original aim of this experiment was to try and find whether frosting caused limited lesions to spread, and if frosted limited lesions continued to spread at lower than 100% humidity. However, it was impossible to obtain limited lesions because the greenhouse temperatures became too high in the summer for inoculations to infect. Therefore the effect of frosting alone on plants of different varieties was carried out. Dijkstra (1964) had suggested that perhaps frost resistance in a healthy leaf could provide a standard for clover rot resistance in that leaf. If this was so then fungus would not have to be used at all in testing varieties against clover rot.

The main problem was how to measure the damage to the leaf caused by frosting them. Electrolyte loss from leaf disks was used, basically because it was a simple method that could easily have been used if a large number of varieties were being tested.

<u>Experiments on frosting of clover leaves</u>

Two month old plants of 4 varieties, 2nS/2, 2nR, 4nR and 4n/RB, were pretreated at a low temperature, 4°C, and a short day-length, 8 hours, to simulate conditions present in the field at times when frosting would occur. It was hoped that this would also harden the plants to frosting, as is known to occur in other plants. The pretreatment varied from 5 to 7 days. Plants to be frosted were then transferred to a cooled incubator (Gallenkamp, 1H - 280) which was kept in a cold room at an ambient temperature of 4°C, so that temperatures below zero could be obtained. The actual frosting temperature varied from -5 to -1°C and the time from 6 to $8\frac{1}{2}$ hours. The plants were then removed and 5 leaf disks cut from each plant using a No. 4 cork borer. The disks from each plant put into 10 ml of water of known conductivity, each plant being used as a replicate. The conductivity of the water was measured 30, 90, 150 and 210 minutes after addition of the disks with a 'Chandos' conductivity meter, and the change in conductivity

noted. Controls were disks cut from pretreated plants that had not been frosted.

There were three experiments, differing slightly in their frosting temperatures and times and length of pretreatment. These were :

Experiment 1 - Pretreatment for 5 days, followed by frosting at $-5^{\circ}C$ for $8\frac{1}{2}$ hours.

Experiment 2 - Pretreatment for 5 days, followed by frosting at $-4^{\circ}C$ for 6 hours.

Experiment 3 - Pretreatment for 7 days, followed by frosting at $-1^{\circ}C$ for 6 hours.

The results of the 210 minutes conductivity measurements are given in Table 19, along with the standard errors. The data was very variable, especially in the frosted treatment.

In experiments 1 and 2, there were clear differences between the frosted and the controls, the frosted disks losing far more electrolytes. Therefore frosting did have an effect at -4 and $-5^{\circ}C$. At $-1^{\circ}C$ (experiment 3) there was little differences between the frosted and the controls although some of the differences were significant because of the small amount of variability e.g. 2nS/2 frosted and control.

In comparing the varieties, the controls of 2nS/2 were far higher than the other controls (t significant at 0.01 level) in experiments 1 and 2 but not in experiment 3 that had a slightly longer pretreatment time. Perhaps the longer pretreatment time allowed the 2nS/2 variety to recover from the shock of removal from 20 - $25^{\circ}C$ with long day lengths to $4^{\circ}C$ with short day lengths.

In comparing the frost treatments using the t test, the following results were obtained :

Table 19 The effect of frosting on conductivity (μmhos) of leaf disks in 10 ml of water after 210 minutes

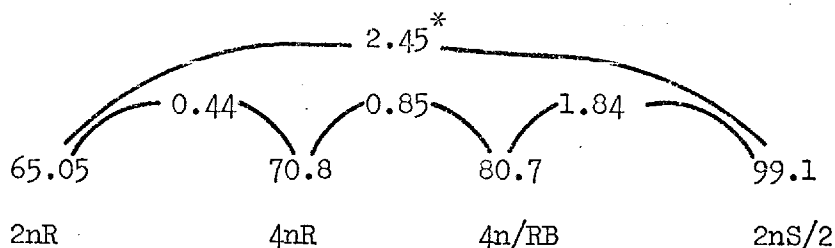
Experiment (described in text)	2nR		4nR		2nS/2		4n/RB	
	Controls	Frosted	Controls	Frosted	Controls	Frosted	Controls	Frosted
1	24.3	65.05	27.85	70.8	41.6	99.1	27.7	80.7
	(1.3)	(9.7)	(2.9)	(9.3)	(1.6)	(9.5)	(0.6)	(7.4)
2	32.63	111.8	46.75	93.94	88.5	157.0	55.66	128.4
	(4.2)	(13.5)	(6.5)	(15.6)	(1.8)	(21.4)	(7.9)	(9.9)
3	25.9	30.8	21.4	24.4	21.3	28.3	26.3	27.8
	(2.9)	(2.7)	(4.6)	(2.5)	(1.0)	(1.6)	(3.1)	(3.4)

Controls expressed as mean of at least 4 replicates

Frosted expressed as mean of at least 8 replicates

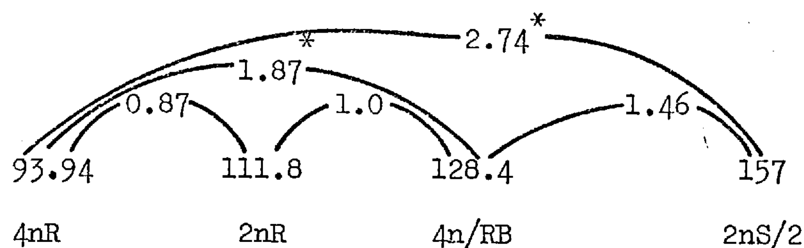
Numbers in brackets are the standard errors

Experiment 1



* Significant at 0.025 level

Experiment 2



* Significant at 0.05 level

Experiment 3 No significant differences to 0.05 level.

Apart from experiment 3, the trend was for the more susceptible varieties to have larger electrolyte losses, although these differences were not always significant.

The main problem with this experiment was the variability of the data. Perhaps the use of a different method of measuring the frost damage could eliminate this, e.g. measurement of a specific ion using flame photometry, or measuring leakage of amino acids, although such methods would probably be just as variable. Frosting certainly did have an effect at -4 and -5°C and there was a tendency for more susceptible varieties to show larger losses of electrolytes, but the results were far from conclusive.

Some studies on the toxin produced by S. trifoliorum

Held (1955) found that S. trifoliorum pathogenic to clover produced a wilt inducing toxin in liquid medium cultures. Varieties of clover differing in their susceptibility to clover rot did not show differences in their reaction to the toxin. No work has since been published on the toxin. Because the toxin appeared to be present after inoculations for limited lesions, some studies were carried out on it.

a) Confirmation of the presence of toxin in liquid medium cultures

The mycelium from six-week old SCA cultures of S. trifoliorum was removed by centrifugation at 3000 rpm. 20 ml of this culture medium was put into each of eight 50 ml flasks. Equal amounts of unused SCA medium were put into another eight 50 ml flasks. Healthy, turgid leaves with their petioles were cut from clover plants and one leaf put into each of the above flasks so that the end of the petioles were immersed in the medium. To obtain high transpiration conditions, a fan was placed 5 feet from the flasks so that a steady breeze blew over the leaves, and a light ('Osram' I.R.R., 230 v, 250 w) was put 3 feet above the leaves. These were left for 16 hours (overnight). After this time, all the leaves in the culture filtrate showed some signs of wilting, whereas those in uninoculated media appeared quite turgid.

When the leaves were transferred to water, some of the lesser wilted leaves recovered, but leaves most effected by the toxin, which were brown and desiccated, never recovered.

Clearly, toxin was present in the culture filtrates. Held (1955) found no differences between different varieties against the toxin using a method similar to the one used above. However, it was decided that perhaps another method would show differences and could possibly be used as a method for testing resistance of clover varieties against S. trifoliorum. Changes in tissue permeability measured by electrolyte loss was used.

b) The effect of toxin on electrolyte loss from leaf disks of different varieties

The fungus from one month old SCA cultures of *S. trifoliorum* was removed by centrifugation at 3000 rpm. The culture medium was divided into two halves, one of which was autoclaved at 15 p.s.i. for 15 minutes. This was a check against the toxin being thermolabile. Leaf disks were cut from four varieties of red clover (2nR, 2nS/2, 4nR and 4n/RB) using a No. 3 cork borer and disks from each variety were put into three vessels containing inoculated culture medium, autoclaved inoculated culture medium, or unused medium. The disks were kept totally immersed in the media by use of plastic netting. The vessels were placed into a desiccator which was fitted to a pump which was run for one hour to allow vacuum infiltration of the media into the leaf disks. The disks were removed from the media and washed once in water, then 5 disks were placed into a vial containing 10 ml of water, of known conductivity, for each replicate. There were five replicates of each treatment with each variety. Conductivity was measured with a 'Chandos' conductivity bridge at 30, 90, 150 and 210 minutes after addition of the disks to the water. The water blank readings were subtracted from these readings, the means being given in Table 20.

The 210 minute data was subjected to statistical analysis using the t test, as shown in Table 21. The different treatments within each variety were compared to see if the toxin had any effect, and whether autoclaving the toxin made any difference to its activity. Autoclaving of the inoculated medium made no significant difference to the toxin, but the inoculated medium always caused significantly more loss of electrolytes than the control. Comparisons between varieties showed that the control treatments did not differ significantly from each other, but the inoculated medium caused significantly more electrolyte loss in the 4nR variety than the other varieties.

As Held (1955) found, there appears to be no correlation between resistance to clover rot and resistance to the toxin.

Table 20 Conductivity measurements (μmhos) of leaf disks (in 10 ml of water) from different varieties treated with culture media containing a S. trifoliorum toxin

Time	Variety											
	2nR			4nR			2nS/2			4n/RB		
(minutes)	I.M.	A.M.	C	I.M.	A.M.	C	I.M.	A.M.	C	I.M.	A.M.	C
30	5.9	6.5	4.7	12.9	5.7	7.3	6.8	7.2	6.2	7.2	8.2	5.9
90	10.9	13.0	7.8	15.8	13.8	11.0	10.4	11.1	8.8	12.2	12.3	9.4
150	14.0	17.6	9.6	23.0	17.7	13.0	14.8	13.6	10.6	15.0	15.2	11.6
210	16.0	21.0	10.4	26.0	20.6	13.9	17.1	15.6	12.1	17.4	16.9	13.1

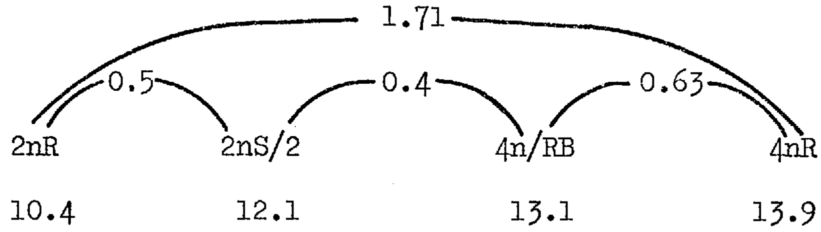
I.M. = inoculated culture medium A.M. = autoclaved inoculated culture medium

C = control medium

Each figure is the mean of 5 replicates

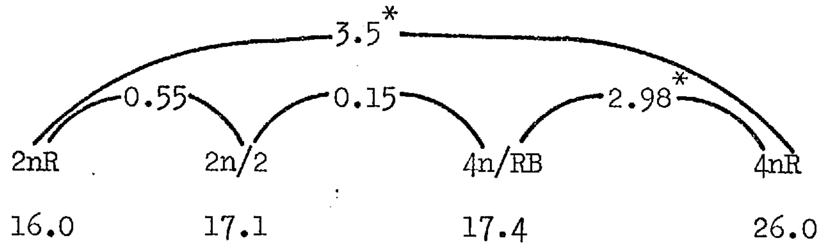
Table 21 Results of statistical analysis using T test of the 210 minute data of Table 20

a) Comparison of control treatments



None significant at 0.05 level

b) Comparison of I.M. treatments



* 0.01 probability

c) Comparison of C and I.M. treatments within each variety

Variety	T value	Probability
2nR	2.85	0.01
2nS/2	1.84	0.05
4n/RB	2.494	0.025
4nR	4.130	0.01

d) Comparison of I.M. and A.M. treatments within each variety

Variety	T value	Probability
2nR	1.46	-
2nS/2	0.48	-
4nR	0.276	-
4n/RB	1.806	-

None significant at 0.05 level

I.M. = inoculated culture medium A.M. = autoclaved inoculated culture medium and C = control medium

DISCUSSION

What is it that allows a pathogen to infect a plant whereas the same plant is resistant to a non pathogen ? Why are some varieties of a plant less susceptible to a pathogen than others ? These two basic questions can be asked about most diseases and an attempt has been made to answer them in this work. The pathogen used was Sclerotinia trifoliorum, the plant was red clover, and there were four different varieties, which vary in their susceptibility. Much of the work was concerned with the role of antifungal compounds in disease resistance, but other factors, such as frosting and the effect of a toxin produced by S. trifoliorum were also studied in order to try and determine why resistance differs in varieties.

In leaves, on which this work was exclusively carried out, four antifungal compounds had already been reported; the two isoflavones biochanin A (Bredenberg, 1961) and formononetin (Virtanen and Hietala, 1958), and the two pterocarpans medicarpin and maackiain (Higgins, 1971; Higgins and Smith, 1972). More antifungal compounds were found, however, using Cladosporium TLC bioassays, some of which were pterocarpans, one an isoflavone and the others of unknown identity. Very little work was carried out on the latter compounds, some of which were preformed, whereas at least one was induced by the non-pathogen B. cinerea. The UV spectra taken of three of the preformed compounds and of the B. cinerea induced compound were all very similar, which suggests that they were similar types of compounds. It was unusual that two of the preformed compounds were not found in any of the infected tissue. This could have been due to further metabolism upon infection, for if the compounds were phenolic, then with increase in certain enzymes upon infection - e.g. polyphenol oxidase - the compounds could have been converted to polyphenols. No work was carried out on concentrations of these compounds in tissue, or on their fungitoxicity to the pathogen or non-pathogen and therefore little can be

said about their role in disease resistance. Further work on these compounds is necessary to determine their importance.

The remainder of the work on antifungal compounds was concerned exclusively with the pterocarpans and isoflavones. This was for two reasons : firstly because of the supposed importance of pterocarpans as antifungal compounds and the uncertain significance of the isoflavones, and secondly because of the practical point that they were easily located on TLC plates under UV light, unlike many of the other antifungal compounds found.

That so many different antifungal compounds were present, six or more in infected tissue, shows that the resistance reaction was not dependant on a single phytoalexin. This was the early classic theory, e.g. pisatin in peas and phaseollin in beans. More recent work has shown that many antifungal compounds are present in several plants, e.g. French beans are now known to have phaseollin, phaseollidin, phaseollinisoflavan, kievitone and 2^1-methoxyphaseollinisoflavan (Burden, Bailey and Dawson, 1972; Van Etten and Smith, 1975). Further work will probably show that a great number of plants will also have many antifungal compounds. That there are so many different antifungal compounds means that it is more difficult for a fungus to adapt as a pathogen of a particular plant. The fungus has to cope with each of the antifungal compounds so that their antifungal activity does not affect it, otherwise the pathogenicity will be weakened or lost.

Although reported as antifungal in the late 50's (Virtanen, Hietala and Wahlroos, 1957; Virtanen and Hietala, 1958; Bredenberg, 1961), little work had been carried out on the role of the isoflavones biochanin A and formononetin in disease resistance. Large amounts of isoflavones were known to occur in other legumes, but in at least two of them - lucerne which has formononetin and daidzein (Olah and Sherwood, 1971) and chickpea which has biochanin A and formononetin (Wong, Mortimer and Geissman, 1965) - no antifungal activity has been attributed to them. Instead pterocarpans appear to be more important fungal inhibitors; the phytoalexin medicarpin in

lucerne (Smith, McInnes, Higgins and Millar, 1971), and medicarpin and maackiain as preformed inhibitors in chickpea (Smith, 1971a, 1971b; Keen and Sims, 1973). Therefore a doubt existed as to the importance of isoflavones in disease resistance.

It was known that isoflavones existed in healthy tissue as glycosides (Francis, Millington and Bailey, 1967; Schultz, 1967). This was found in this work but only two glycosides were distinguished, although three, the mono-, di- and tri-glycosides had been previously reported. As shown in lucerne (Olah and Sherwood, 1973) large proportions of the glycosides of both biochanin A and formononetin were hydrolysed upon infection to their respective aglycones. This was important as other isoflavonoids were found to be more antifungal as aglycones than as glycosides (Perrin and Cruickshank, 1969). Upon infection with either a pathogen or non-pathogen, a third isoflavone, genistein, was found in quite large concentrations, and this was also present as predominantly the aglycone. Although present in healthy tissue, as first shown by Pope and Wright (1954), genistein increased upon infection at least three-fold (in 4nR variety) and as much as ten-fold (in 4n/RB variety).

On Cladosporium TLC plate bioassays, both biochanin A and genistein caused inhibitory zones, but not formononetin or daidzein (which was found at low concentrations in S. trifoliorum-infected tissue). The latter two compounds differ from the first two by the absence of a hydroxyl group. The presence or absence of oxygen-containing substituents were thought to alter the antifungal activity of certain isoflavanoids (Perrin and Cruickshank, 1969), and perhaps this can explain the phenomenon.

When the isoflavones biochanin A, formononetin and genistein were bioassayed against S. trifoliorum none of them had any effect on the mycelial dry weight after 10 days at concentrations of up to 650 µg/ml. These results are in contrast to those of Virtanen, Hietala and Wahlroos (1957) who reported inhibition of S. trifoliorum on glucose-agar incorporating 0.04% biochanin A (400 µg/ml) although concentrations of formononetin that

were inhibitory were not quoted. These differences in results could be due to the different methods of bioassaying; a liquid medium being used in this work because of the difficulties found in evenly distributing biochanin A into agar media. Another factor could have been the use of different isolates of the fungus. S. trifoliorum is known to differ, and the isolate used in this work was believed to be an aggressive strain. Perhaps the aggressiveness of different strains of the fungus depends on their reactions to antifungal compounds in red clover, and further work could be carried out along these lines.

In bioassays against the non-pathogen, B. cinerea, none of the isoflavones significantly decreased spore germination, although there was a reduction in germ-tube length, to 70% of the control with biochanin A, to 40% of the control with formononetin, and to 50% of the control with genistein. The lower concentrations, 10 and 20 μg/ml, caused the most reduction and higher concentrations of up to 650 μg/ml had little or no further effect. Therefore the maximum reductions of germ-tube length obtained by the isoflavones were at concentrations far lower than found in either healthy or B. cinerea-inoculated tissue. That there was a concentration at which maximum inhibition is caused, and higher concentrations have no further effect, suggests that there are a limited number of sites upon which the isoflavones can act. Bioassays against B. cinerea were carried out in water, but when inoculated onto clover, B. cinerea spores were in 5% (w/v) glucose-water, and this glucose could well have overcome the inhibitory affects of the isoflavones. Perhaps spore germination tests in 5% glucose-water should also have been carried out.

From the results of the bioassays, it appears that the isoflavones are not particularly antifungal. This is in agreement with the results of Smith (1971b) who, in using chickpea, found no antifungal activity associated with the isoflavones, but is in contrast to the results of Virtanen, Hietala and Wahlroos (1958). There is other indirect evidence that formononetin does not have much affect on microorganisms. Barz (1969) found in chickpea

and Phaseolus aureus that the turnover of formononetin and daidzein was quite fast, the biological half-life being about 50 hours, although biochanin A turnover was quite slow. This was later found (Barz, Adamek and Berlin, 1970) for formononetin to be almost entirely due to the rhizosphere microorganisms degrading it. As the isoflavones are not themselves very fungitoxic they are probably not directly important in disease resistance, a point further emphasised by there being no correlation between higher concentrations of isoflavones and resistance in the varieties analysed.

Although in themselves not important, isoflavones may play an indirect role in resistance as part of the biosynthetic pathway to the pterocarpan phytoalexins. Recently Dewick (1975) found that seedling roots of red clover treated with $CuCl_2$ produced medicarpin and maackiain, and formononetin was a good precursor, although daidzein was not so good. Daidzein was shown to be a precursor of glyceollin (formerly called hydroxyphaseollin but now known to be different) in soybeans (Keen, Zaki and Sims, 1972). Formononetin (see figure 1) is closer structurally to medicarpin and maackiain (see figure 2) than daidzein and it is not surprising, therefore, that it was the better precursor, whereas daidzein is closer structurally to glyceollin.

When the concentrations of the isoflavones were compared in healthy, S. trifoliorum-infected and B. cinerea-inoculated tissue, basically the same picture was seen in all 4 varieties. Both biochanin A and especially formononetin concentrations dropped whereas genistein concentrations rose in S. trifoliorum-infected tissue as compared to healthy tissue. In B. cinerea inoculated tissue, formononetin concentrations dropped and genistein concentrations rose, although neither to the extent of S. trifoliorum-infected tissue, whereas biochanin A rose in two varieties and dropped in the other two varieties. When the total isoflavones were studied, there was significantly less in S. trifoliorum-infected tissue than either healthy or B. cinerea-inoculated tissue. This drop in isoflavone concentration upon

infection with S. trifoliorum was apparently not due to fungal degradation as S. trifoliorum was shown to do nothing to isoflavones in vitro. Therefore the host's metabolism must have caused the drop. That this 'lost isoflavone' had been used in the biosynthesis of pterocarpans was therefore a possibility. Dewick (1975) found, however, that it was de novo synthesised formononetin which was the precursor of the pterocarpans, as the normal root formononetin level stayed stable. But in the disease situation, hydrolysis of the existing isoflavones from the glycosides to aglycones may free them for further synthesis. This hydrolysis would probably not take place in the $CuCl_2$ treatment and the existing isoflavones may remain bound up in some way. Formononetin and biochanin A are known to remain mostly unassimilated in healthy chickpea and Phaseolus aureus (Barz, Adamek and Berlin, 1970). However, the importance of the concentrations of the existing isoflavones is in doubt as there was no correlation between resistance and healthy tissue isoflavone concentrations. Also, far higher concentrations of the pterocarpans were found in B. cinerea inoculated tissue than in S. trifoliorum tissue, although this may not be associated with more synthesis of pterocarpans but rather with fungal degradation. Nevertheless, there was no drop in total isoflavone concentration upon inoculation with B. cinerea. De novo synthesis of isoflavones must have accounted for most of the pterocarpan produced. As concentrations up to 2550 μg/gm fresh weight of maackiain alone were found, this would mean a similar amount of isoflavone, possibly as formononetin or perhaps genistein, was synthesised, as both the isoflavones and pterocarpans have similar masses (maackiain 284, formononetin 268).

The concentrations of isoflavones in healthy tissue and their possible role in pterocarpan biosynthesis are important, especially with regard to the oestrogenic properties of isoflavones. If de novo synthesis of isoflavones accounts for all or most of the precursor for pterocarpans, then existing levels of isoflavones are not important in disease resistance. In theory, it is therefore possible to breed red clover with low healthy

isoflavone concentrations, and consequently lower eostrogenic activity, without loss of the existing disease resistance bestowed by the phytoalexins.

Although trifolirhizin, the glycoside of maackiain, is present in the roots of red clover (Bredenberg and Hietala, 1961a, 1961b), there were no pterocarpans detected in healthy clover leaves in this work. Trifolirhizin was reported to be present in dry foliage of red clover (Chang, Suzuki, Kumai and Tamura, 1969) but this could have been due to some induction by fungi or perhaps induction by the method of extraction. Therefore most, if not all, of the pterocarpans detected in infected tissue had been induced by fungal infection and hence qualify as phytoalexins.

Five different pterocarpans were found, including those already reported in red clover, maackiain and medicarpin (Higgins, 1971; Higgins and Smith, 1972). The other pterocarpans had not previously been reported in red clover, and were pisatin, homopisatin and 6a-hydroxymaackiain, although the latter was probably a degradation product and will be discussed later. Pisatin is the well documented phytoalexin of Pisum sativum (Perrin and Bottomly, 1962) and other Pisum species (Cruickshank and Perrin, 1965). In P. sativum, Stoessl (1972) has also found small quantities of maackiain. It may be coincidantal that these two compounds occur together, or perhaps the biosynthetic pathway for the two is so similar that the minor component (maackiain in pea, and pisatin in red clover) is a biproduct from the main biosynthetic pathway. The two compounds are structurally very similar (see figure 2), both having the 8, 9-dioxymethylene group. The same situation may also occur with homopisatin and medicarpin. Pisatin and homopisatin differ from maackiain and medicarpin respectively by being methylated at the 3 position and hydroxylated at the 6a position. (For the numbering system, see figure 2). Homopisatin has not previously been reported as occurring naturally, although it has been synthesised and shown to be strongly antifungal against Monilinia fructicola (Perrin and Cruickshank, 1969). As with medicarpin and maackiain, the polarity of pisatin and homopisatin is the same and therefore they ran together on TLC. This

made it impossible to separate the two compounds with this method, but separation could be obtained by gas-liquid chromatography (GLC) for quantitative purposes, although this is usually a destructuve method and could not be used for preparative work. Dewick (1975) used GLC for separating medicarpin and maackiain.

Mechanisms of induction of the phytoalexins were not studied, although several chemicals were used to induce phytoalexins. This was in order to try and find some standard method for producing phytoalexins so as to compare the different varieties in their capacity for phytoalexin production. Induction of phytoalexins is a controversial point and there are several theories on the induction mechanisms. When pyrogalliol red was used as an inducer, no phytoalexin was produced, although Hadwiger and Schwochau (1971) found this was a good inducer of pisatin in immature pea pods. Perhaps these are unusual in this respect, and therefore results from such material cannot be generally applied to other plant-fungi systems.

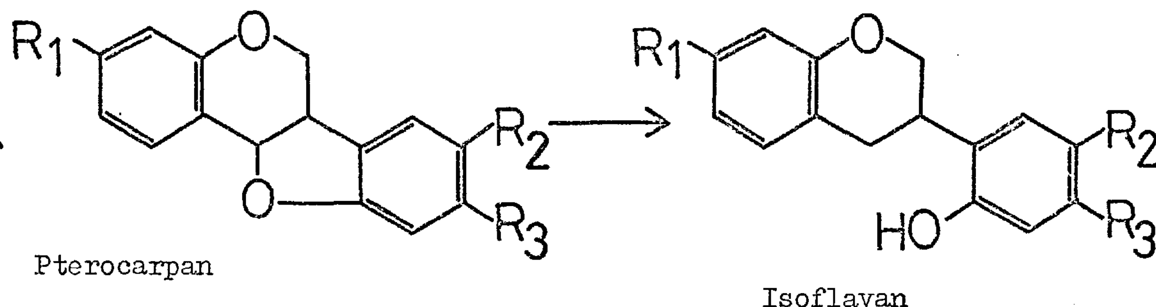
Only medicarpin and maackiain of the pterocarpans were bioassayed against S. trifoliorum because of the large amounts needed for the technique used. Only medicarpin had any effect, with an ED_{50} against mycelial dry weight of 50 - 60 μg/ml. Against B. cinerea, medicarpin had an ED_{50} for spore germination of 30 μg/ml, and for germ-tube length of 20 μg/ml. Neither maackiain or a mixture of pisatin and homopisatin had much effect against spore germination, but both reduced germ-tube length. Low concentrations of maackiain reduced germ-tube length quite considerably but higher concentrations caused little or no further reduction. The pisatin/homopisatin mixture had an ED_{50} for germ-tube length of about 90 μg/ml (using the extinction coefficient of pisatin for the mixture). In bioassays against other fungi, medicarpin had an ED_{50} of 10 - 15 μg/ml against germ-tube length of Helminthosporium turcicum (Smith et al., 1971), maackiain and homopisatin had ED_{50} for growth of less than 10 μg/ml against Monilinia fructicola, and pisatin an ED_{50} of less than 16 for growth inhibition of M. fructicola (Cruickshank and Perrin, 1969). Therefore,

against B. cinerea, the results obtained in this work are of the same order of magnitude as previous reports, except for the pisatin/homopisatin mixture which did not seem to be so active.

When the spreading lesions caused by S. trifoliorum were extracted they were found to contain proportionately far lower concentrations of medicarpin and maackiain than did the restricted lesions caused by B. cinerea. That there were such high concentrations of phytoalexin with B. cinerea could explain why the lesions were restricted, the fungal growth having been stopped. Although the amounts of phytoalexins were very high (2550 µg/gm fresh weight in 4nR variety), such high concentrations have been found in other plants. Bailey and Deverall (1971) estimated concentrations of over 3000 µg/ml cell volume of phaseollin in bean hypocotyls inoculated with Colletotrichum lindemuthianum, and Keen (1971) found concentrations of 10 - 100 mg/gm dry weight of glyceollin (hydroxyphaseollin) in soybean hypocotyls inoculated with Phytophthora megasperma var. sojae (equivalent to about 1000 to 10,000 µg/gm fresh weight). Such high concentrations of phytoalexins have only been found in hypersensitive reactions, that is reactions caused by an incompatible host-pathogen combination, which result in rapid necrosis of the infected region. This causes prevention of spread of the pathogen, apparently due to the high concentrations of antifungal compounds produced.

That S. trifoliorum-infected tissue had lower concentrations of medicarpin and maackiain could be due to less induction of phytoalexins and/or degradation of the phytoalexins by the fungus. Degradation by S. trifoliorum in vitro was shown to occur, but B. cinerea also degraded maackiain in vitro, though degradation studies on medicarpin were not possible because of lack of material. Both of these phytoalexins have been shown to be degraded by other fungi. Stemphylium botryosum, a lucerne pathogen, was shown to degrade medicarpin (Higgins and Millar, 1968, 1969a, 1969b) to the isoflavan vestitol (Higgins, Stoessl and Heath, 1974). It can also degrade maackiain in the same way to dihydromaackiain (Higgins et al.,

1974; Higgins, 1975) as well as phaseollin (Heath and Higgins, 1973) to phaseollinisoflavan (Higgins *et al*., 1974). Degradation of pisatin appears to be of a different type. For medicarpin and maackiain, the degradation was therefore as follows :



Pterocarpan

Isoflavan

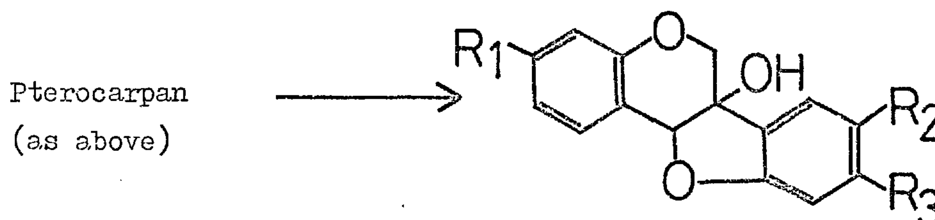
Medicarpin R_1 = OH, R_2 = H, R_3 = OCH_3 — Vestitol R_1 = OH, R_2 = H, R_3 = OCH_3

Maackiain R_1 = OH, R_2+R_3 = O-CH_2-O — Dihydromaackiain R_1 = OH, R_2+R_3 = O-CH_2-O

The isoflavan is as antifungal as the parent phytoalexin, but this in turn is further converted. Other fungi are also known to degrade medicarpin, but the conversion products have not been identified.

However, the conversion of the phytoalexins by *S. trifoliorum* was not of this kind, but was by the addition of oxygen to the 6a position to form a hydroxyl at this point, as follows :

Pterocarpan (as above) ⟶



Maackiain ⟶ 6a-hydroxy maackiain R_1 = OH, R_2+R_3 = O-CH_2-O

Medicarpin ⟶ 6a-hydroxymedicarpin R_1 = OH, R_2 = H, R_3 = OCH_3

This type of conversion has also been found with *Colletotrichum lindemuthianum* and phaseollin (Burden, Bailey and Vincent, 1974), where the first conversion product was 6a-hydroxy phaseollin, but further

hydroxylation followed to form 6a,7-dihydroxy phaseollin. Both of these metabolites were still antifungal, but *in vitro* studies showed that these in turn disappeared.

6a-hydroxymaackiain in spore germination tests had no effect against spore germination but did reduce germ-tube length, but this was less than caused by maackiain. So, in this case, hydroxylation may be a detoxifying step. 6a-hydroxymaackiain did begin to disappear by 24 hours, although this was not evident with 6a-hydroxymedicarpin. Further studies on the disappearance of these two compounds are needed.

6a-hydroxy maackiain was found in tissue infected with *S. trifoliorum* at higher concentrations than maackiain, but was not detected in *B. cinerea*-inoculated tissue. Van Etten and Smith have similarly shown that the phaseollin degradation product, la-hydroxyphaseollone, accumulates in bean tissue infected with *Fusarium solani* f. sp. *phaseoli*. If *S. trifoliorum* induced as much maackiain formation as did *B. cinerea*, and if all the 6a-hydroxymaackiain came from maackiain, then, on average, three-quarters of the induced maackiain had been degraded further than 6a-hydroxy maackiain. The lesions extracted were 5 days old, and so a large proportion of the lesion was older than 24 hours, the time taken for total conversion of maackiain to 6a-hydroxymaackiain *in vitro*. It would perhaps be possible to follow the course of degradation by extracting concentric rings from lesions, so that the oldest to the youngest part of the lesions were analysed separately. One can envisage the youngest part having some maackiain and some 6a-hydroxymaackiain, the next part containing no maackiain but just 6a-hydroxymacckiain, and the oldest part of the lesion containing neither compound.

6a-hydroxy medicarpin was not detected in *S. trifoliorum*-infected tissue because it had the same R_f value as medicarpin which was not known at the time of the analysis. Therefore the concentrations given for medicarpin may well be for both medicarpin and 6a-hydroxymedicarpin. Further investigation of this would have to be carried out using different solvent

partitioning, as used in the degradation studies, to separate 6a-hydroxy maackiain, or by using GLC. Medicarpin was found to inhibit mycelial growth of S. trifoliorum with an ED_{50} of 50 - 60 μg/ml, and total inhibition at about 100 μg/ml. Red clover can produce higher levels of medicarpin as shown by inoculation with B. cinerea, but lower concentrations were present in S. trifoliorum-infected tissue probably due to degradation. Presumably, during infection, S. trifoliorum can maintain medicarpin at low concentrations by degrading it as it is being produced so that inhibitory concentrations are never reached. However, when put into an environment of high medicarpin concentration, as in the bioassay, inhibition results which possibly stops degradation occurring . From these results, a plant capable of producing high concentrations of medicarpin quickly would be resistant to S. trifoliorum. Both lucerne and white clover produce medicarpin, and it was estimated that the cell concentration of medicarpin in leaf tissue after inoculation with Monilinia fructicola was 324 μg/gm fresh weight (Cruickshank, Veeraraghavan and Perrin, 1974). This was less than found in the 4nR variety of red clover. As white clover can be as badly infected with clover rot as red clover (G.R. Dixon, personal communication) a similar situation concerning medicarpin degradation must also occur in white clover.

That B. cinerea could degrade maackiain was not surprising as it is closely related to S. trifoliorum, but the degradation appeared to be slightly different, 6a-hydroxy maackiain being converted into some other compound. There is the need for further work here, and also to try and explain why such high concentrations of maackiain existed in B. cinerea-inoculated tissue, even though degradation could take place. There are several possible explanations. The presence of other antifungal compounds, especially medicarpin, which B. cinerea may be unable to degrade, could inhibit the fungus and thus prevent it from degrading maackiain. It was unfortunate that an experiment to find out if B. cinerea could degrade medicarpin could not be carried out, but in spore germination tests medicarpin was quite active against B. cinerea. Alternatively the speed of

degradation may be important, B. cinerea being incapable of degrading the maackiain fast enough, so high inhibitory levels of maackiain are formed. Perhaps induction of maackiain is different, being less or slower with S. trifoliorum than with B. cinerea, so that maackiain is not only degraded faster but less is synthesised with S. trifoliorum infection. From this work it is not possible to determine whether if any of these suggestions are what in fact happens in tissue. The original aim was not concerned with these points but on the more general point of the relative importance of the antifungal compounds in disease. In this respect it is quite clear why B. cinerea is a non-pathogen of red clover, because more than enough antifungal compounds, especially medicarpin and maackiain, were present to prevent its growth. However, with S. trifoliorum, none of the compounds studied were present in high enough concentrations to have much inhibitory effect.

It is clear that antifungal compounds in tissue can explain the failure of B. cinerea to infect red clover leaves. When comparing varieties of red clover differing in their susceptibility to S. trifoliorum, however, these compounds do not seem important for these differences. The more resistant varieties were found to have higher concentrations of medicarpin, maackiain and pisatin/homopisatin mixture, especially the 4nR variety, when inoculated with B. cinerea. The concentrations were all far too low, however, to cause any inhibition in S. trifoliorum-infected tissue of all varieties. Perhaps some of the other antifungal compounds which were not studied, or possibly other factors, are more important in this respect. Frost resistance and resistance to a toxin produced by S. trifoliorum were therefore also studied.

A toxin produced by S. trifoliorum in culture was found by Held (1955) who showed that when it was taken up by petioles into the leaves under high transpiration conditions, wilting occurred. This did not happen with the uninoculated, control culture medium. This observation was confirmed, and high toxin treatment was also found to produce irreversible wilting with browning and desiccation of the tissue. Held (1955) had found

no correlation between resistance to S. trifoliorum and resistance to the toxin using the above method. By using a different method i.e. measuring damage caused by the toxin by the loss of electrolytes, again no correlation was found - the 4nR variety showing the most damage. From this it seems the toxin is not a primary factor in infection of S. trifoliorum. The nature of the toxin is unknown and its presence in naturally infected tissue has not been shown. Wilting is regarded as one of the symptoms of clover rot, although this would only take place under high humidity conditions. Because the toxin did not show differences in resistance, it could not be used in a method for testing varieties against S. trifoliorum, which was one of the aims of the experiment.

The effect of frosting on the different varieties was tested because of the work of Dijkstra (1964, 1966). He found that limited ascospore lesions spread after having been frosted and that varieties more resistant to frost also seemed to be those most resistant to S. trifoliorum. As the frost resistance of the varieties used in this work was not known, it was hoped to correlate resistance with less damage caused by frosting. As a measurement of frost damage, electrolyte loss from leaf disks was used. The plants used were glasshouse grown and therefore, had been in an environment that was warmer than occurs at times of natural frosting. As plants can apparently become hardened to frost by low temperatures and daylengths (Mazur, 1969), a pretreatment was given (at 4°C and 8 hour daylength) so that frost hardiness hopefully would be gained. However, the results obtained on frost damage were not conclusive, basically because of the variability of the data, although there was a tendancy for the more resistant varieties to be less damaged by frosting. Frost hardiness has been shown to be under hormonal control, abscissic acid inducing hardiness under conditions at which it would not normally be present e.g. long daylengths (Mazur, 1969). If frost resistance and resistance to S. trifoliorum are correlated, then treating plants with abscissic acid could possibly make them more resistant to S. trifoliorum under natural

conditions. Frosting does not have to occur, however, for ascospore lesions to spread, as lesions kept in 100% relative humidity will also spread (Loveless, 1951b). Perhaps frosting is not primarily important in initiating lesion spread, but is just a further weakening factor to the plant. However, frosting, as it causes water soaking of the tissue, possibly allows spread from ascospore lesions when there is not 100% relative humidity. Water soaking has been shown to initiate ascospore lesion spread (Dijkstra, 1966). The important factor may merely be the presence of water, either as 100% relative humidity or water soaking of the leaf. The whole question of spread from ascospore lesions is one in which further work could be carried out, but there is one drawback. This is the inability of S. trifoliorum to regularly produce apothecia in the laboratory, and hence the production of ascospore lesions throughout the year, is impossible.

Inoculating detached leaves from greenhouse-grown plants had little success in showing difference between the varieties, the amount of rotting being the same for each variety. However, on one occasion when using field-grown material, differences were obtained, but it was impractical to use field-grown material all the year round. Therefore a method of producing artificially limited lesions was devised and these lesions used to detect varietal differences. That such differences were obtained again points to the importance of limited lesions in this disease, as the artificially limited lesions were crude approximations of natural ascospore lesions.

Looking at the conditions under which detached leaves were inoculated it is not surprising that different varieties did not show any differences in the rate of infection. There are no red clover varieties that are truly resistant to S. trifoliorum, the so called resistant varieties being less susceptible. In conditions of heavy inoculum and good environmental conditions for infection, then all the varieties would be seriously attacked. Such conditions were present when inoculating detached leaves, with an inoculum drop with a food base of culture medium and with incubation

in conditions of 100% relative humidity. That field-grown plants did show differences between varieties, and also that the infection rate was slower, especially in the more resistant varieties, suggests that natural conditions can somehow increase the resistance of the plants. One possible way in which this could be done has already been suggested, that frost hardiness may be correlated to resistance through the action of abscissic acid. Perhaps the action of other hormones induced by the environmental conditions are also important, and hormonal action has certainly been implicated in other diseases especially concerning disease resistance mechanism That field-grown plants exhibit resistance not detected in glasshouse grown plants is not uncommon and is related to the polygenic character of the resistance in some way. The terms 'field resistance' and 'polygenic (or horizontal) resistance' have now become almost synonymous. Because of the difficulties involved little is known about the phenomenon.

That the artificially limited lesion method did show up varietal differences was encouraging as there is at present no simple method for testing clover varieties against S. trifoliorum. However, to prove its usefulness the method would have to be used against a large number of different varieties varying in their resistance. Perhaps the limited lesions could also be used for studying the conditions that prevent, or allow, spread of infection from limited lesions as occurs with ascospore lesions, thus making it possible to have limited lesions for experimental work throughout the year without the need for ascospores. It had been one of the aims of this work to look at frosting in such a way, but because of other reasons this was not done.

Although the basic aim of this work, i.e. the relative importance of various antifungal compounds in disease resistance of red clover, was answered to a certain extent, much about the nature of disease resistance remains unanswered. Disease resistance is fundamental to the study of plant pathology but much of it still remains a mystery. That the study of disease resistance will occupy the minds of many plant pathologists for many years to come is in little doubt.

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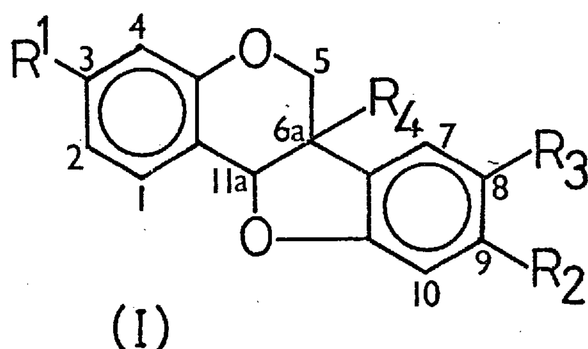
APPENDIX

The identification of pterocarpans from red clover

(A report by Mr. J.N. Bilton of the Chemistry Department, Imperial College, on the chemical data and identification of the unknown pterocarpan compounds found in this work).

Compounds containing the pterocarpan skeleton (I) have wide occurrence in the tissues of leguminous plants that have been subjected to stress or to infection by pathogens (1 - 12).



(a) R^1 = OH, R_2, R_3 = O-CH_2-O

(b) R^1 = OH, R_2 = OMe, R_3, R_4 = H

(c) R^1 = OMe, R_2, R_3 = O-CH_2-O,
R_4 = OH

Red clover is known to contain (-) Maackiain ((Ia) = 6a R, 11a R), and (-) Medicarpin ((Ib) = 6a R, 11a R), as well as isoflavones and coumestans.

Fraction A

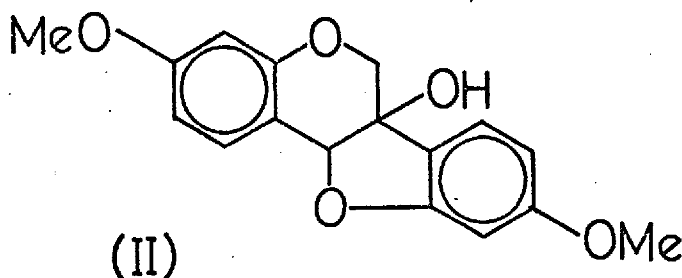
When red clover was subjected to stress by treatment with a non-pathogen it gave a mixture of two compounds which ran together on TLC and which had a UV spectrum very similar to methoxy-maackiain. The material was also very sensitive to acid. The mass spectrum of the mixture showed the presence of two molecular species, $\frac{m}{e}$ 314 = $C_{17}H_{14}O_6$, and $\frac{m}{e}$ 300 = $C_{17}H_{16}O_5$ Since the components lacked any phenolic hydroxyl groups (negative Gibbs Test). The extra oxygen atom over those present in maackiain and medicarpin must have been present as a hydroxyl group.

When the sample was chromatographed on silica plates employing multiple elution with petroleum ether/diethyl ether mixture (40 - 60), then the UV absorbing bands could be sufficiently broadened to allow a clean mass spectrum of the slower component to be obtained when the bottom edge of

the band was eluted with Analar acetone (cf. 7). This spectrum was virtually identical to that of an authentic sample of (+) pisatin (IC 6a S, 11a S) run at the same temperature.

Low electron voltage mass spectra (20 ev) of this material enabled an assignment of the substituent distribution between the two aromatic rings to be made. Additional evidence in confirmation of the structure was obtained when the sample and pisatin were irradiated on TLC plates under an intense UV light. Both spots quickly turned an orange-brown colour. The characteristic photolability of pisatin has been studied by Perrin [2].

Attention was now turned to the minor constituent of the mixture which ran slightly faster than pisatin on TLC. The presence of two peaks at 281/287 nm and the absence of the longer wavelength maximum of maackiain at 310 nm (in ethanol) in the UV spectrum suggested a structure (II).



Also known as homopisatin [18]

This gross structure has been reported in a review [13], but no data was available from the journal literature. The compound called variabilin had a positive rotation $[\alpha_D]^{20} = +211^{\circ}$ (methanol), this being indicative of the 6a S, 11a S absolute configuation [14].

The structural assignment was helped by analysis of the mass spectrum of (II) by metastable defocusing and by the observation of second field-free metastable ions. The overall pattern of the mass spectrum as well as the detailed fragmentation pathways were very similar to pisatin.

Fragment ions containing rings A and B were unshifted in mass relative to those of pisatin, whilst ions containing ring D were shifted fourteen mass units lower (Ring D-OMe versus O-CH_2-O).

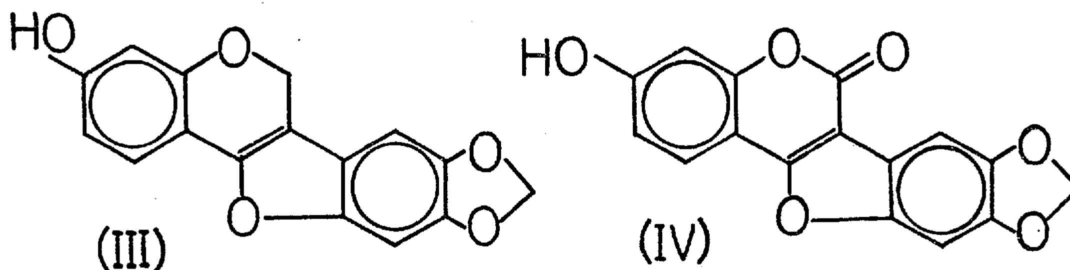
Fraction 4

Extracts from clover infected with Sclerotinia contained a new substance closely related to maackiain but which showed acid lability similar to pisatin.

Careful chromatography afforded a phenolic substance which turned reddish on exposure to laboratory lighting. Exposure to a strong UV source rapidly generated a deep reddish colour [cf. 2].

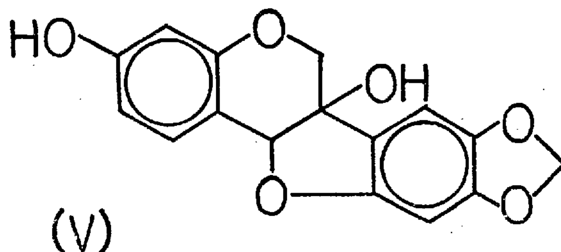
The UV spectrum λ_{max}^{EtOH} 233, 281, 286, 310 nm, showed shifts with base to 251 and 300 nm[10].

The NMR of this substance was run in deuterochloroform, but this proved to be a particularly unfortunate choice of solvent since traces of acid present caused the sample to decompose and turn yellowish within about fiv minutes of preparation. The resulting NMR pattern resembled that of maackiain but showed two sets of methylenedioxy protons. Aromatic signals could be assigned on the basis of there being a mixture of mainly anhydrosophorol[2] (III) and some medicagol (IV) present [17].



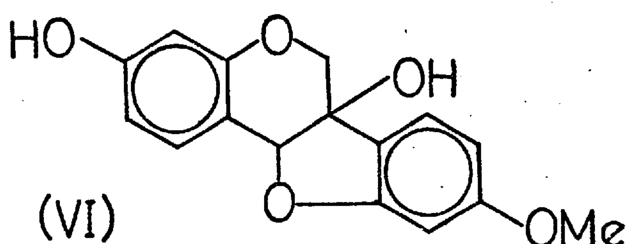
The UV spectrum of this dehydrated/autoxidised material and its mass spectrum confirmed the assignment.

The mass spectrum of the original compound $\frac{m}{e}$ 300 = $C_{16}H_{12}O_6$ clearly indicated a 3-demethylated pisatin structure (V).



Treatment of the phenol with diazomethane converted it to pisatin. Two groups [10, 11] have reported the occurrence of this compound and the UV/MS data presented are in accord with these findings.

Some samples of (V) contained significant amounts of a closely related substance (VI) ($C_{16}H_{12}O_S$) which was clearly the 3-demethylated analogue of 6a-hydroxyhomopterocarpin (II) found in clover samples treated with the non-pathogen.

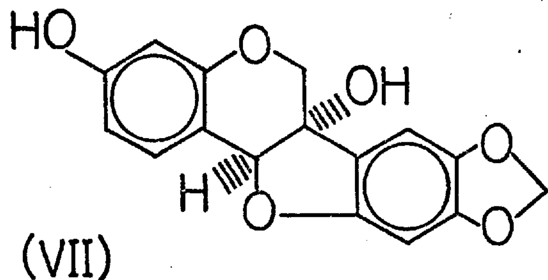


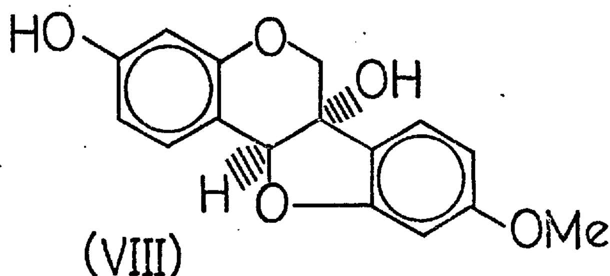
Maackiain and Medicarpin Metabolites

Maackiain and medicarpin were metabolised by *Sclerotinia in vitro* to afford 6a-hydroxylated derivatives.

Since the maackiain used was obtained from trifolirhizin of known absolute stereochemistry (6a R, 11a R), it is possible to assign the absolute stereochemistry of the 6a-hydroxy maackiain (VII) a 6a R, 11a R, because no transfusion of the B/C ring junction is allowed for reasons of strain [15].

Similar arguments can be applied to the medicarpin metabolite (VIII). The medicarpin was obtained from Jackbean [16] and is known to have the (-) 6a R, 11a R configuration [3].





Fungal transformations of (-) 6a R, 11a R pterocarpans to (-) 6a R - OH, 11a R pterocarpans have been reported [9].

The absolute stereochemistry of the products (I) (C) and (II) is less certain.

If the clover enzyme system involves (-) maackiain as an intermediary metabolite without converting it to its enantiomer, then the as yet unreported entantiomer of (+) pisatin would result. This seems a reasonable possibility. If, however, the biosynthetic sequence excludes (-) maackiain, then by analogy with other legume systems (+) pisatin would be formed.

In the case of the pathogen products (V) and (VI), then they may have the absolute configuration as shown in (VII) and (VIII), if they are considered as fungal hydroxylated metabolites of the plant phytoalexins. A complicating feature is the recently reported fungal mediated demethylation of (+) pisatin [10, 11].

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