STUDIES ON PHYTOALEXINS

Thesis submitted to the University of Glasgow

for the degree of Doctor of Philosophy

in the Faculty of Science

by

JOHN ANDREW BAILEY, B.Sc.

September, 1968

ACKNOWLEDGEMENTS

The work described in this thesis was carried out at the Garscube Research Laboratory, in the Department of Botany of the University of Glasgow.

The author wishes to express his thanks and appreciation to the following:

Professor P. W. Brian, who supervised the work, for his interest and helpful advice throughout, and for placing the facilities of the department at the disposal of the author.

Dr. B. A. Knights, for his guidance in the various chromatographic techniques used in the work.

The other members of the department who helped in so many ways, and in particular Miss M. B. Wood for typing this thesis, and Mr. N. Tait for supplying the photographic prints.

The Science Research Council who provided a Research Studentship, without which the work would not have been possible.

CONTENTS

GENERAL INTRODUCTION

The biochemical and physiological changes which occur when two metabolic systems interact have long interested biologists. This has primarily involved work on human disease, i.e. the interaction of the human body with a pathogenic agent; but more recently interactions of plants with various agents have been studied. These have included symbiotic relationships, e.g. nitrogen fixing root nodules, lichens and mycorrhizae, and pathogenic relationships with bacteria, fungi and viruses.

With fungal diseases of plants as with the other relationships each participant, the plant and the fungus, plays a vital role towards the final effect. A disease will result when a fungus gains entry into a plant, establishes itself, and by interrupting or disturbing normal metabolic processes causesdetrimental effects to the whole plant or to a localised region of that plant. The principal factors which will determine the successful establishment of a disease are the aggressiveness of the fungus, a term used to describe the capacity of the fungus to colonise and damage the host, and includes such factors as the growth rate of the fungus, the production of hydrolytic enzymes by the fungus, and the tolerance of the fungus to toxic substances produced by the host; the presence and availability of the required nutrients at the infection site; and the presence of inhibitors and toxic substances originating from either the host or the parasite (Hare, 1966). It is the latter aspect which

has increasingly attracted the attention of many plant pathologists.

Attempts have been made to explain resistance and susceptibility solely in terms of the inhibitors or toxins produced by the plant and the fungus respectively. Proof that a chemical is responsible for resistance or susceptibility is difficult to obtain. Koch's postulates have been adapted by several workers as a guide to obtaining such proof (Chester 1933; Allen 1959; Ludwig 1960; Wood 1967). The major criteria can be summarised as:

1. The chemical must be present at the regions showing the disease reaction.
2. The chemical must be obtained pure and characterised chemically.
3. The pure chemical must be shown to have a similar effect to that observed naturally.
4. The chemical must be present at concentrations which show this effect.

Toxins produced by the fungus which act against the host

Pathogenic and saprophytic fungi have long been known to produce toxic products when grown in artificial media (de Bary 1886). These toxins have been invoked as the cause of the disease syndrome: Gäumann stated in 1954 that "The microorganisms responsible for disease act by virtue of the toxins they produce" and that "microorganisms are pathogenic only if they are toxigenic".

Factual evidence for a role for such toxins in the etiology of a disease has been reported in only a few instances. In recent years

toxins have been described with the distinctive property that they are host-specific, i.e. they are only toxic to the species or variety of plant to which the fungus, producing the toxin, is pathogenic. These include the toxins produced by Helminthosporium victoriae, pathogenic to certain strains of Avena sativum; Periconia circinata pathogenic to Sorghum vulgare var. subglabrescens; Alternaria kikuchiana pathogenic to Pyrus serotina (Pringle and Scheffer 1964) and by Helminthosporium carbonum pathogenic to Zea mais (Scheffer and Ullstrup 1965). The use of such toxins will enable diseases to be studied under conditions where the role of a major variable, in this case the fungus, is defined and hence it should be possible to determine the biochemical changes producing the disease syndrome. The production and interaction of more than one host-specific toxin may make actual explanations more complex (Pringle and Scheffer 1967).

Toxins produced by the host plant which act against the fungus

In an analogous way attempts have been made to explain resistance to disease on a similarly purely chemical basis. These attempts can be divided into three categories.

1. Fungitoxic materials may be present in healthy plants at concentrations which inhibit the growth of fungi, (pre-formed resistance factors).
2. Changes in the amounts and/or the distribution of materials normally present in the healthy plant following infection could be responsible for the resistance of that plant.

3. Inhibitory substances, which are not normally present in healthy tissues, may be produced as the result of infection. These substances may be formed:

   (a) by the host as a result of a modification of the host's metabolism by the fungus (Phytoalexins), or

   (b) by the fungus, converting non-toxic precursors occurring in the host.

It has been suggested that many examples of resistance can be explained in these terms, but critical evidence is lacking in many cases.

1. Preformed Resistance Factors

Macerated plant tissues and substances isolated from these can often be shown to have antifungal activity. Gilliver (1947) investigated the antifungal action of extracts from 1,915 angiosperms: 23% of these species gave extracts which were inhibitory to Venturia inaequalis. Inhibition of fungi by such extracts does not necessarily signify that they are important in a resistance mechanism. Many artefacts can arise during maceration due to the mixing of substrates and enzymes which in the intact cell are prevented from doing so. This is particularly well demonstrated in tissues with a highly active polyphenol-oxidase system, when maceration results in the production of dark pigments formed by the condensation of oxidised phenolic compounds. It is of prime importance that any suspected inhibitor occurs free at some site where it could be expected to influence an invading fungus.

Other difficulties often associated with work on preformed inhibitors are that they do not differentiate between resistance and susceptibility; they do not necessarily occur at the site of infection; and that they occur at concentrations too low to show an effect.

(a) Toxins produced by dead tissues

The earliest case in which chemicals were shown to be involved in resistance is also the most well documented report of preformed chemicals being responsible for resistance. Walker and his colleagues working on the resistance of onion bulbs to Colletotrichum circinans and Botrytis allii established that pigmented onions were resistant to C. circinans and B. allii, while varieties which lacked these pigments were highly susceptible (Walker 1923). They found that associated with the water insoluble pigments, which occurred in the dead outer scales, were water soluble phenols (Link, Angell and Walker 1929). Catechol (Fig. 1.1) and protocatechuic acid (Fig. 1.2) were identified as being present in the water soluble fraction and were shown to inhibit germination of both C. circinans and B. allii (Link, Angell and Walker 1929; Link and Walker 1933).

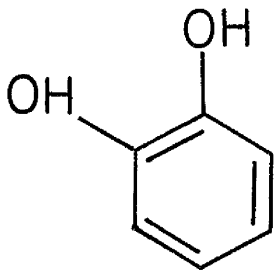


Fig. 1.1

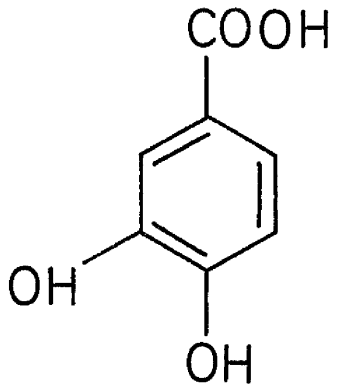


Fig. 1.2

Disease of susceptible bulbs resulted after the fungus had established infection in the outer scales and had grown through these outer regions into the centre of the bulb. The establishment of infection in the outer scales was prevented in resistant varieties. When the fleshy scales from resistant onions were exposed to a pathogen in the absence of the dead scales, they were invaded and became diseased as readily as susceptible varieties. It was suggested that this phenomena was probably due to the fact that the phenols occurred in a bound non-toxic form, e.g. as glycosides, in the living cells, and were only released when the cells died.

Phenolic compounds from other dead protective regions of plants also have a fungistatic effect. Martin and Batt (1957) reported that the ether soluble acidic constituents (phenolic acids) of the wax from the peel of apples resistant to Podosphaera leucotricha, conferred resistance to susceptible leaves when deposited thereon. The seed coats of peas resistant to foot rot diseases caused by Ascochyta pisi, A. pinodella, and Mycosphaerella pinodes contained higher amounts of phenolic compounds than less resistant ones. The leucoanthocyanins present were shown to have fungistatic activity (Clauss 1961).

(b) Toxins diffusing from living tissues

Fungistatic activity has been demonstrated for substances which diffuse from living plant tissues. Timonin (1941) demonstrated that exudations from roots of resistant varieties of flax were either more toxic or less stimulatory to Fusarium oxysporum f. lini than exudates

from susceptible varieties. The exudates from the roots of resistant varieties of pea, grown under sterile conditions, depressed the rate of germination of spores of Fusarium oxysporum f. pisi, more than exudates from peas which are susceptible to wilt (Buxton 1957).

The ability of substances diffusing from leaves to inhibit the germination of fungal spores is well documented (Kovacs 1955; Kovacs and Szëoke 1956; Topps and Wain 1957). Kovacs correlated the low incidence of local lesions on the leaves of sugar beet, resistant to Cercospora beticola, with the presence of inhibitors from healthy leaves. The inhibitors were detected in drops of water removed from the surface of the leaf.

Such inhibitors act at the surface of plant tissues to prevent penetration of the fungus. Having successfully overcome the initial barriers to infection, the fungus must now overcome the defences of the living plant cells.

(c) Inhibitors present in living cells

Phenols, their oxidation products (quinones), and their further condensation products have tended to dominate attempts to explain resistance in chemical terms. In 1929 Newton and Anderson reported work on the resistance of varieties of wheat to black stem rust caused by Puccinia graminis f.sp. tritici. They suggested that resistance was due to the liberation of phenols upon penetration by the fungus, and were able to relate phenolic content to resistance. Although Anderson (1934) failed to obtain a correlation between the phenolic content of wheat and resistance, interest was maintained by other workers.

Kargopolova (1937) indicated a definite correlation between phenolic content and rust resistance of several wheat varieties. More recently, Király and Farkas (1962) were able to repeat the work of Newton and Anderson confirming the results of Kargopolova, that the resistant variety Khapli has a higher phenolic content than susceptible varieties. Other varieties of wheat failed to conform to this idea. In fact, they showed that strains of rust pathogenic to Khapli were not able to cause disease in some varieties with a low phenolic content. They were, however, able to correlate the rate of induced phenolic synthesis with resistance: the resistant varieties accumulated phenolic compounds at a faster rate.

A similar situation exists regarding the resistance of potatoes to Verticillium albo-atrum. Histochemical tests have indicated that O-dihydroxyphenols, particularly chlorogenic acid, occur at higher concentrations in resistant tubers than in susceptible tubers. The phenols have been found at the site of infection at concentrations which were much greater than the concentrations known to inhibit germination and mycelial growth of V. albo-atrum. The increased susceptibility of senescing potato plants was associated with a parallel fall in the concentration of phenolic compounds (Lee and Le Tourneau 1958; McLean, Le Tourneau and Guthrie 1961). Patil (1966) has indicated that the rate of synthesis of chlorogenic acid is greater in the resistant than in the susceptible varieties, and that this rate decreases with age.

The distribution of toxic phenolic compounds in the potato has been correlated with their resistance to scab, Streptomyces scabies.

Tubers of resistant varieties contained more chlorogenic acid than susceptible ones and the regions where pathogens are likely to gain entry, e.g. lenticels and injured tissues, again contained greater amounts of chlorogenic acid. This was only demonstrated for the periderm of the tuber; no similar differences occurred in the flesh (Johnson and Schaal 1952; Schaal and Johnson 1955).

Other evidence regarding the importance of preformed resistance factors present in living plant tissues has been reported by Virtanen (1957) and Turner (1960). Virtanen showed that fungitoxic oxazolinones were present in extracts from rye, wheat and maize. In the healthy plant oxazolinones generally occurred as non-toxic glycosides; the toxic aglycones being released during extraction. The concentrations of free oxazolinones found in the intact plants were much less than those shown to be fungitoxic. Turner emphasised the importance of a toxic glycoside, Avenacin, in the resistance of oats to Ophiobolus graminis; the aglycone in this case was non-toxic.

Resistance may be due to the presence of preformed compounds which are not necessarily directly fungitoxic, but which inactivate the extracellular enzymes produced by the fungus in the process of primary invasion of the plant. Since de Bary (1886) first put forward the view that the extracellular enzymes of fungi act in pathogenesis many other workers have emphasised the importance of these enzymes (Husain and Kelman 1959; Wood 1960). Chona (1932) showed that pectinase activity of several plant pathogenic fungi was inhibited by the juice expressed from apples and potatoes. Many other plant juices have been shown to

possess similar activity against a range of extracellular enzymes (Byrde 1963). Water extracts from cucumber hypocotyls inhibited the activity of commercial pectinase and the endopolygalacturonase, produced by Cladosporium cucumerinum. The extracts from resistant varieties showed greater inhibition than susceptible varieties and, whereas after infection the susceptible varieties produced less inhibitory extracts, the resistant varieties yielded extracts which became increasingly inhibitory following infection (Mahadevan, Kuć and Williams 1965).

2. Quantitative changes of materials present in normally healthy plant tissues

Thus far, only passive immunity has been considered where the condition of the plant constitutes its resistant reaction; no changes in the metabolism of the plant being involved. In 1959 Allen stated that "there is now mounting evidence that the health of many plants is preserved, not by virtue of mechanical barriers, nor escapes from infection, but through an active metabolic initiative which destroys or immobilises the pathogen at some stage before it can produce serious disease. There is even unassailable evidence that for many plants, defence is not prepared in advance but depends upon metabolic events brought into play and substances produced upon the approach of the pathogen to its prospective host". Since 1959 evidence in favour of an actively induced resistance mechanism has accumulated. In the examples given in section 1.(c) it appears likely that the quantitative

changes which occur after infection may well be the more important (Farkas and Kiraly 1962; Patil 1966).

Many changes in the metabolism of plant tissues can be detected after infection, the most readily demonstrated being a marked increase in the oxygen uptake of the infected tissue. The accumulation of phenolic compounds and coumarins are other widespread phenomena associated with infected tissues. Chlorogenic acid and isochlorogenic acid accumulated in roots of Ipomea batatas (sweet potato) infected with Ceratocystis fimbriata. However, resistant and susceptible varieties behaved very similarly in this respect, and little in vitro fungitoxic activity could be demonstrated for these acids against C. fimbriata (Uritani and Akazawa 1959). Chlorogenic acid (Fig. 1.3) and caffeic acid (Fig. 1.4) accumulated in tissue from potato tubers infected with Helminthosporium carbonum. Again the concentrations of these acids present in the extracts could not wholly explain the inhibitory activity shown by these extracts (Kuć 1956).

Fig. 1.3

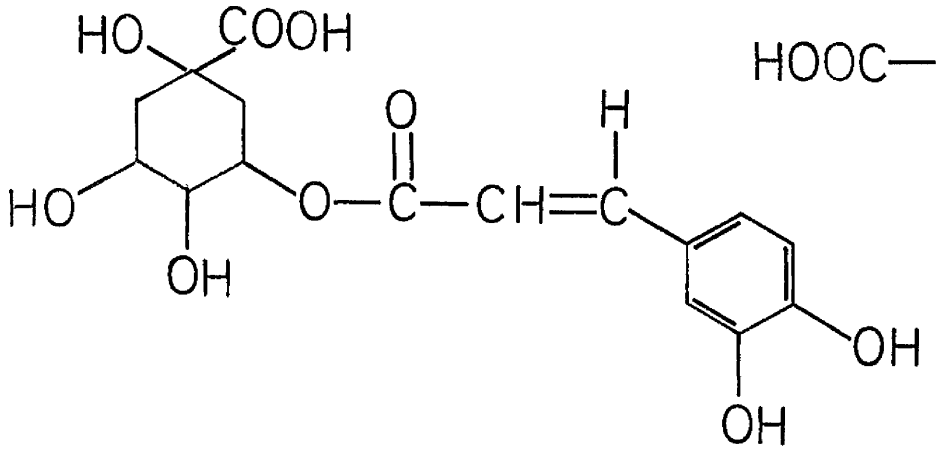
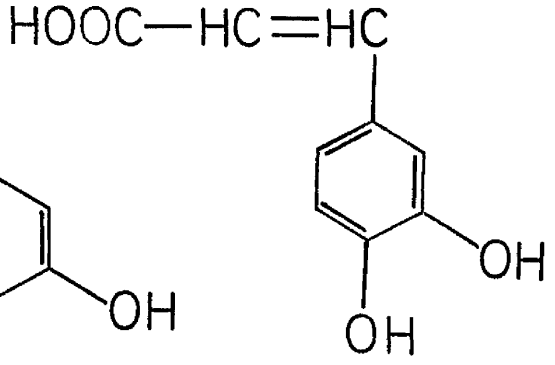


Fig. 1.4



In addition, several coumarins, e.g. umbelliferone (Fig. 1.6) and its glycoside skimmin, scopoletin (Fig. 1.5) and its glycoside scopolin,

and esculetin, accumulated in the roots of sweet potato following infection. This accumulation occurred to a greater extent in resistant varieties than in susceptible varieties (Minamikawa, Akazawa and Uritani 1963). Hughes and Swain (1960) reported a 10-20 times increase in scopolin content in potato tubers infected with Phytophthora infestans.

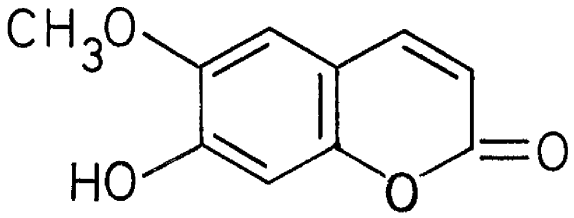


Fig. 1.6

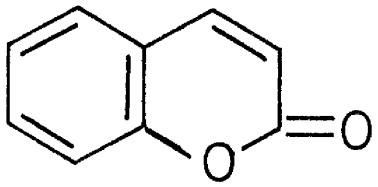


Fig. 1.5

It is of interest that both Solanum tuberosum and Ipomea batatas have, in addition, been shown to produce phytoalexins. These phytoalexins, abnormal metabolites formed as a result of an alteration to the host's metabolism induced by fungal infection, may afford the basic explanation of resistance in these tissues; the changes described above, resulting from the alteration of the host's metabolism, playing a minor part in the resistance mechanisms.

3a. Phytoalexins

Although Ward (1902) was probably the first to suggest that resistance was due to "internal factors" in the plant, Bernard's initial work on the mycorrhizae of orchids may well be the foundation of recent

work (Bernard 1902). He observed that the endophytic mycorrhizae failed to penetrate beyond the roots into the tuber. He studied the interaction of pieces of tissue with the fungus on a solid medium. Using Loroglossum hircinum and the fungus Rhizoctonia repens he demonstrated that the fungus grew out from the point of inoculation on the medium until adjacent to the tissue, where further growth was prevented. This inhibitory effect was destroyed both by grinding up the tissue and by heating it at 55°C for thirty-five minutes. Magrou (1924) found that if tuber tissue was left for two weeks on a medium in the absence of R. repens and then removed, the regions from which the tissue had been removed failed to support the growth of the fungus. Nobécourt (1929) demonstrated little inhibitory activity in healthy killed tuber tissue; however, tissue which had been exposed to R. repens and then killed, showed a marked inhibitory effect. More recently Gäumann and his colleagues have determined the cause of such results. Using Orchis militaris and Loroglossum hircinum, they have identified two fungitoxic compounds, Orchinol (Fig. 1.7) (Gäumann and Kern 1959) and the closely related compound Hircinol (Gäumann, Nüesch and Rimpau 1960).

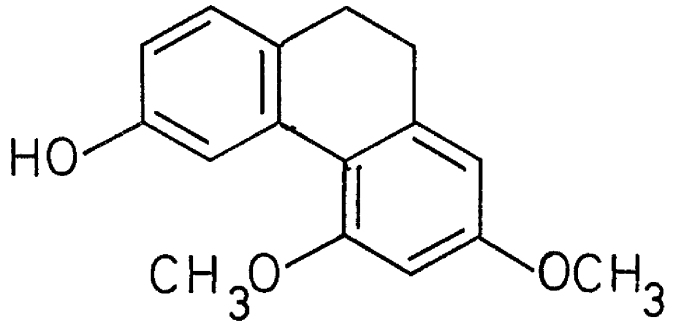


Fig. 1.7

Orchis militaris produced predominantly Orchinol, while Loroglossum

hircinum produced predominantly Hircinol.

The results with Loroglossum hircinum are typical (Gäumann 1963). Gäumann and his colleagues were unable to detect hircinol in 1 kg. of fresh tissue, and concluded that it was not formed during normal metabolism. Tissue fragments which were incubated under sterile conditions for six days contained 2.7 µg. of hircinol per gram of fresh weight of tissue; indicating that injurious effects induced the formation of small quantities of hircinol. Under similar conditions tissue which was exposed to Rhizoctonia repens produced 57 µg. of hircinol per gram of fresh weight of tissue. The production of hircinol as a result of injury may represent a barrier to infection, for although the net concentration produced by the tissue was low, the concentration in the outer cells in which it was produced would have been much higher.

The formation of orchinol has been shown to be induced by a number of plant parasitic fungi and bacteria found in association with the roots. Twenty-four saprophytes were ineffective. Orchinol is an unspecific fungitoxin, but bacteria are not affected.

This work is a well documented example of infection by fungi inducing the production of compounds which are not normal metabolites of the plant. Although the work was carried out on a symbiotic relationship, evidence for similar situations existing in pathogenic relationships is considerable.

In 1933 Chester had stated "most plants are immune to most fungi", i.e. that resistance to disease development is the most common state. A pathogenic fungus which is able to utilize one host can be found to

be quite unable to utilize a second. A study of this situation has figured greatly in work on the actively induced resistance mechanisms in plants.

The awareness of the possible induced production of fungitoxic compounds arose after work by Müller and Borger (1940) on the resistance of varieties of potato to Phytophthora infestans. They showed that potato tuber slices, which had previously been inoculated with an avirulent strain of P. infestans were found to be resistant to infection by a virulent strain; slices which were only exposed to the virulent strain became infected. They implicated the formation of compounds by the interaction of the potato and the avirulent strain, which inhibited the growth of the virulent strain. This inhibitory principle they termed a Phytoalexin. They defined phytoalexins as " 'antibodies' which are produced as a result of the interaction of two metabolic systems, the host and the parasite, and which inhibit the growth of microorganisms pathogenic to plants" (Cruickshank 1963).

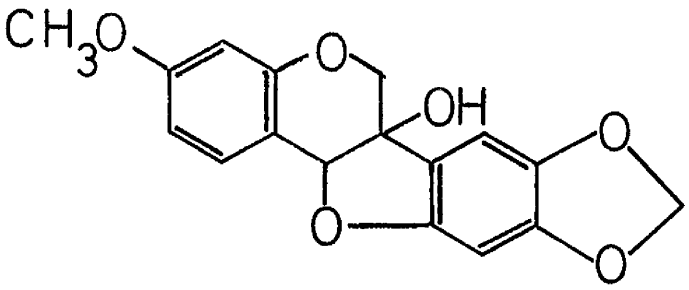
Müller attempted to explain why a fungus which is pathogenic to one host is non-pathogenic to another. He investigated the interaction of Phaseolus vulgaris with Sclerotinia fructicola, a fungus pathogenic to plum, but non-pathogenic to beans. The induced production of fungitoxic compounds, not present in the healthy plant, was again demonstrated. The technique he used was a simple one termed the "drop-diffusate technique". Drops of a fungal spore suspension are placed in contact with plant tissues in such a way that the suspension can be recollected after a suitable time. The suspension which has been called the "diffusate"

is collected, the spores removed by centrifugation and then tested for antifungal activity. This idea was adapted by Müller (1958) and by Cruickshank and Perrin (1960) for their work on phytoalexins produced by species of the Leguminosae. There are several advantages in this method as compared with the extraction methods used previously. Firstly, the tissue that Müller used, the inner surface of bean pods, presents no major physical barrier to infection by the spores, and allows diffusion of low molecular weight compounds to and from the cells of the pod. Finally the diffusate is readily obtained, probably avoiding the production of artefacts which could arise from macerating the tissue.

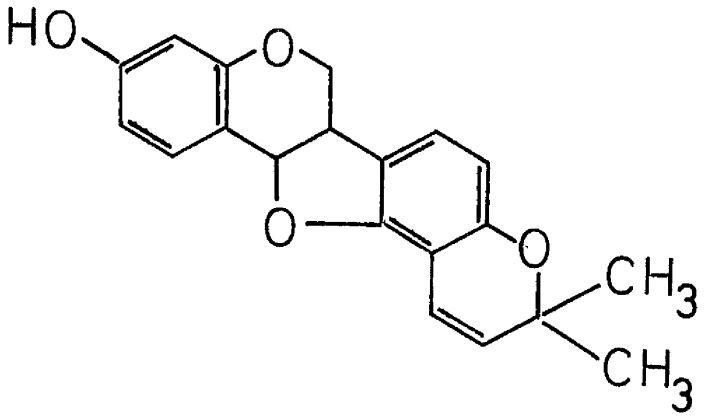
The induced formation of fungitoxic materials has been demonstrated in several plant-fungus interactions. The most well documented examples include Pisatin, produced by Pisum sativum in the presence of Sclerotinia fructicola (Cruickshank 1960); Phaseollin, produced by Phaseolus vulgaris in the presence of Sclerotinia fructicola and Phytophthora infestans (Müller 1958); Ipomeamarone, produced by Ipomea batatas in the presence of Ceratocystis fimbriata (Uritani, Akazawa and Uritani 1954); an isocoumarin, produced by Daucus carota in the presence of Ceratocystis fimbriata (Condon and Kuć 1960); and Rishitin, produced by Solanum tuberosum in the presence of Phytophthora infestans (Tomiyama, Sakuma, Ishizaka, Sato, Katsui, Takasugi and Masamune 1968). See Fig.1.8. The formation of phytoalexins has been implicated for soybean, broadbean and several other plant species, (see Cruickshank 1963; Wood 1967).

The important aspects of the phytoalexin theory of disease resist-

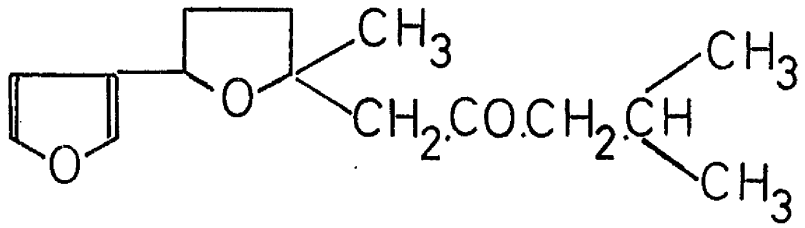
Fig. 1.8



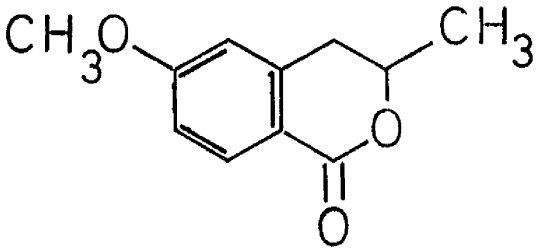
Pisatin



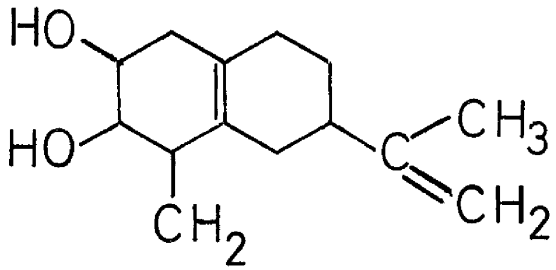
Phaseollin



Ipomeamarone



Iso-coumarin



Rishitin

ance were put forward by Müller and later modified by Cruickshank (1963). They can be summarised:

(1) Phytoalexins are produced as a result of the alteration of the normal metabolism of the host cell. This change is induced by germinating fungal spores, the metabolic products secreted by such spores and by certain chemicals, especially metabolic inhibitors.

(2) Phytoalexins are produced only by living plant cells.

(3) A high degree of host specificity occurs.

(4) The fungitoxic action of the phytoalexin is non-specific, i.e. all fungi are affected. However, different fungi may show varying sensitivity to the phytoalexin.

(5) Müller restricted the concept of phytoalexins to inhibitors which are not normally present in healthy tissues. However, the important factor as regards a resistance mechanism is that substances are produced as a result of infection at a concentration which inhibits further growth of the fungus: whether or not they are "normal" metabolites is of minor importance.

(6) The formation of the phytoalexins known at present seems to involve the synthesis of aromatic compounds.

(7) The importance of phytoalexins in disease resistance is not completely established. Fungi which are pathogenic to pea are not as sensitive to pisatin as non-pathogens. The concentration of pisatin formed in response to infection by

(7) these fungi also varies, and it seems from this work that to be pathogenic, a fungus must be resistant to the concentration of pisatin it induces (Cruickshank and Perrin 1963).

The isolation, chemical characterisation and hence the quantitative estimation of the toxins produced either by the fungus against the plant or by the plant in response to the fungus should enable the host-pathogen relationship to be studied in great detail.

3b.

Recently an example of fungitoxic materials being produced directly by the action of the fungus on non-toxic precursors present in the host has been described (Fawcett and Spencer 1967). Using a variety of apple showing a degree of resistance against Sclerotinia fructigena, they were able to demonstrate that infected fruit tissue inhibited the growth and germination of a non-pathogen, Alternaria brassicola. Healthy tissue showed no antifungal activity (Fawcett and Spencer 1966). Six phenolic compounds were isolated from the ether extracts of the juices from infected fruit. Two phenolic acids, 4-hydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid, were identified and shown to be fungitoxic. In contrast to phytoalexins the living apple cells were not required for the production of these compounds: S. fructigena when grown in the juice from healthy apple flesh

produced similar compounds in the medium after incubation at 23°C for ten days. These compounds were only produced in the presence of this juice; no phenolics were detected when S. fructigena was grown in the presence of the peel nor in the presence of the pulp, which had previously been extracted with water and ethanol. They concluded that non-toxic precursors were being converted to anti-fungal phenolic acids by the direct action of the fungus. The importance of such phenolics in the resistance mechanisms of apple fruits is, as yet, unknown.

Objectives

The main objectives of the work to be described here were:

(1) to investigate the resistance mechanisms in apple fruits in relation to the possible production of phytoalexins;

(2) to utilize a system where the phytoalexin is known, to study physiological factors which may affect the production of the phytoalexin. The system used was the production of pisatin by Pisum sativum.

The initial work with apple fruits emphatically demonstrated the problems involved when extracting plant tissues. Exposed or macerated apple flesh is very rapidly oxidised with the production of a complex brown pigmentation. In addition, the thick waxy cuticle of the intact fruits presents a barrier to penetration by

the fungal spores. Inoculation of the fruits with fungal spores requires that this barrier be removed or overcome by inoculations directly into the flesh, through the cuticle. Plant tissue cultures derived from the apple mesocarp, (and also from the root and stem of pea seedlings and from the stem of the mature pea plant) were established in an attempt to overcome these problems. A further advantage when using tissue cultures is that they provide actively dividing cells growing under a controlled chemical and physiological environment.

The main use of tissue cultures by plant pathologists has been to compare the growth characteristics of healthy and diseased tissues. Tissues originating from "Galls" have been widely used. A second and increasing application has been in the study of obligate parasitic fungi grown in their host tissue cultures. This has enabled such parasites to be grown in vitro and it may enable them to be grown on a defined artificial medium. Little work has been done to compare the resistant reaction of a plant with tissue cultures derived from that plant. Mathes (1967) has shown that tissue cultures obtained from plants known to produce fungitoxic products secreted fungitoxic compounds into the medium in which they were grown. Saad and Boon (1966) reported that tissue cultures derived from the leaf and petiole of apple shoots both resistant and susceptible to Venturia inaequalis did not support the growth of this fungus. V. inaequalis was able to grow on tissue cultures derived from tobacco and tomato, plants which are not normally host to V. inaequalis. Ingram and Robertson (1965) and Ingram (1967) have used tissue cultures derived from the potato to study the expression

of R-gene resistance to Phytophthora infestans. They showed that tissue aggregates from susceptible plants supported extensive growth of P. infestans whereas those from resistant plants allowed only rudimentary growth. The production of post-infectional toxins by suspension cultures of potato was demonstrated.

Physiological factors acting on the host which are known to influence phytoalexin production include high and low temperatures, oxygen supply and the state of maturity of the tissue (Jerome and Müller 1958; Chamberlain and Gerdemann 1966). The more specific work pertinent to the investigations which follow will be discussed later in the relevant sections.

CHAPTER 2. MATERIALS AND METHODS

List of materials used in the work reported here.

Material	Grade	Source
Agar	Oxoid No.3	Oxoid Ltd., England.
6-Benzylaminopurine (BA)	-	Sigma Chemical Co., U.S.A.
Chloramphenicol	-	Parke, Davis & Co. England.
Coconut milk	-	Covent Garden Fruit Co., Glasgow.
Cycloheximide	-	Calbiochem Ltd., U.S.A.
2,4-Dichlorophenoxy-acetic acid (2,4-D)	-	British Drug Houses Ltd.
Ethyl alcohol	Absolute	J. Burroughs Ltd., England.
Ferric chloride	Spectrographically standardised substance	Johnson, Matthey & Co. Ltd., England.
DL-p-fluorophenylal-anine (FPA)	-	Sigma Chemical Co. Ltd., U.S.A.
Gibberellic acid (GA_3)	91.4%	Imperial Chemical Industries Limited.
Indolylacetic acid (IAA)	Horticultural quality	British Drug Houses Ltd.
Inorganic reagents	Analar when available	British Drug Houses Ltd.
Kinetin (K)	-	Sigma Chemical Co. Ltd., U.S.A.
L-Malic acid	-	Sigma Chemical Co. Ltd., U.S.A.
Malt extract	-	Oxoid Ltd., England.

Material	Grade	Source
Organic reagent	Analar when available	British Drug Houses Ltd.
Patulin	-	Professor P. W. Brian.
Petroleum spirit (b.p. 40°-60°C)	Analar, free from aromatic hydrocarbons. Distilled before using up Vigreux column and partial take-off head (Vogel 1962 p.98)	British Drug Houses Ltd.
Pisum sativum cv. Greenfeast	-	C. Sharpe, Lincs., England.
Puromycin dihydrochloride	-	Nutritional Biochemicals Corporation, U.S.A.
Yeast extract	-	Difco Laboratories, U.S.A.

Table 2.1. The composition of the fungal culture media

Medium I

Ammonium tartrate	1.0g.
KH_2PO_4	1.0g.
$MgSO_4.7H_2O$	0.5g.
Glucose	12.5g.
Distilled Water	to 1,000 ml.

Medium II (Malt extract agar)

Malt extract	50g.
Peptone	1g.
Glucose	20g.
Agar	25g.
Distilled Water	to 1,000 ml.

Medium III (Czapex Dox agar)

$NaNO_3$	3.0g.
K_2HPO_3	1.0g.
$MgSO_4.7H_2O$	0.5g.
KCl	0.5g.
$FeSO_4.7H_2O$	0.01g.
Sucrose	30g.
Agar	20g.
Distilled Water	to 1,000 ml.

A. Culture Techniques

Strictly sterile conditions were maintained during all cultural procedures.

(a) Culture of fungal species

The fungi required in this work were from the following sources.

Botrytis allii	:	Professor P. W. Brian.
Botrytis cinerea	:	Isolated from a soft rot of apple cv. Cox's Orange Pippin. Pathogenicity of this isolate was confirmed by re-inoculation.
Penicillium expansum	:	C.M.I., 37, 128.
Cylindrocarpon heteronema	:	Isolated from contaminated culture (See Chapter 3).

These fungi were maintained on either Czapex Dox agar (Thom and Raper, 1945) or on 5% Malt extract agar. The cultures were incubated at 20°C in the dark. Sporulating cultures were obtained by incubating them in the dark at 24°C in the cases of P. expansum and C. heteronema, but for B. allii and B. cinerea, regularly sporulating cultures could only be achieved by growing them at ambient temperatures (20-25°C) in the light. The spore suspensions used in the assays of spore germination were obtained from these cultures after ten days incubation. The media used for culture of the fungi are shown in Table 2.1.

(b) Culture of callus tissues

Callus cultures, which had been established from pea and apple tissues, were grown on artificial medium. Pea root callus cultures have been established by Torrey and Shigemura (1957) and apple mesocarp callus cultures have been established by Letham (1958) and Nitsch (1959).

Table 2.2. The composition of tissue culture media

Medium IV

Salts and micronutrients

$Ca(NO_3)_2.4H_2O$	288 mg.	Na_2SO_4	200 mg.
KNO_3	80 mg.	$MgSO_4.7H_2O$	740 mg.
KCl	65 mg.	H_3BO_3	1.5 mg.
$NaH_2PO_4.2H_2O$	21.5 mg.	H_2MoO_4	0.0017 mg.
$MnCl_2.4H_2O$	6 mg.	$CuSO_4.5H_2O$	0.02 mg.
$ZnSO_4.7H_2O$	2.65 mg.	$FeCl_3$	3.1 mg.
KI	0.75 mg.	Na.E.D.T.A.	0.8 mg.

Solutions were maintained so that a final stock solution was produced. 100 ml. was used in 1l. of medium.

Vitamins and amino acids		Additional growth factors (Letham, 1958)	
Aneurin HCl	0.1 mg.	Cysteine	10 mg.
Pyridoxine HCl	0.1 mg.	Pantothenic acid	0.2 mg.
Nicotinic acid	0.5 mg.	Inositol	0.2 mg.
Glycine	3.0 mg.	Biotin	0.2 mg.

Concentrated stock solutions were prepared so that 1 ml. of stock solution was required for 1l. of medium.

2,4-Dichlorophenoxyacetic acid	6 mg.
Coconut milk	130 ml.
Sucrose	20 g.
Glass distilled water	to 1,000 ml.
Agar, when required	6 g.

Medium V As for medium IV, but lacking the additional growth factors.

Medium VI As for medium V, plus 1g. yeast extract.

The cultures used in this work were maintained on 30 ml. of medium, solidified with agar in 100 ml. Erlenmeyer flasks and incubated at 26°C in a growth-room illuminated with a single 40 watt tungsten lamp. Callus cultures were routinely subcultured every twenty-eight days. In the experiments using callus growing in liquid medium, the Erlenmeyer flasks were incubated at 26°C in the dark in a shaking incubator, rotating at 150 revolutions per minute.

The different media used to culture these tissues are shown in Table 2.2. No adjustment of pH was necessary as the pH of these media was 5.5 - 6.0. All media were sterilized by autoclaving at 121°C for 10 minutes.

The establishment and growth of callus tissue cultures

Pea tissues

(1) Young stem and root tissues

Pea seeds were washed in 70% ethanol for five minutes and then treated with 0.1% mercuric chloride for ten minutes. After washing these seeds once in absolute ethanol and in ten changes of sterile distilled water, they were placed on moist absorbent cotton wool in 100 ml. Erlenmeyer flasks, which had previously been sterilized at 121°C for thirty minutes, and incubated in the dark for four days at 15°C. The final 1/4" of the root was discarded and the next 1/4" of the root was transferred on to solid medium VI to initiate callus growth. Segments of pea stem 1/4" long were removed from above the cotyledons of young seedlings

which had been incubated for thirteen days, and transferred to medium VI to initiate callus growth. When callus tissue had been formed on the surface of the segment, it was transferred to fresh medium VI. From these initial isolations faster growing strains were selected.

The four types of callus each originated from a single source. The callus tissues obtained from the pea root showed great variations and it was possible to distinguish and establish three visually distinct types. The pea root calluses were maintained on medium VI, as yeast extract seemed essential for rapid growth. The callus tissue obtained from the pea stem was maintained on medium V.

(2) Mature stem tissues

Pea seeds, which had been sterilized as described above, were sown in pots containing sterilized soil and grown for eight weeks in a greenhouse. After this time, lengths of stem 6" long were collected, the leaves removed and the stems sterilized in 0.1% mercuric chloride for five minutes. They were then rinsed in 70% ethanol and washed in five changes of sterile distilled water. Segments of stem, 1/4" long, were taken from the middle of these stems and placed on medium VI to initiate callus growth. Growth of this tissue was very slow and, as a result, no experiments could be carried out with this isolate.

Apple tissues

(1) Mature fruit tissues

The fruits of cultivars Golden Delicious, Granny Smith and Cox's

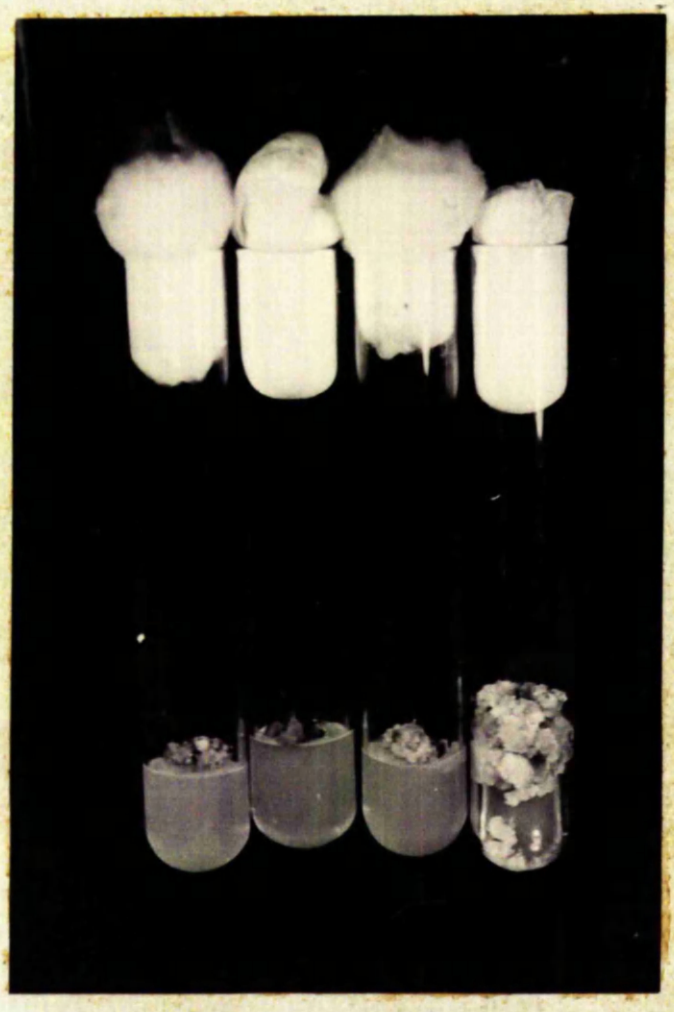


Fig. 2.1. Effect of 2,4-D on the growth of Granny Smith callus.

1. Medium V, callus subcultured from medium V.
2. Medium V - 2,4-D, callus subcultured from medium V - 2,4-D.
3. Medium V, callus subcultured from medium V - 2,4-D.
4. Medium V - 2,4-D, callus subcultured from medium V.

Orange Pippin were sterilized as described for pea seeds. After washing the fruits in distilled water, the peel was removed and plugs of tissue 8 mm. in diameter were obtained using a cork borer. The plugs were cut into segments 1/4" long and placed on medium IV to initiate callus growth. By selecting a fast growing strain, successful callus cultures were established from the cultivars Golden Delicious and Granny Smith. The segments of Cox's Orange Pippin fruit tissue failed to produce any callus growth.

The cultures were maintained on medium V.

On this medium Golden Delicious callus grew very rapidly, producing large quantities of tissue. Granny Smith callus, although producing healthy tissues, grew at a slower rate. Letham (1958) reported that in the medium on which he maintained his apple callus tissues he reduced the concentration of 2,4-D to 1 mg./l.

Granny Smith callus produced growth which was very similar to that of Golden Delicious callus if transferred to a medium lacking 2,4-D. However, further subculturing on to this medium failed to maintain the growth of this callus (Fig. 2.1). It would appear that the concentration of 2,4-D in medium V was preventing rapid growth of this callus. Thus when the callus was transferred to a medium lacking 2,4-D, the inhibitory effect was removed and rapid callus growth occurred. Nevertheless, 2,4-D is necessary for growth since further subculturing on to a medium lacking 2,4-D failed to maintain the growth of the callus. No attempt was made to obtain active growth by adjusting the concentration of 2,4-D in the medium.

(2) Apple fruitlet tissue

Fruitlets (1/2" in diameter) of Granny Smith were sterilized as for pea seeds. The fruitlet was cut into four pieces and each piece was placed with a cut surface on medium IV to initiate callus growth. The contamination rate was very high and any growth which was produced was poor. No fast growing callus tissue was formed on this medium.

B. Assay Techniques

(1) Spore germination assay

The fungus was grown on a suitable medium to produce a heavily sporulating culture. The spores were removed from the culture by shaking with sterile distilled water. The suspended spores were centrifuged at 4,000 r.p.m. for two minutes and the supernatant was discarded. The spores were resuspended in a volume of sterile distilled water. This volume was adjusted so that when two drops of this spore suspension were added to 1 ml. of the test-solution and mixed thoroughly using a Fisons Whirlimixer, the final concentration of spores in the test suspension produced between 10-20 spores when viewed down the microscope at 400 X magnification, (the diameter of the field of view being 280μ). Two drops of this test suspension were applied to each end of a clean sterile slide. The slide was supported on glass rods over moist filter paper in a petri-dish and incubated at 24°C in the dark. Each suspension was tested on two slides. The percentage germination was estimated by confirming the uniformity of germination on both slides and in one drop counting the germination of 100 spores from widely separated fields. (Usually 6-8 fields were required).

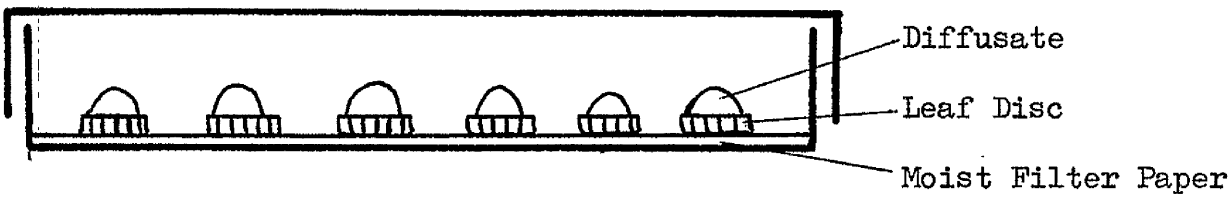


Figure 2.2. Drop-Diffusate Technique (Cruickshank, 1960)

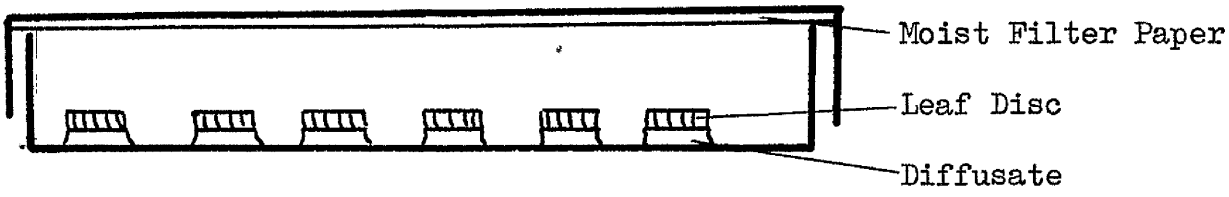


Figure 2.3. Modified Drop-Diffusate Technique.

The basic assay was used for most of the following work. The details of any differences will be indicated at the appropriate time.

(2) Pisatin production by pea pod endocarp surfaces and by pea leaf discs.

The production of pisatin by pea pod endocarps and by pea leaves has been reported by Cruickshank (1963) using the "drop-diffusate technique" as described by Müller (1958). This technique, as used in this work, is described opposite. The endocarp surfaces of opened, detached peas were directly inoculated with the test-solution. The pods used in an experiment were chosen with respect to age and size. Pea leaves obtained from peas of the same age were also selected for uniformity. Only leaves which were removed from the same node of plants sown at the same time were used in any one experiment. The leaves were rubbed between the fingers, and discs 8 mm. in diameter were cut from the laminas using a cork-borer. Sufficient leaf discs for the entire experiment were cut and bulked together. These discs were placed at random on moist filter paper in 9 cm. glass petri-dishes (Fig. 2.2). The test-solution was applied in small drops on to the adaxial surfaces of the leaf discs. After a suitable period of incubation at 24°C in the dark, the test-solution, now termed the diffusate, was removed and the concentration of pisatin in the diffusate was measured.

This technique was shown to be readily carried out when using healthy leaf discs. However a slight rearrangement was necessary in order to carry out the work on senescing leaf discs. Using the original

technique it was noticed that, as a result of changes in permeability, the more senescent discs did not retain the diffusate, which was absorbed by the underlying filter paper. Thus it was impossible to measure the pisatin production by senescing pea leaf discs. The improved procedure is shown in Fig. 2.3. Drops of the test-solution were placed on the bases of 9 cm. plastic disposable petri-dishes. The leaf discs were floated with their adaxial surfaces in contact with the drops. Very little coalescence of the drops occurred when their number was maintained at twenty per dish. A piece of moist filter paper was placed in the lid, and the petri-dishes were incubated as above.

This modified technique exhibits several important advantages:

(1) The volume of the drop can be standardised.

(2) Each disc yields a diffusate irrespective of its physical condition.

(3) The volume of the drop can be increased considerably when compared with that volume which can be maintained on the surface of a leaf disc. Thus the labour and time involved in cutting large numbers of discs can be reduced.

These three advantages, in addition to the uniformity of the leaf discs used, standardise this technique to a high degree.

Unfortunately, the early work, i.e. on the induction of pisatin, was carried out using the original technique. However, all the important experiments with senescing leaf discs were done using the modified technique.

Generally these procedures were designed to yield at least 5 ml.

of diffusate so that 5 ml. was available for assaying pisatin content. (When volumes differed from 5 ml. it will be stated.. The difference in volume was, of course, allowed for when calculating the pisatin concentration of the diffusate).

The labour and time required to cut large numbers of discs rarely allowed the replication of treatments. To compensate for this, experiments were repeated at least twice to confirm the original results, and all the data on drop-diffusates presented in this thesis have been checked in this way.

(3) The assay of pisatin content in diffusates

As described by Cruickshank (1961), the characteristic ultraviolet absorption spectrum of pisatin and the partition between light petroleum and water can be adapted to a physical assay for pisatin in diffusates. 5 ml. of diffusate, was extracted four times with an equal volume of light petroleum (b.p. 40-60°C) and the combined extracts were taken to dryness at 40°C under vacuum. The residue was dissolved in 5 ml. of absolute ethanol, and the optical density (O.D.) of the resulting solution was measured between 200 and 380 mμ in a Unicam S.P.800 spectrophotometer using absolute ethanol as the control. The concentration of pisatin in diffusates was calculated from the O.D. at 309 mμ, taking an O.D. value of 1.0 for a 5 ml. solution as equivalent to 43.8 μg./ml. of the original solution (Cruickshank, 1961). All values for pisatin concentration of diffusates given in the results which follow are based on this calculation.

(4) The confirmation of the identification of pisatin in diffusates from pea pod endocarp surfaces.

Pea pod endocarp surfaces were inoculated with a culture filtrate of Penicillium expansum. (See Chapter 4). After seventy two hours the diffusates were removed and the pisatin was extracted in light petroleum. This was taken to dryness at 40°C under vacuum. The pod tissues were bulked and macerated in 90% ethanol in a Waring blender. The ethanolic extract was taken to dryness at 50°C. under vacuum and the pisatin was obtained from the residue by dissolving it in large quantities of 20% ethanol. The residue obtained after the 20% ethanolic solution had been taken to dryness at 60°C was dissolved in redistilled ether, and this was added to the extract from the diffusate. A preliminary experiment showed that, by subjecting the ether soluble materials to chromatography on 0.25 mm. layer of activated silica developed in chloroform, good separation of several ultra-violet absorbing spots and one spot which stained brown when sprayed with 1% ceric ammonium nitrate dissolved in 4N. sulphuric acid (ceric sulphate reagent) could be achieved. This was repeated with a greater amount of the ether soluble material on plates with 1.00 mm. of celite, which had been pre-washed in methanol and dried for one hour at 105°C. After repeated developing in chloroform, to obtain better separation, the separated bands were removed from the plate and eluted with ether. The ether was removed at 40°C under vacuum and the residue was redissolved in absolute ethanol. The optical densities of the ethanolic solutions were measured between 200 and 380 mμ. Pisatin was identified as the only compound which stained

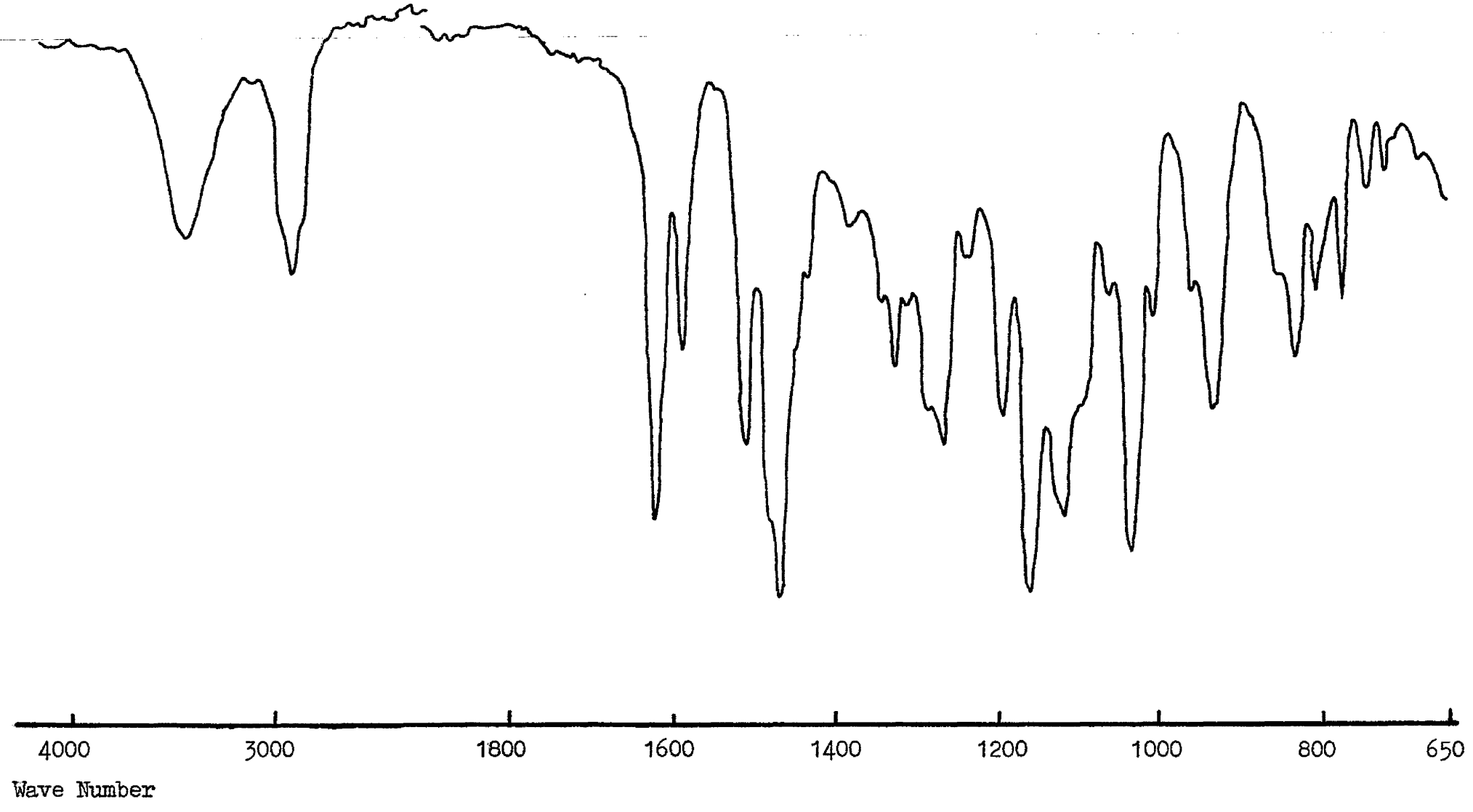


Figure 2.4. The infra-red spectrum of purified pisatin.

when sprayed with the ceric sulphate reagent.

Pisatin, which was purified in this way, was subjected to Infra-red and Ultra-violet spectrophotometry. The spectrophotometric traces obtained are shown in Fig. 2.4 and 2.5 respectively. There is a very close similarity between the spectrophotometric traces obtained here and the results published by Perrin and Bottomley (1962). The conversion of pisatin to anhydropisatin in the presence of hydrochloric acid was demonstrated with unpurified pisatin (Fig. 2.5).

The results indicate that there is a strong justification to believe that the material obtained in this work was the compound pisatin as described by Cruickshank and his co-workers.

(5) The assay of senescence of pea leaf discs

The parameter used to determine the state of senescence of leaf discs was their chlorophyll content. This is a commonly used measurement and evidence would suggest that it is a good indicator of the occurrence of the other processes associated with senescence, i.e. protein and nucleic acid breakdown (Osborne, 1962).

Leaf discs were cut from leaves obtained from similar nodes of pea plants grown outside. Fully expanded leaves removed from the third, fourth and fifth node were most suitable because of their larger size and also their more uniform senescence behaviour. The rate of senescence of 8 mm. leaf discs, cut from the lamina of the leaf, was investigated by floating the discs on various solutions in 9 cm. petri-dishes containing 20 ml. of the test solution. The method of selecting the discs was identical to that used in obtaining discs for the assay of pisatin production. In experiments where both pisatin production and senescence

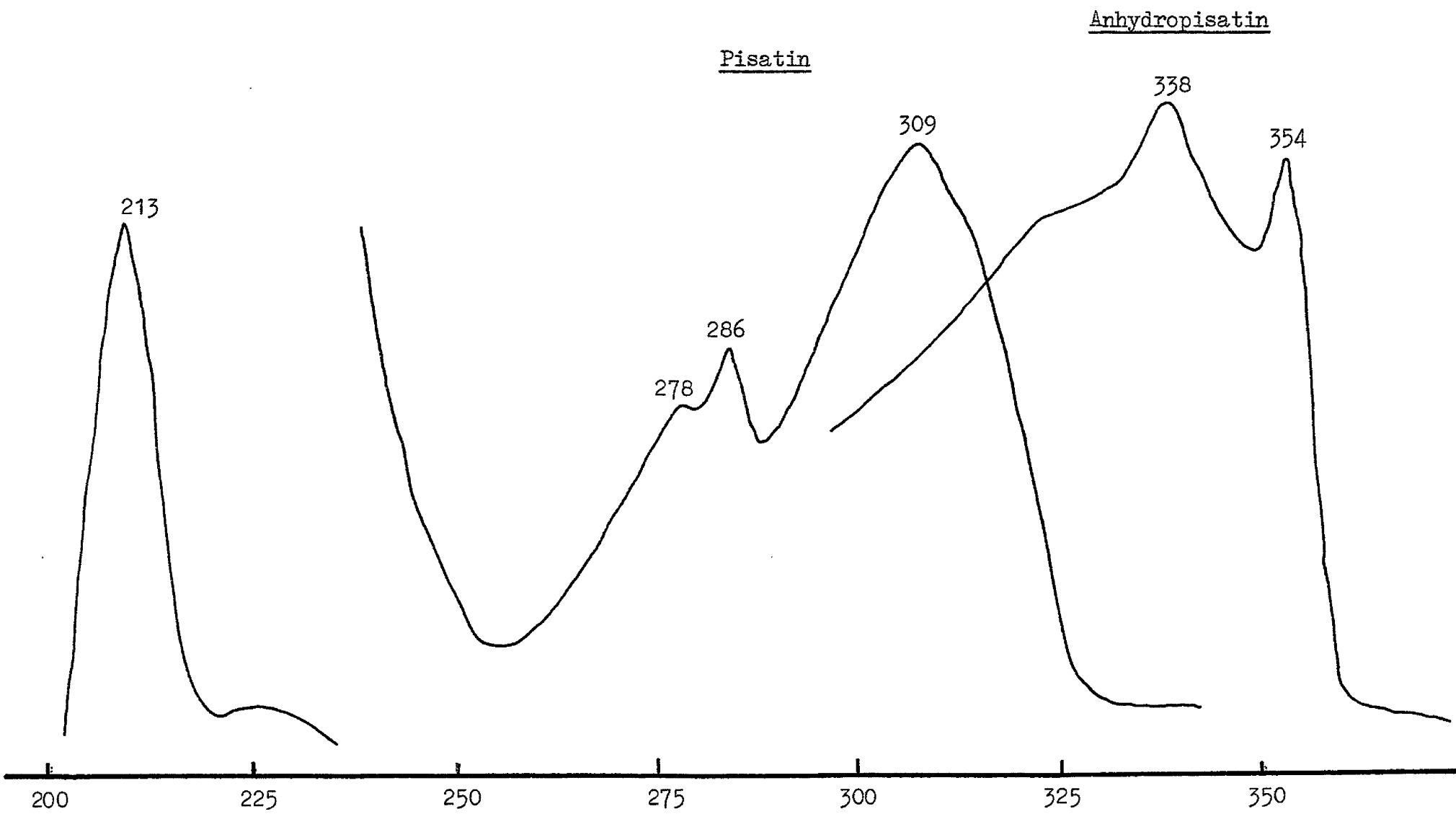


Figure 2.5. The ultra violet spectrum of Pisatin and Anhydropisatin

were measured, twenty leaf discs, chosen at random, were floated in each petri-dish. After the discs had been floating for the required length of time, they were individually transferred to test-tubes containing approximately 2.5 ml. of 70% ethanol. The chlorophyll was extracted by standing these tubes overnight in the dark. The extracted disc was removed and the volume of ethanol was carefully adjusted to 3.0 ml. and the O.D. at 665 mμ of the ethanolic extract was measured using 70% ethanol as the control. The mean value of the twenty discs was used as a measure of the chlorophyll content of the discs.

The rate of senescence of leaf discs obtained from leaves of different ages was found to vary greatly. The rate of senescence of these discs was generally slower than other experimental material used to study senescence, e.g. Xanthium pennsylvanicum (Osborne, 1962), Brassica rapa (Kuraishi, 1968) and Phaseolus vulgaris (Leopold and Kawase, 1964). By using discs of leaves obtained from the same node of a plant, good uniformity of senescence was achieved within an experiment. However, it will be noticed that great variations in the rate of senescence of these discs occurred between different experiments.

CHAPTER 3. AN INVESTIGATION INTO THE PRODUCTION OF PHYTOALEXINS IN APPLE FRUIT TISSUES

Susceptibility to diseases caused by pathogenic fungi is a major problem encountered during the storage of apple fruits. The varying resistances to disease encountered in these fruits pose interesting physiological and biochemical problems.

Resistance varying between different varieties

The rate of rotting of various cider apples, infected with Sclerotinia fructigena, has been shown to be inversely related to the rate of browning of damaged fruit tissue (Byrde, 1957). Glutathione was shown to prevent the production of brown products and to increase the susceptibility of fruits to disease. The oxidised juices from fruits had little effect on the germination of the spores in vitro, but diminished the macerating activity of enzyme preparations from Sclerotinia fructigena. It was suggested that the resistance of the cider apples examined was due, in part, to the occurrence of polyphenols, which were oxidised to compounds of high molecular weight by the host's enzymes and these, in turn, inactivated the extracellular enzymes of the fungus (Byrde, Fielding and Williams, 1960).

Varying resistance associated with the physiological state of the fruit

The resistance of particular apple varieties to fungal pathogens has been demonstrated to vary greatly in relation to the maturity of

the fruit. This has been shown to be true for fruit attached to the tree and more particularly for fruit during storage. Gloeosporium perrenans inoculated into fruitlets attached to the tree failed to produce immediate rotting. A rot was produced only after the fruitlets had attained a certain level of maturity. However, fruitlets which were detached from the tree before inoculation became diseased more readily. Fruits which were inoculated immediately after harvesting were rotted by G. perrenans after ten weeks, while similar fruits which had been stored for five months before inoculation became rotted after only four weeks (Edney, 1956). It was suggested that substances were present in the resistant fruits which inactivated the extracellular enzymes of the fungus. As the fruits matured the amounts of these materials decreased and the fruits became susceptible (Edney, 1958). When Edney (1964) compared unripe (resistant) fruit with ripe (susceptible) fruit, he found that the unripe fruit yielded extracts which inactivated the polygalacturonase activity of the fungus, whereas no inactivation occurred using extracts from ripe fruit.

A remarkable change from resistance to susceptibility has been demonstrated in apples infected with Botryosphaeria ribis (Lewis and Shay, 1953; Kuć, Williams, Maconkin, Ginzel, Ross and Freedman, 1967). Sitterly and Shay (1960) emphasised the nutritional requirements of B. ribis, showing that the change to susceptibility occurred at or just before the climacteric; and factors which hastened the climacteric, e.g. treatment with maleic hydrazide or ethylene, also hastened the change to susceptibility. Susceptibility was also increased by the

infusion of sucrose or fructose into the fruits prior to inoculation. More recently it has been demonstrated that resistant apples can supply the nutrients required for growth of B. ribis, and although no direct fungitoxic effects have been demonstrated, resistant apples were found to yield extracts which inhibited pectinase activity. As the apples matured this inhibition decreased, and extracts from mature susceptible fruits failed to reduce pectinase activity (Kuć et al., 1967).

Fungitoxic substances have been detected in healthy fruits by Cole (1958) and by Hulme and Edney (1960). As discussed previously, inhibitors have also been shown to be present in fruits infected with Sclerotinia fructigena (Fawcett and Spencer, 1967). Evidence indicating that a direct fungitoxic effect is responsible for the resistance of apples to infection comes from Swinburne (1964) who showed that fruits were resistant to rotting if inoculated with Cylindrocarpon mali immediately after harvesting. Stored fruits were completely susceptible. In a resistant fruit only a small restricted area at the site of infection was affected. After seven weeks of incubation growth of the fungus recommenced and a rot developed. In a quarter of the fruits examined, however, no further rotting occurred and he found that he was unable to reisolate the fungus. He concluded that during the development of the infection in unripe fruits a toxic chemical was elaborated which, by inhibiting fungal growth, restricted the immediate development of the rot. In the minority of fruits the fungus was killed and no further growth occurred, but in the remainder, this toxicity was not complete and eventually the fungus was able to recommence growth and produce the rot.

There is little evidence in favour of an active resistance mechanism operating in apples, but the increased susceptibility which the fruits show as they mature and senesce could be explained in such terms, although other explanations are possible.

The phytoalexin theory of disease resistance postulates that plant tissues react to fungal infection by the formation of antifungal compounds at levels inhibitory to all but the more aggressive pathogens of the host concerned. Most work heretofore has been concerned with such substances diffusing into infection drops. However, it could be expected that infected tissues themselves, e.g. soft rots, might develop a phytoalexin content which, though not severely inhibitory to the primary pathogen, might prevent or delay the entry of secondary invaders. Partly to look into this possibility and partly because a massive fleshy organ such as an apple fruit would provide ideal experimental material for work involving extraction of large quantities of tissue, initial work was directed to a search for phytoalexins in rotted apple fruits.

Experiment 1. The effect of juice from apple fruit rots on the germination of Botrytis allii

The juice of apple fruit rots was tested for the presence of inhibitors by measuring its effect on the germination of spores of a non-pathogen, Botrytis allii. Dense spore suspensions of B. allii, B. cinerea and Penicillium expansum were prepared from ten day old cultures incubated at 24°C in the dark. Fruits of Cox's Orange Pippin were inoculated under sterile conditions by removing a plug of tissue, applying

0.1 ml. of spore suspension into the gap left, replacing the plug and sealing with Vaseline. The fruits were incubated for four days, during which time substantial rotting had been produced in the apples inoculated with B. cinerea and P. expansum. B. allii failed to rot any tissue (Apples inoculated with B. allii had previously been shown to be resistant to rotting after five weeks incubation). The rotted tissue was removed and squeezed through muslin. Only the region in close association with the site of infection was removed from apples inoculated with B. allii. Healthy flesh was extracted in a similar way. All the extracts were cleared by centrifuging at 4,000 r.p.m. for thirty minutes. The germination of spores of B. allii in these extracts after twenty four hours was tested.

Table 3.1 The percentage germination of B. allii in various apple fruit extracts

Extract Concentration (Dilutions in distilled water)	Healthy tissue	Tissue inoculated with B. allii	B. cinerea rot	P. expansum rot
10^{0}	81	100	100	100
10^{-1}	100	100	100	100
10^{-2}	100	100	100	100
10^{-3}	100	100	100	100
10^{-4}	100	92	100	100
Distilled Water	100	91	100	100

No effect on the germination of spores of B. allii could be demonstrated with these extracts.

The application of the "drop-diffusate technique" to the study of the production of inhibitors by apple tissues

Attempts were made to adapt the "drop-diffusate technique" for use with apple tissues. Using segments of apple fruit tissue it was found impossible to re-collect the diffusate, although it was possible to collect diffusate from apple peel; however, the major problem encountered here was the probable failure of the germinating spores or their metabolites to penetrate through the peel into the internal tissues of the fruit. Methods used to overcome this barrier, e.g. pricking the peel with a needle, made the recovery of the diffusate impossible.

The results of this work failed to produce any information regarding the production of inhibitors by apple fruits.

The problems associated with the extraction of inhibitors and the penetration of fungi into tissues, stimulated the establishment of callus tissue cultures from apple fruit tissue. Callus provides a uniform mass of sterile, actively dividing cells to which fungal spores can be applied without damaging any of the cells. In this way large volumes of fruit tissue could be exposed to a non-pathogen and the production of post-infectional toxins might be more readily detected.

The resistance of apple callus cultures to infection by *Cylindrocarpon heteronema*

During the course of establishing callus cultures from apple fruits on a solid medium, it was observed that a contaminating fungus made extensive growth on the medium surrounding the callus but no growth was

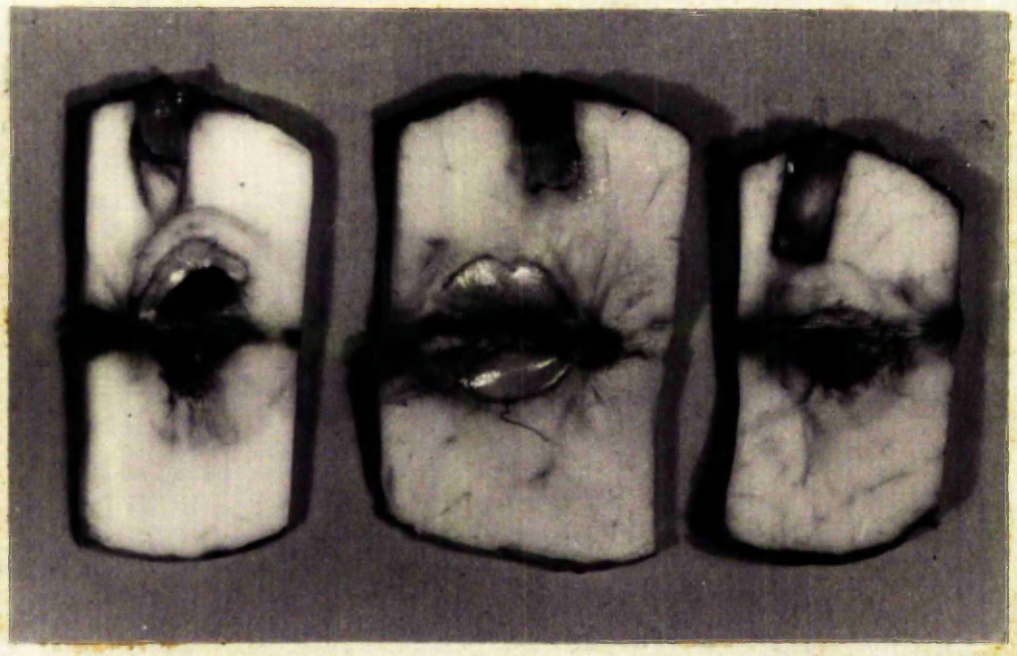


Fig. 3.1 Apple fruits inoculated with C. heteronema.

Left : Dunn's Seedling

Centre : Golden Delicious

Right : Cox's Orange Pippin

seenadjacent to the callus and no growth was visible on the callus itself. This phenomenon was investigated further.

The fungus was sent to the Commonwealth Mycological Institute for identification. It was identified as the microconidial stage of Nectria galligena Bres. Cylindrocarpon heteronema, Berk. and Br. (Wollenw). C. heteronema has been reported to be pathogenic to various apple fruits (See Swinburne, 1964). The pathogenicity of the isolate used in this work was investigated.

Experiment 2. To investigate the pathogenicity of Cylindrocarpon heteronema

(a) Apple fruits

Fruits of cultivar Golden Delicious were obtained from a commercial source on the 10th of October, 1967 and stored in the dark either at 3°C or at 20°C. Five apples were inoculated with a dense spore suspension of C. heteronema on the 12th of October, 1967 and at regular intervals until the 21st of February, 1968. The inoculated fruits were incubated for at least three months at 26°C in the dark. On no occasion was it possible to demonstrate the production of a rot as a direct consequence of the infection by C. heteronema.

Similarly, inoculation into the fruits of other cultivars (Dunn's Seedling, Cox's Orange Pippin and another sample of Golden Delicious) failed to produce a single rot which could be shown to be caused by C. heteronema (Fig. 3.1).

Segments of apple fruit tissue were also shown to resist invasion by C. heteronema. Discs of tissue 1/8" thick and 3/8" in diameter

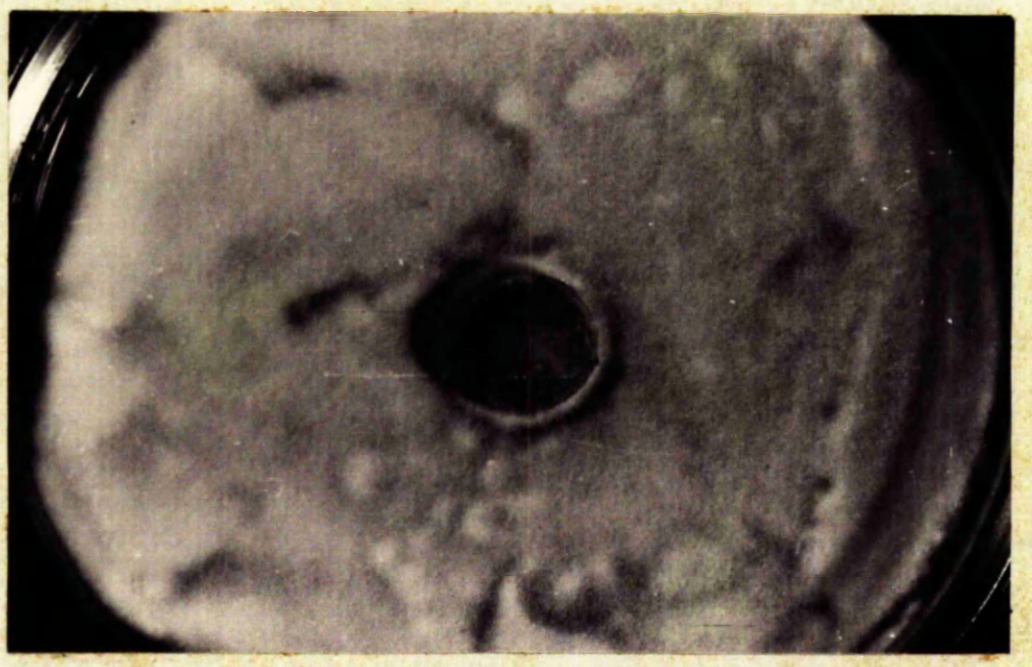


Fig. 3.2 Segment of Golden Delicious apple fruit inoculated with C. heteronema and incubated for seven days.

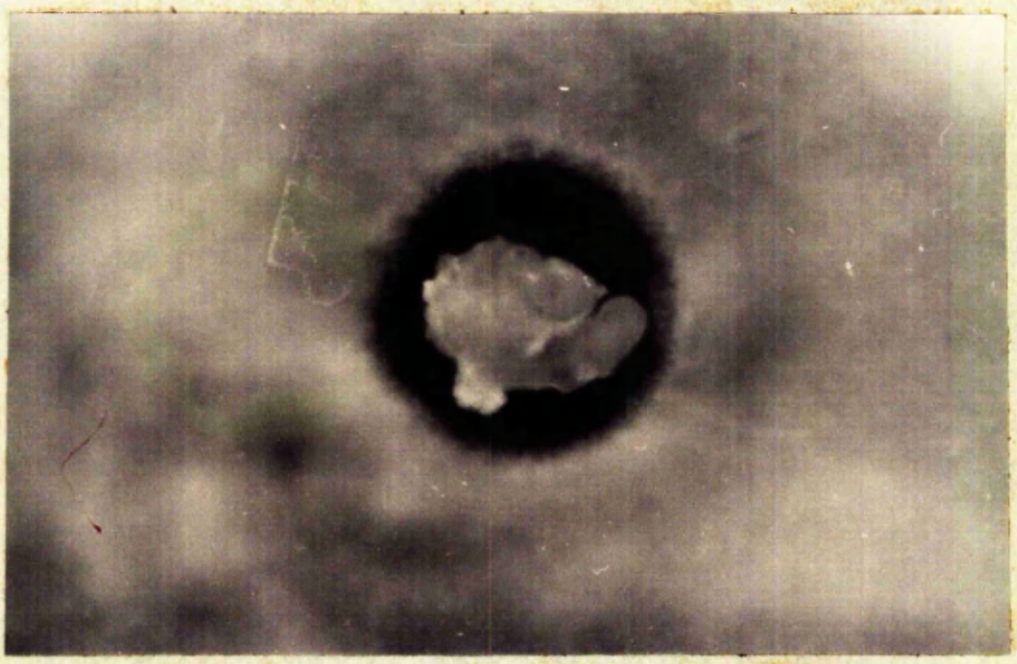


Fig. 3.3. Golden Delicious callus inoculated with *C. heteronema*.

Viewed through the base of the petri-dish.

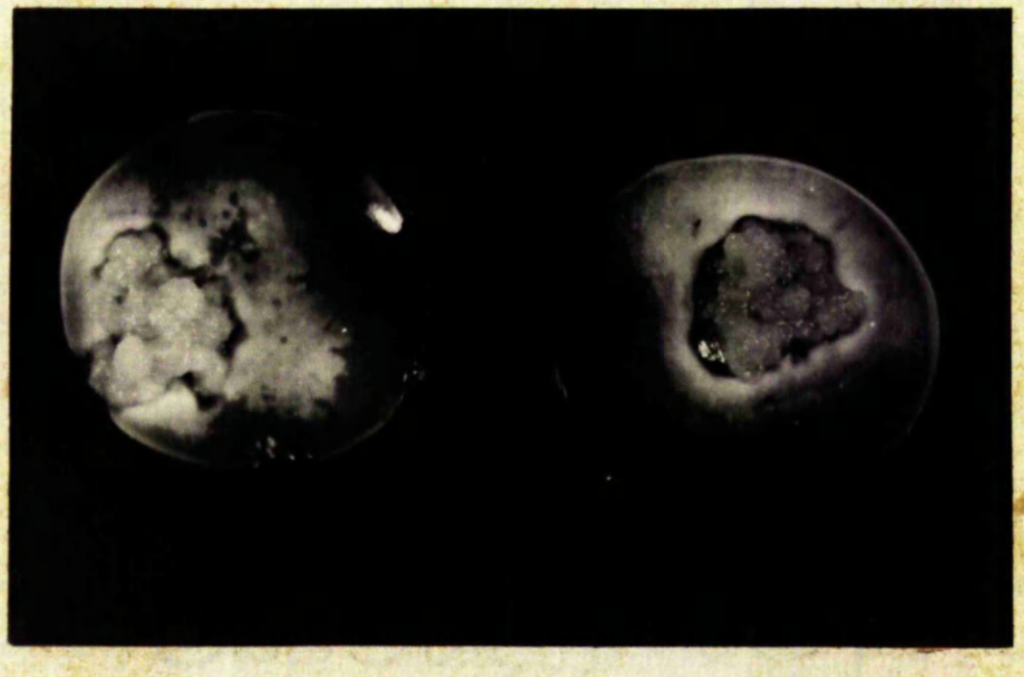


Fig. 3.4. Apple callus inoculated with C. heteronema.

Left : Granny Smith

Right : Golden Delicious

were removed from apple fruits (Golden Delicious) under strictly sterile conditions and transferred to petri-dishes containing medium V. The discs and the surrounding medium were inoculated with a dense spore suspension of C. heteronema and incubated for seven days at 26°C. Twenty discs were set up; twelve remained firm and showed no signs of rotting; the remaining eight were rotted within two days. However, after being incubated a further two days, spores of a species of Penicillium were produced on the surface of the tissue (Fig. 3.2). It was concluded that C. heteronema is unable to invade isolated apple tissue.

(b) Callus cultures derived from the mesocarp of apple fruits

Small explants of callus were transferred on to medium V contained in petri-dishes. Four explants were placed on each plate and were incubated for twenty-one days. 0.2 ml. of a dense spore suspension of C. heteronema was applied over each callus and also spread across the medium. The plates were then incubated for seven days. Extensive growth of the fungus occurred on the medium away from the callus tissues, but no growth occurred on the callus or on the medium directly adjacent to the callus (Fig. 3.3). Callus of both Golden Delicious and Granny Smith behaved in a similar fashion (Fig. 3.4).

Calluses of Golden Delicious which were four months old at the time of inoculation were still highly resistant to infection. However, Granny Smith callus showed some degree of susceptibility after this time. As indicated in Chapter 2, Granny Smith callus does not grow rapidly on a medium containing 6 mg./l. 2,4-D. This may be responsible for the decrease in resistance as the callus ages. No experiments were done using

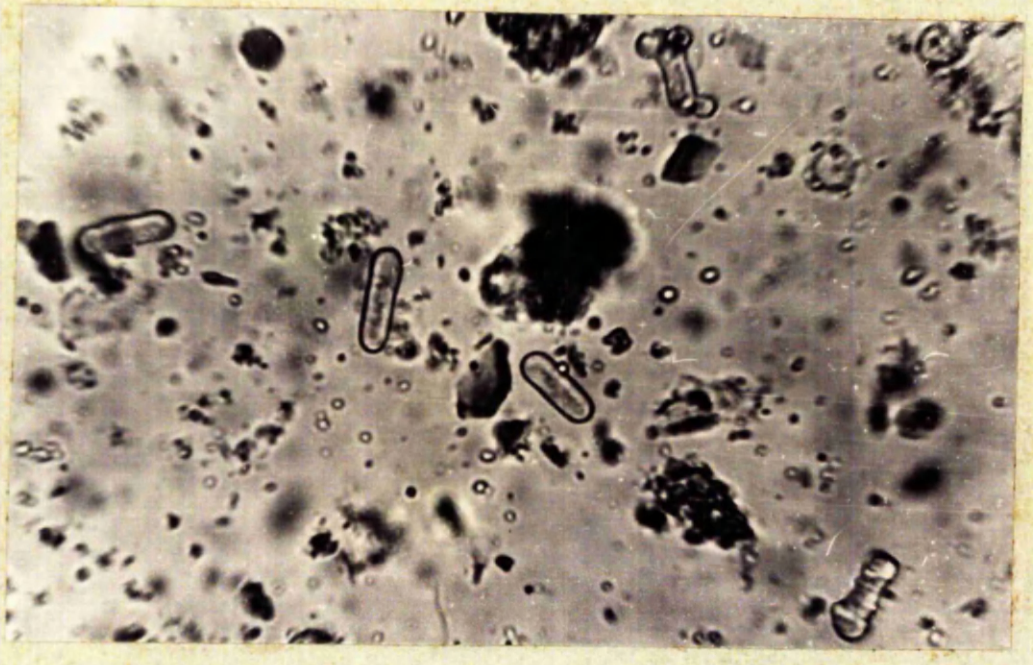


Fig. 3.5. Inhibition of germination of spores of C. heteronema inoculated on to Golden Delicious callus.

Granny Smith callus on a medium in which the concentration of 2,4-D had been adjusted to allow rapid growth.

Experiment 3. The morphology of C. heteronema during the infection of Golden Delicious callus

Explants of Golden Delicious callus were transferred on to Medium V and incubated for twenty-one days. The callus was inoculated with a dense spore suspension of C. heteronema and incubated for a further seven days. After this time C. heteronema was seen to be growing on the agar surface. Small pieces of callus were removed, macerated, stained with Trypan Blue and investigated microscopically.

Generally all the spores failed to germinate. Hyphae, which were present in the spore suspension, also failed to grow and tended to become highly septate with thick walls. The ungerminated spores produced small swellings on their surfaces and, although no direct measurements were made, they also seemed to be reduced in size (Fig. 3.5).

Experiment 4. To demonstrate the diffusion of the inhibitory principle from callus of Golden Delicious and Granny Smith

As a result of the initial observation that zones where inhibition of growth of C. heteronema occurred around the growing callus, petridishes of Medium V were simultaneously inoculated with mycelium of C. heteronema at the centre of the plate and with four explants of callus at the extremities of the plate. They were incubated for twelve days. Control plates were inoculated with only C. heteronema.

On the test plates, mycelium of C. heteronema grew steadily from the

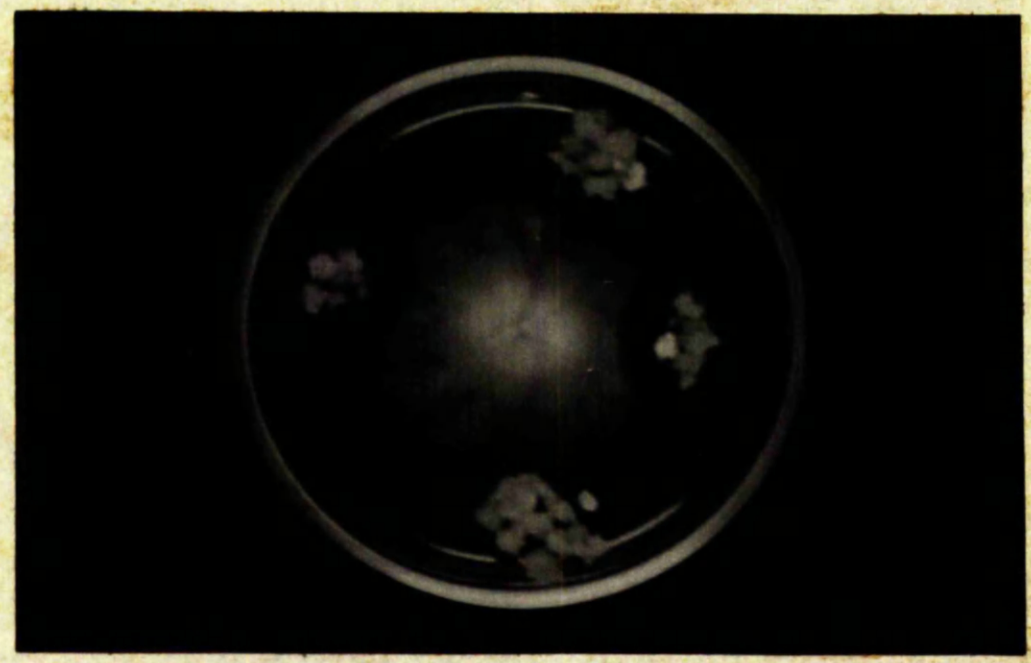


Fig. 3.6. The inhibition of growth of C. heteronema adjacent to Golden Delicious callus.

central inoculum producing abundant conidia until it extended to a region adjacent to the callus where growth and sporulation were prevented (Fig. 3.6). On the control plates the mycelium of C. heteronema had extended to the edges of the plate.

Experiment 5. The effect of heat on the resistance of Golden Delicious callus to infection by C. heteronema

Callus of Golden Delicious was grown on medium V for twenty-one days in 6" x 1" Pyrex boiling tubes. Thirty cultures were selected with regard to uniformity; half of these were individually transferred to petri-dishes containing either medium II or medium V. The remainder were individually transferred into sterile boiling tubes, immersed in boiling water for ten minutes and then transferred to either medium II or medium V. The thirty transferred cultures were immediately inoculated with a dense spore suspension of C. heteronema and incubated for a further ten days. The situation shown at this time is described in Table 3.2 and Fig. 3.7.

Experiment 6. To examine the production of an inhibitor of spore germination by Golden Delicious Callus

The marked resistance of Golden Delicious callus and the demonstrated in vivo inhibition of spore germination led to an examination of the presence of such an inhibitor in the juice obtained from callus, this being the necessary preliminary to a possible identification of the inhibitory principle. During preliminary experiments, the presence

Table 3.2. The effect of heat on the resistance of callus to infection by C. heteronema

Treatment	Reaction
Unheated callus transferred to medium II.	Resistant: No growth on the callus. A zone of inhibition surrounded the callus.
Unheated callus transferred to medium V.	Resistant: No growth on the callus. A zone of inhibition surrounded the callus.
Heated callus transferred to medium II.	Susceptible: C. heteronema sporulated over the surface of the callus.
Heated callus transferred to medium V.	Susceptible: Growth of C. heteronema on the callus was slower, but sporulation occurred after fourteen days.

Fig. 3.7. The effect of heat on the resistance of Golden Delicious callus to *C. heteronema*.

A. Uninoculated callus.

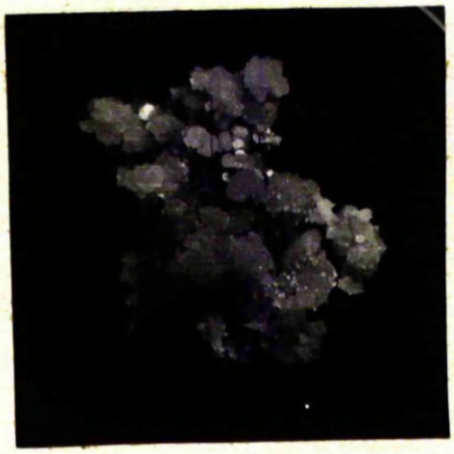
B. Callus transferred to medium V and inoculated with *C. heteronema*.

C. Callus transferred to medium II and inoculated with *C. heteronema*.

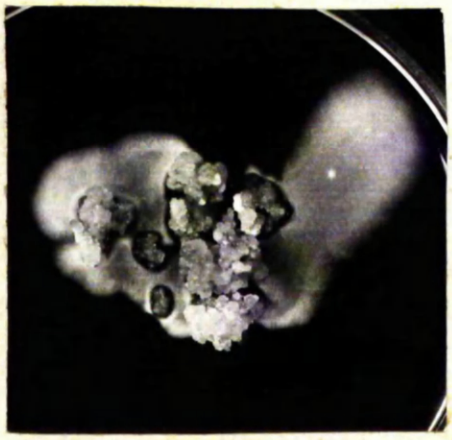
D. Callus heated and then transferred to medium V and inoculated with *C. heteronema*.

E. Callus heated and then transferred to medium II and inoculated with *C. heteronema*.

A



B



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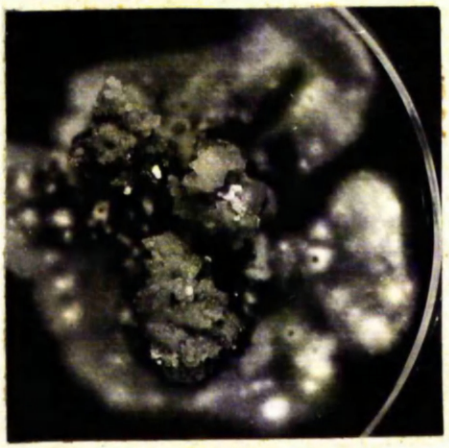


E

of an inhibitor of C. heteronema spore germination was detected in juice from both healthy and infected callus. However, the inhibitory effect varied and a further experiment was designed to investigate this.

Golden Delicious callus was grown on medium V in 6" x 1" Pyrex boiling tubes for twenty-eight days. 1.0 ml. of a dense spore suspension of C. heteronema was applied to each of half of the calluses. The other half received 1.0 ml. of distilled water and acted as uninfected controls. The cultures were incubated until those flasks which contained C. heteronema showed evidence of its growth at the edge of the medium (six days). The following callus tissue extracts were prepared.

1. The calluses were squeezed through muslin.
2. The calluses were transferred to boiling tubes which were immersed in a water bath at 90°C for twenty minutes. The heated callus was removed and squeezed through muslin.
3. The calluses were squeezed through muslin and the juice obtained was allowed to stand for twenty minutes. It was then immersed in a water bath at 90°C for twenty minutes.

Clear extracts were obtained by centrifuging the juices at 50,000 g for fifteen minutes. These extracts were tested for an ability to inhibit the germination of spores of C. heteronema immediately after being centrifuged and also after being stored overnight at 3°C.

The initial percentage germination was measured after twenty-four hours and a further investigation was made after four days. Extracts were tested as extracted and also at the following dilutions in either distilled water or medium I : 0.5, 0.25 and 0.1.

Table 3.3. The inhibition of germination of spores of C. heteronema by extracts obtained from Golden Delicious callus

Extract	Callus inoculated with distilled water		Callus inoculated with a spore suspension of C. heteronema	
Dilution	% germination after 24 hrs.	Growth after 4 days	% germination after 24 hrs.	Growth after 4 days
1. 1.0	< 1	No germination	< 1	No germination
0.5 in medium I	< 1	No germination	< 1	No germination
0.25 " " I	< 1	No germination	< 1	No germination
0.1 " " I	< 1	No germination	< 1	No germination
Medium I	> 90	Mycelium	> 90	Mycelium
1.0	< 1	No germination	< 1	No germination
0.5 in D.W.	< 1	No germination	< 1	No germination
0.25 " "	< 1	No germination	< 1	No germination
0.1 " "	< 1	No germination	< 1	No germination
Distilled Water	0	No germination	0	No germination
2.	> 90	Mycelium	> 90	Mycelium
3.	> 90	Mycelium	> 90	Mycelium

When the above tests were repeated using the extracts which had been stored at 3°C overnight, no inhibitory effects could be demonstrated. This instability of inhibitory extracts had been observed in previous preliminary experiments.

Extracts obtained from Granny Smith callus similarly inhibited the germination of spores of C. heteronema and this inhibitory effect was destroyed after the extract had been heated at 90°C for twenty minutes.

The pH of the callus or the extracted juice is not responsible for the inhibition. Agar plates, on which callus of Golden Delicious and Granny Smith had been grown for twenty-one days, did not show any colour variations when flooded with a Universal pH indicator. Expressed juice had a pH between 6.0 and 6.5: spores of C. heteronema were shown to germinate in medium I through a range of pH between 3.5 and 8.5.

Discussion

Following the failure to demonstrate the presence of inhibitors in apple fruits, the fortuitous observation was made that apple callus appeared to inhibit C. heteronema. C. heteronema although regarded as a pathogen of apple fruits, would not invade any of the apple fruits tested in these experiments. The investigation was then directed to determine whether this lack of pathogenicity was related to the observed inhibition by the apple callus.

It was established that diffusible substances produced by the callus prevented the germination and growth of C. heteronema. In Experiment 4 it was demonstrated that if an agar plate was simultaneously inoculated with callus and with C. heteronema, and then incubated for two weeks, the growth of the fungus was arrested approximately 5 mm. away from the callus. Whereas, if callus was allowed to grow on agar for three weeks and then inoculated, although the callus was resistant, fungal growth occurred on the agar very close to the callus. This

might imply in the first case that the fungus secreted diffusible metabolites which affected the callus, and, as a result, the callus produced inhibitors which diffused out and inhibited the growth of the fungus. In the absence of this interaction, diffusion of inhibitors did not occur and although no growth was possible on the callus itself, growth did occur closely adjacent to the callus.

The demonstration that juice from healthy and inoculated apple callus was inhibitory to C. heteronema would rule out post-infectionally produced inhibitors as the sole explanation of the results obtained. The amounts of inhibitor present in healthy and inoculated callus was not determined. However, the zones of inhibition found in Experiment 4 could be explained by an increased production of inhibitors.

It seems likely that the juice from the callus has a fungicidal effect since no growth of spores on test slides occurred in the presence of the inhibitory juice after seven days, although the inhibitory effect of these juices was lost within twenty-four hours. Swinburne (1964) has reported a similar fungicidal effect in mature apple tissues infected with C. heteronema.

In similar experiments with mature apple fruits, the resistance of these fruits to invasion by C. heteronema was also most marked. However, it was not possible to demonstrate any inhibitory effect of juice expressed from apple fruits on any of the fungi tested. This was shown in the early experiments. Later, juice from apple fruits was shown to have no effect on the germination of C. heteronema. Similarly, no evidence was obtained of diffusion from apple tissue segments of a substance inhibitory to C. heteronema.

Therefore it must be stated that any extrapolation of the results obtained with callus tissues to the situation occurring in natural apple fruits is not fully justified. Nevertheless, it may be of value to indicate how the results obtained with callus tissues could aid the elucidation of resistance mechanisms in apple fruits. It has been possible to demonstrate very readily the production of inhibitors using the tissue culture system; no such inhibitors have been reported to have been detected in healthy apple fruits. The inhibitory principle in juice from apple callus is highly unstable and similar inhibitors may be present in apple fruit tissues either before or after infection, but due to their instability were not detected in juice from such fruits. It will be shown later that tissue cultures of Pisum sativum produce the phytoalexin, pisatin, when grown under sterile conditions in artificial media. Thus the possibility cannot be ruled out that the inhibitory effect found with apple callus is due to the presence of the natural phytoalexin(s) of apple fruit. If the inhibitors found in apple callus could be isolated and identified, it may then be possible to utilize this information in an attempt to detect the presence of similar compounds in apple fruits. This would be particularly useful if the inhibitors were unstable, present in low concentrations or produced only very locally at the site of infection.

CHAPTER 4. THE PRODUCTION OF THE PHYTOALEXIN, PISATIN, BY PISUM SATIVUM

Introduction

The production of a phytoalexin by Pisum sativum was first reported by Müller (1958). He detected phytoalexin production by the inner epidermis of the pod after inoculation with a spore suspension of Sclerotinia fructicola. The chemical structure of this phytoalexin, pisatin, has been determined as 3-hydroxy-7-methoxy-4'5' methylenedioxy-chromanocoumarone (Perrin and Bottomley, 1962). (see Fig.1.8).

Cruickshank and Perrin have dealt with various aspects of the biology of pisatin, e.g. the induced formation of pisatin (Cruickshank and Perrin, 1961; Cruickshank and Perrin, 1963); the antimicrobial spectrum of pisatin (Cruickshank, 1962); and the relationship of pisatin to disease resistance (Cruickshank and Perrin, 1963).

In these experiments pisatin was only detected in diffusates when fungal spores were present. Only very small traces of pisatin were detected when drops of sterile distilled water were recovered from the pods and pisatin could not be detected in healthy pod tissue. Fungi which had been shown to induce pisatin formation did not produce pisatin when grown in artificial media. The host origin of the phytoalexin has since been confirmed by work which showed that various chemicals induce the formation of pisatin in the absence of fungal spores. Sterile

culture filtrates, various metabolic inhibitors and amino acids were shown to induce pisatin formation (Perrin and Cruickshank, 1965). Work by Van den Ende (1964), with the related phytoalexin phaseollin produced by Phaseolus vulgaris, would indicate that the fungus acts as a trigger to the biosynthesis of phaseollin and is not directly involved in its biosynthesis. Radioactive phaseollin was only obtained if the bean plant had been previously labelled; if the fungus had been grown on a labelled medium and the radioactive spores applied to the plant no activity could be found incorporated into the phaseollin. It was concluded that the inducing agent, if produced directly by the fungus, was not incorporated into phaseollin.

Hadwiger (1966) has obtained evidence regarding the induction and biosynthesis of pisatin. L-phenylalanine-C^{14} and cinnamic acid-COOH-C^{14} were incorporated into pisatin when supplied to the pod in the presence of a synthetic inducing agent; suggesting that pisatin was formed as a result of an alteration in the normal shikimic acid pathway of the pea. Using radioactively labelled phenylalanine-C^{14}, of high specific activity, he has obtained evidence which he considers indicates that pisatin is a normal metabolic product of healthy peas and that infection causes an increase in the activity of the biosynthetic pathway leading to pisatin formation (Hadwiger, 1967).

The related non-toxic compound anhydropisatin has been shown to be present in healthy pea tissues (MacMillan, private communication).

The nature of the inducing agents produced by infecting fungi are unknown. Spore free supernatants were shown to induce phytoalexin

formation (Uehara, 1959; Cruickshank and Perrin, 1963; Klarman and Gerdemann, 1963). Uehara reported that the ability of the supernatant to induce phytoalexin formation was destroyed after it had been heated at 60°C for 10 minutes and suggested that fungal spores secrete extracellular enzymes which act on the host to produce phytoalexins. Cruickshank and Perrin have been reported to have isolated a heat stable chemical of low molecular weight from the culture filtrate of Sclerotinia fructicola, which induces pisatin formation when applied to uninfected pea pods (Falk, 1967).

Physiology of phytoalexin production

The susceptibility of a plant to infection can be affected by several factors. Various treatments, e.g. heat, abrasion, pressure and inoculation with other fungi, applied to the plant before inoculation, have been shown to predispose plants to infection. Leaves of Phaseolus vulgaris which were immersed in hot water (40-55°C) became more susceptible to infection by Uromyces phaseoli, Erysiphe polygoni and Colletotrichum lindemuthianum (Yarwood, 1956). Phaseolus vulgaris, which is normally highly resistant to Sphaerotheca fuligena and Erysiphe cichoracearum, also became susceptible to these pathogens when treated in this way (Yarwood, 1963; Yarwood, 1965). Similar treatments have been demonstrated to reduce phytoalexin production and, in a few cases, this has been correlated with increased susceptibility.

Jerome and Müller (1958) studied the influence of temperature on the resistance of Phaseolus vulgaris to Sclerotinia fructicola and the

relationship of this resistance to phytoalexin production. Plants which were exposed to 44°C for two hours and immediately inoculated with Sclerotinia fructicola showed a complete loss of resistance, associated with a production of phytoalexin which was only 6-15% of the untreated controls. If, after this heat treatment, the pods were allowed to recover for three days at 20°C they were found to be resistant to infection and phytoalexin production was higher than in the untreated controls. Analogous results were obtained using Glycine max: plants which were treated at 44°C for one hour became susceptible to the non-pathogens Phytophthora cactorum and Helminthosporium sativum. This loss of resistance was correlated with a decrease in the production of phytoalexin (Chamberlain and Gerdemann, 1966).

The maturity of infected plant tissue is reported to affect the concentration of phytoalexin produced. Mature pods of Phaseolus vulgaris were shown to produce more than twice as much phytoalexin as younger ones (Müller, 1958); however, other workers have failed to repeat this finding (Cruickshank, 1963). Pea pods have been shown to produce maximal concentrationsof pisatin when very young. As they matured the concentration of pisatin produced decreased. The most mature pods tested produced very low concentrations of pisatin and were often found to have become susceptible to invasion by Sclerotinia fructicola (Cruickshank and Perrin, 1963). A similar relationship has been demonstrated to exist for the phytoalexin produced by Glycine max (Uehara, 1958).

Storage of pea pods has also been shown to affect pisatin production. Pods stored at 4°C showed an initial increased production of pisatin to a

level which was maintained for over four weeks. A similar initial increase occurred at 20°C but this was followed by a rapid decrease until no pisatin was produced by pods which had been stored for only six days (Cruickshank and Perrin, 1963). The results of these workers indicate that high phytoalexin production is associated with young actively metabolising tissues. As these tissues mature and senesce the production of phytoalexin decreases and they become less resistant to infection.

The production of pisatin is not restricted to pod tissues. Pisatin has been detected in naturally diseased tissues and also in artificially infected stems and leaves (Cruickshank and Perrin, 1961). The leaf of a plant has a finite life. Throughout its life the activities of specific metabolic processes in the leaf change. Such changes might affect phytoalexin production, while factors which influence these processes might also influence phytoalexin production. The rate of photosynthesis (Richardson, 1957) and the activity of certain enzymes (McKee, 1962; Wallace and Pate, 1965) showed a maximum prior to maturity and decreased as the leaf matured and finally senesced. The activities of several other enzymes, e.g. peroxidase (Racusen and Foot, 1966) and cellulase (Horton and Osborne, 1967), increased at maturity and at senescence respectively. Carr and Pate (1967) have reported work on the metabolic changes occurring in pea leaves during ageing. The nitrogenous reserves of the leaf built up during the first two weeks, remained constant for a longer period, and rapidly disappeared later in life. Similar changes in the amounts of soluble nitrogen (amino acids and amides) were also demonstrated.

The life span of a leaf is determined by the conditions to which it is subjected. This span can be increased or decreased in a number of ways. If a plant is reduced to a single leaf, this leaf will survive for a much greater time than a similar leaf attached to an intact plant. Physiological factors which can affect senescence include the production of storage organs, flower and fruit formation, alterations in the light regime or nutrient status, and drought. Several of these factors are also associated with hormonal changes occurring in the plant (Osborne 1967).

Physiology of senescence

Senescence has been studied using whole leaves attached to the plant, whole leaves detached from the plant and leaf discs. The major changes which occur in leaves during senescence have been shown to be falls in the levels of nucleic acids, (primarily ribonucleic acid), proteins and chlorophyll (Osborne, 1962).

Plant hormones, e.g. auxins, cytokinins and gibberellin, and plant growth retardants have been shown to delay the senescence of a variety of plants. The senescence of detached leaves of Xanthium pennsylvanicum was prevented when the petioles were stood in a solution of kinetin (Richmond and Lang, 1957). When petioles of detached tobacco leaves were placed in water the leaves lost soluble nitrogenous material and senesced very rapidly. Leaves which were sprayed with kinetin showed areas which remained green and did not senesce. These areas were restricted to the regions which had received the kinetin spray. Mothes, Engelbrecht and Schutte (1961) showed that the kinetin caused a migration of soluble

nitrogenous materials to the sprayed region where they accumulated. In the light this was associated with an increased synthesis of protein. An amino acid which is not incorporated into plant protein was also accumulated in the kinetin sprayed regions. It was considered that the primary response of the leaf to kinetin, when delaying its senescence, was the accumulation of the soluble nitrogenous materials (Mothes and Engelbrecht, 1959).

Other researchers have emphasised the importance of enhanced protein synthesis occurring when hormones delay senescence. Kinetin delayed the senescence of leaf discs of many plants (Osborne and McCalla 1961; Dennis, Stubbs and Coultate 1967; Beevers and Guernsey 1967; Kessler, Spiegel and Zolotov 1967). The rate of protein synthesis of leaf discs from Xanthium pennsylvanicum decreased when the discs senesced floating on water, with a resulting fall in the levels of nucleic acid, protein and chlorophyll. This senescence was delayed in discs floated on a solution of kinetin; protein synthesis was enhanced and the breakdown of nucleic acid, protein and chlorophyll was prevented (Osborne, 1962). The synthetic kinin ^{6}N-Benzyladenine has been shown to delay the senescence of many green vegetables, especially the Brassicas (Zink 1962; Halevy, Dilley and Wittwer 1966; Dennis, Stubbs and Coultate 1967; Tsugita and Andrews 1967). Benzyladenine also stimulated protein synthesis in leaves when growth was enhanced, (Pozsár, Hammady and Kiraly 1967).

The senescence of autumn leaves of Prunus serrulata was delayed by applications of the synthetic auxin, 2,4-dichlorophenoxyacetic acid (Osborne and Hallaway, 1960). Treated leaves showed a slower fall off of protein level and a stimulation of the incorporation of leucine-C^{14} into the leaf

(Osborne and Hallaway, 1964). Other auxins have been shown to delay the senescence of other plants (Sacher, 1965). Senescence of leaf discs has been similarly delayed by floating them on solutions of gibberellic acid (Fletcher and Osborne 1966; Whyte and Luckwill, 1966).

Many other hormonal responses have been reported to involve the stimulation of protein synthesis. A major approach to the problem has been to study the effect of chemicals, which inhibit protein synthesis, on the response caused by the hormone. The chemicals used have included actinomycin D, chloramphenicol, p-fluorophenylalanine, puromycin and actidone. These inhibited ethylene production induced by auxins (Abeles, 1966); green pea segment elongation induced by indoleacetic acid (Penny and Galston, 1966); the expansion of artichoke tissue slices induced by auxin (Masuda, 1966); cell enlargement in Avena coleoptile segments induced by auxin (Nooden and Thiman, 1966); and the formation of α-amylase and protease in barley seeds induced by gibberellin (Varner and Chandra Ram, 1964).

Sacher (1963) has presented evidence that the cause of senescence is a decrease in the level of ribonucleic acid (RNA) and that hormones delay senescence by maintaining or enhancing the synthesis of RNA. He showed that the stimulation of RNA synthesis by bean pods in the presence of auxin, as indicated by the incorporation of orotic acid-C^{14}, was inhibited by 79% by actinomycin D, which inhibits deoxyribonucleic acid (DNA) dependent-RNA synthesis (Sacher, 1965).

Growth hormones have also been shown to enhance senescence of certain leaves (Halevy and Wittwer, 1965). Hormones which are effective in

delaying senescence of one species can be found to be ineffective against another (Whyte and Luckwill 1966; Mishra and Misra 1968). The response of the leaves to treatment with hormones varies greatly, depending on their physiological state. The stimulation of RNA synthesis by bean pods treated with auxin varied from 24% - 263% compared with the untreated control (Sacher, 1965). Osborne and Fletcher (1966) took great care to use leaves in which they could expect similar responses; subjecting them to low light intensities to enable them to obtain leaves in a similar state and hence produce a uniform response to hormone treatment.

Plant growth retardants are also known to delay the senescence of a variety of plants (Harada 1966; Kessler, Spiegel and Zolotov 1967). The rate of senescence of excised bean leaves was markedly delayed if they were maintained with their petioles dipped in 2-chloroethyltrimethylammonium chloride (CCC) or N.N,dimethylaminosuccinamic acid (B.995) (Halevy and Wittwer 1965). These growth retardants were also effective on bean leaf discs (Kessler, Spiegel and Zolotov, 1967). It has been suggested as a result of other work that these retardants act by antagonising the synthesis of natural gibberellins (Kende, Ninnemann and Lang 1963; Baldev and Lang 1965; Baldev, Lang and Agetop 1965). B.995 was shown to accelerate the senescence of leaf discs of Taraxacum officinale; this was partially overcome by a simultaneous application of gibberellin. However, kinetin was also effective in overcoming the action of B.995; and the other growth retardant tested, CCC, acted in a similar way to gibberellin and kinetin, i.e. to delay senescence of these leaf discs (Beevers and Guernsey 1967). A third growth retardant 4-hydroxy-5-isopropyl-2-methylphenyltrimethyl ammonium chloride, 1 piperidine carboxylate (AMO 1618) enhanced the

senescence of leaves of Glycine max and Stevia rebaudiana when applied to the roots. The effect on Glycine max was reversed by spraying the plants with either gibberellin or kinetin (Ruddat and Pharis, 1966).

Although emphasis is usually placed on the effects of hormones on protein synthesis (Van Overbeek, 1966), other metabolic processes are often affected. The kinins, in particular, are known to alter the respiratory rates of senescing leaves (Dedolph, Wittwer and Tuli 1961; Wittwer, Dedolph, Tuli and Gilbart 1962). Halevy, Dilley and Wittwer (1966) have shown that a decrease in the respiratory rate was associated with a delay in the rate of senescence and that when senescence was enhanced the respiratory rate was also increased.

Table I lists various chemicals which have been demonstrated to delay senescence in various plants. It will be seen that the kinins are generally more effective.

The general view obtained from these results is that senescence is closely controlled by hormonal regulation and a maintainance of nucleic acid and protein synthesis. Endogenous hormones maintain the required rates of synthesis in a healthy leaf. An alteration of the normal regulatory processes may occur due to an antagonism of the endogenous hormones leading to an enhancement of senescence; or if these hormones become less active, additional application of a suitable hormone may supplement their effect and prevent senescence. The specific action of an exogenous hormone will depend on the constitution of the normal regulatory processes.

Table 1. Plant growth hormones and growth retardants delaying the senescence of various species

Species tested	Chemicals showing activity	Reference
Cytokinins		
Xanthium pennsylvanicum	K	Richmond & Lang, 1957.
Triticum aestivum	Bz; K	Person, Samborski & Forsyth, 1957.
Various species	BA	Zink, 1961.
Xanthium pennsylvanicum	K	Osborne & McCalla, 1961.
Brassica oleracea var. Italica	BA	Dedolph, Wittwer, Tuli & Gilbart, 1962.
Nicotiana tabacum*	K	Suguira, Umemura & Oota, 1962.
Phaseolus vulgaris	BA	Halevy & Wittwer, 1965.
Triticum aestivum	K	Shaw, Bhattacharya & Quick, 1965.
Triticum aestivum	Bz	Mishra D, 1966.
Phaseolus vulgaris*	K	Kessler, Spiegel & Zolotov, 1967.
Tropaeolum majus*	K	Beevers & Guernsey, 1967.
Brassica oleracea L. gemmifera*	K; BA	Dennis, Stubbs & Coultate, 1967.
Brassica oleracea L. capitata	BA	Tsujita & Andrew, 1967.
Oryza sativa	Bz; BA; K	Mishra & Misra, 1968.
Vigna catjung	Bz; BA; K	Mishra & Misra, 1968.
Arachis hypogea	Bz; BA; K	Mishra & Misra, 1968.
Arabidopsis thaliana*	K	Sankha & Sankha, 1968.
Brassica rapa*	K	Kuraishi, 1968.

Species tested	Chemicals showing activity	Reference
Auxins		
Phaseolus vulgaris	IAA; α-NAA	Sacher, 1957.
Phaseolus vulgaris	2,4-D; 2,4,5-T	Osborne, 1959.
Prunus serrulata	2,4-D	Osborne & Hallaway, 1964.
Arachis hypogea	IAA	Mishra & Misra, 1968.
Gibberellins		
Taraxacum officinale	GA	Fletcher & Osborne, 1965.
Rumex obtusifolius*	GA	Whyte & Luckwill, 1966.
Rumex obtusifolius*	GA	Harada, 1966.
Tropaeolum majus	GA	Beevers & Guernsey, 1967.
Arachis hypogea	GA	Mishra & Misra, 1968.
Growth Retardants		
Phaseolus vulgaris	CCC; B995	Halevy & Wittwer, 1965.
Lactuca sativa	CCC; B995	Halevy, Dilley & Wittwer, 1966.
Rumex obtusifolius*	CCC; B995; AMO1618; Phosphon D	Harada, 1966.
Zea mays	CCC; Phosphon D	Knypl, 1967.
Phaseolus vulgaris	CCC; B995	Kessler, Spiegel & Zolotov, 1967.
Tropaeolum majus	CCC	Beevers & Guernsey, 1967.
Arachis hypogea	CCC	Mishra & Misra, 1968.

* Leaf discs used.

Section I An investigation into the production of pisatin by tissue cultures of Pisum sativum

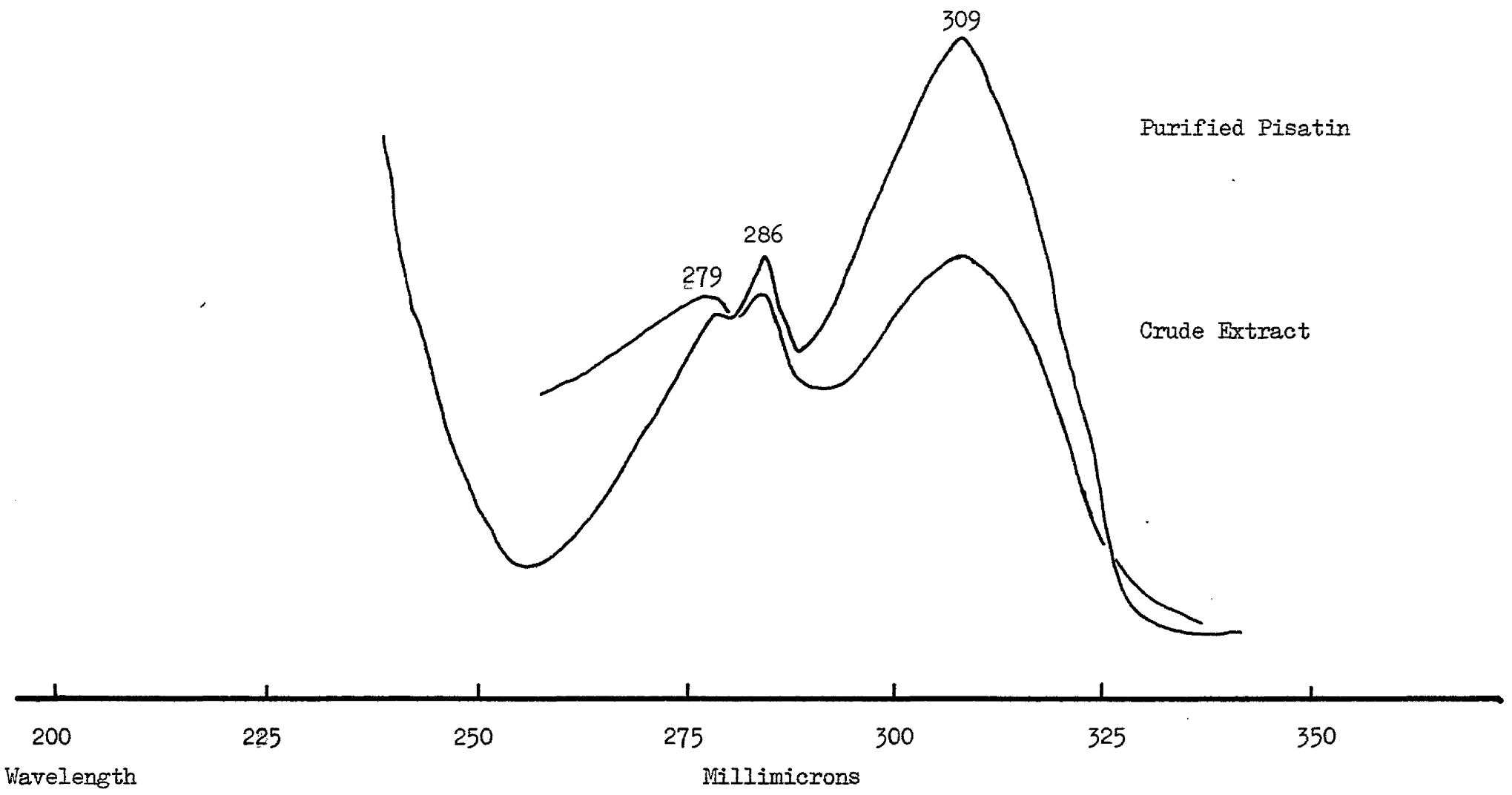
Tissue cultures of pea from various organs were established with a view to investigating the production of pisatin by these tissues after inoculation with various fungi and the relationship of this to the subsequent disease reaction. However, it became clear from early experiments that pisatin was produced by these cultures without infection by fungi. All the callus cultures of pea produced pisatin into the supernatant when grown in liquid medium. As an essential feature of the phytoalexin concept of disease resistance is that the phytoalexin is not a normal metabolic product of plant tissues, this phenomenon was investigated more closely.

In addition, it was observed that the formation of pisatin by callus tissues, when grown in liquid medium, was very variable, varying not only from one experiment to the next within the same culture line (clone) but also between different cultures. The work reported in this section is the result of attempts to explain, firstly, the formation of pisatin under sterile conditions and, secondly, the variations in pisatin production observed.

Experiment 1. The production of pisatin by young stem callus

As described earlier, (Chapter 2), four culture lines of pea callus were successfully established. The majority of the initial experiments were carried out using the young stem callus. The amount of pisatin in the supernatants of this callus growing in liquid medium was shown to change during the period of incubation. This experiment was designed to

Fig. 4.1. A typical Ultra-violet spectrum of an extract of a callus culture supernatant.

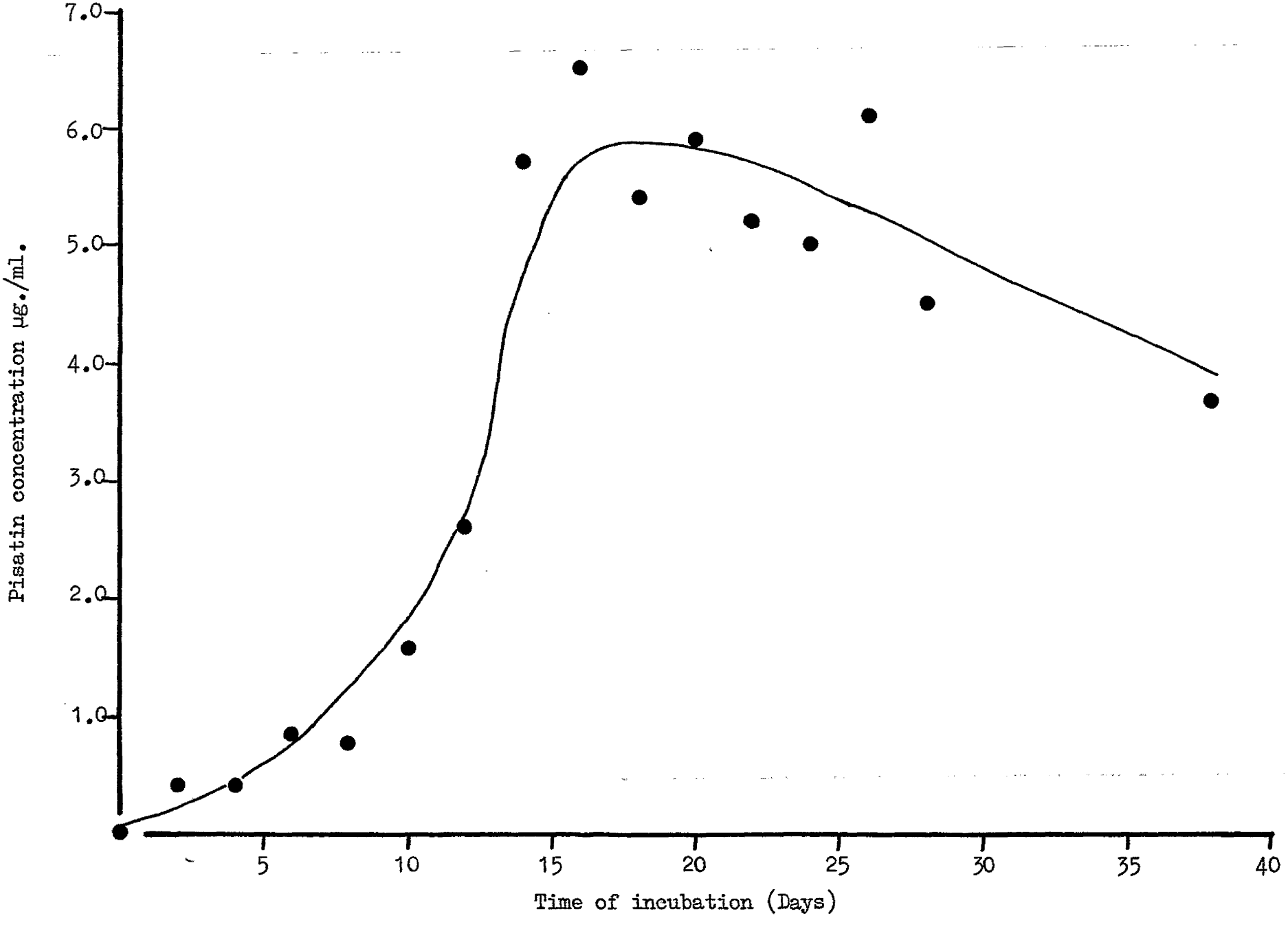


analyse pisatin production by callus over a period of one month after inoculation into liquid medium.

Callus was grown for 29 days on solid medium V. The complete callus from each flask was used as inoculum into 250 ml. Erlenmeyer flasks containing 150 ml. of liquid medium V. 24 flasks were prepared, these being divided into two equal series A and B. Each series was used separately. Series A was divided into three parts (A_{1-3}), each containing four replicated flasks. 10 ml. samples of supernatant from these flasks were assayed in rotation every six days, i.e. samples from A_1 were assayed on days 0, 6, 12 etc.; samples from A_2 were assayed on days 2, 8, 14 etc.; and samples from A_3 were assayed on days 4, 10, 16. Series B was similarly divided, but each part was assayed only once, on days 16, 22, 28.

The assay of pisatin present in the supernatants of liquid callus cultures was basically as described in Chapter 2. 10 ml. of supernatant were removed from the flask and extracted twice with 10 ml. of light petroleum. (This reduced extraction was shown to be highly efficient; only very little pisatin could be obtained by further extraction). The light petroleum was removed under vacuum at 40°C and the residue was re-dissolved in 5 ml. of ethanol. The O.D. at 309 mμ. was used as a measure of pisatin concentration. The purity of pisatin in this ethanol soluble residue was not as great as that obtained from diffusates of pea pod endocarps due to the extraction of materials present in the medium. However, as shown in Fig. 4.1, marked absorption peaks at 279 mμ, 286 mμ. and 309 mμ. were produced. The impurity of the pisatin was indicated by

Fig. 4.2 Pisatin production by young stem callus grown in liquid medium V.



a reduction in the ratio O.D.309 mμ. : O.D. 286 mμ. This impurity was not sufficient to cause any alteration to the assay system described in Chapter 2. Nevertheless, it must be pointed out that the calculated pisatin concentrations may be slightly above the correct values.

The results are shown in Table 4.1. The reduction in volume of the supernatant due to removal of the samples may result in artificially high concentrations of pisatin. The average concentration of pisatin in each part is shown in Table 4.2. The values have been corrected for this change in volume and they are expressed graphically in Fig. 4.2.

The results confirm the fact that the sterile callus produces pisatin when grown in liquid medium. Fig. 4.2 indicates that the production of pisatin exhibits a close similarity to a typical sigmoid growth curve: a period of eight days in which little pisatin was produced, followed by a further period of twelve days in which pisatin was produced rapidly and thereafter production ceased and the concentration of pisatin detectable in the supernatant declined slowly. This decline was probably due to the absorption of the pisatin by the cells rather than a net loss as a result of pisatin breakdown.

Table 4.1 Pisatin concentration in 10 ml. of supernatant from young stem callus growing in liquid medium V.

Supernatant Series	Days after inoculation	O.D. 309 mμ. Replicate				Pisatin conc. μg/ml. Replicate			
		1	2	3	4	1	2	3	4
A_1	0	0.00	0.00	0.01	0.00	0.00	0.20	0.00	0.00
A_2	2	0.02	0.02	0.02	0.02	0.40	0.40	0.40	0.40
A_3	4	0.02	0.02	0.02	0.02	0.40	0.40	0.40	0.40
A_1	6	0.03	0.06	0.04	0.03	0.60	1.30	0.80	0.60
A_2	8	0.04	0.04	0.04	0.04	0.80	0.80	0.80	0.80
A_3	10	0.08	C*	C	0.08	1.70	C	C	1.70
A_1	12	0.06	C	0.23	0.17	1.30	C	5.00	3.70
A_2	14	0.20	0.36	C	C	4.40	7.80	C	C
A_3	16	0.35	C	C	0.34	7.60	C	C	7.40
B_1	16	0.19	0.30	0.28	0.18	4.10	6.50	6.10	3.90
A_1	18	C	C	0.34	0.28	C	C	7.40	6.10
A_2	20	0.34	C	C	C	7.40	C	C	C
A_3	22	0.30	C	C	C	6.50	C	C	C
B_2	22	0.36	0.33	0.31	0.26	7.80	7.20	6.70	5.60
A_1	24	C	C	0.29	0.33	C	C	6.30	7.20
A_2	26	0.38	C	C	C	8.30	C	C	C
A_3	28	0.28	C	C	C	6.10	C	C	C
B_3	28	0.26	0.18	0.18	0.26	6.70	3.90	3.90	5.70
A_1	38	C	C	0.26	0.27	C	C	5.70	5.90
A_2	38	0.24	C	C	C	5.20	C	C	C

* C indicates that the flask was contaminated.

Table 4.2 Pisatin concentration in the supernatant from young stem callus growing in liquid medium V.

Days	Total volume	Average pisatin concentration		Average pisatin concentration corrected for change in volume	
		O.D. 309 mμ.	μg/ml.	O.D. 309 mμ.	μg/ml.
0	150	0.00	0.00	0.00	0.00
2	150	0.02	0.40	0.02	0.40
4	150	0.02	0.40	0.02	0.40
6	140	0.05	0.90	0.047	0.84
8	140	0.04	0.80	0.037	0.75
10	140	0.08	1.70	0.075	1.58
12	130	0.15	3.0	0.13	2.60
14	130	0.28	6.60	0.24	5.70
16	130	0.34	7.50	0.29	6.50
16	150	0.24	5.10	0.24	5.10
18	120	0.31	6.80	0.25	5.40
20	120	0.34	7.40	0.27	5.90
22	120	0.30	6.50	0.24	5.20
22	150	0.32	6.80	0.32	6.80
24	110	0.31	6.80	0.23	5.00
26	110	0.38	8.30	0.28	6.10
28	110	0.28	6.10	0.21	4.50
28	150	0.22	4.80	0.22	4.80
38	100	0.26	5.80	0.17	3.90
38	100	0.24	5.20	0.16	3.50

Experiment 2. To investigate the rate of growth of young stem callus in liquid medium V.

The similarity between the rate of pisatin production shown in the previous experiment and a normal growth curve suggested that the two processes might be inter-related. This experiment was designed to measure both the growth rate and the pisatin production of young stem callus when grown in liquid medium.

Young stem callus was grown for 28 days on solid medium V and transferred to 100 ml. Erlenmeyer flasks containing 40 ml. of liquid medium V. 36 flasks were prepared in this way and incubated in a shaking incubator at 150 revs./min. The flasks were divided into six treatments, each consisting of six replicates. Measurements of growth and pisatin production were made after 0, 6, 12, 19, 26 and 60 days. Growth was expressed as the dry and fresh weights. The tissue mass was filtered through previously weighed standard Oxoid Membrane filters and the weight of tissue after filtration and after drying at 60°C for 4 days was measured. The pisatin concentration of the supernatants was measured as described in Experiment 1. The results are shown in Table 4.3 and expressed graphically in Fig. 4.3.

Table 4.3 The growth of young pea callus in liquid medium V.

Days	Dry wt. grams	Fresh wt. grams	Pisatin conc. µg./ml.
0	0.02098 ± 0.00133	0.362 ± 0.013	0.00
6	0.05445 ± 0.00414	0.533 ± 0.014	0.05
12	0.10843 ± 0.00440	0.964 ± 0.062	1.10
19	0.15914 ± 0.01820	1.360 ± 0.044	1.10
26	0.16785 ± 0.00449	1.546 ± 0.075	1.10
60	0.180405 ± 0.00689	1.573 ± 0.186	1.10

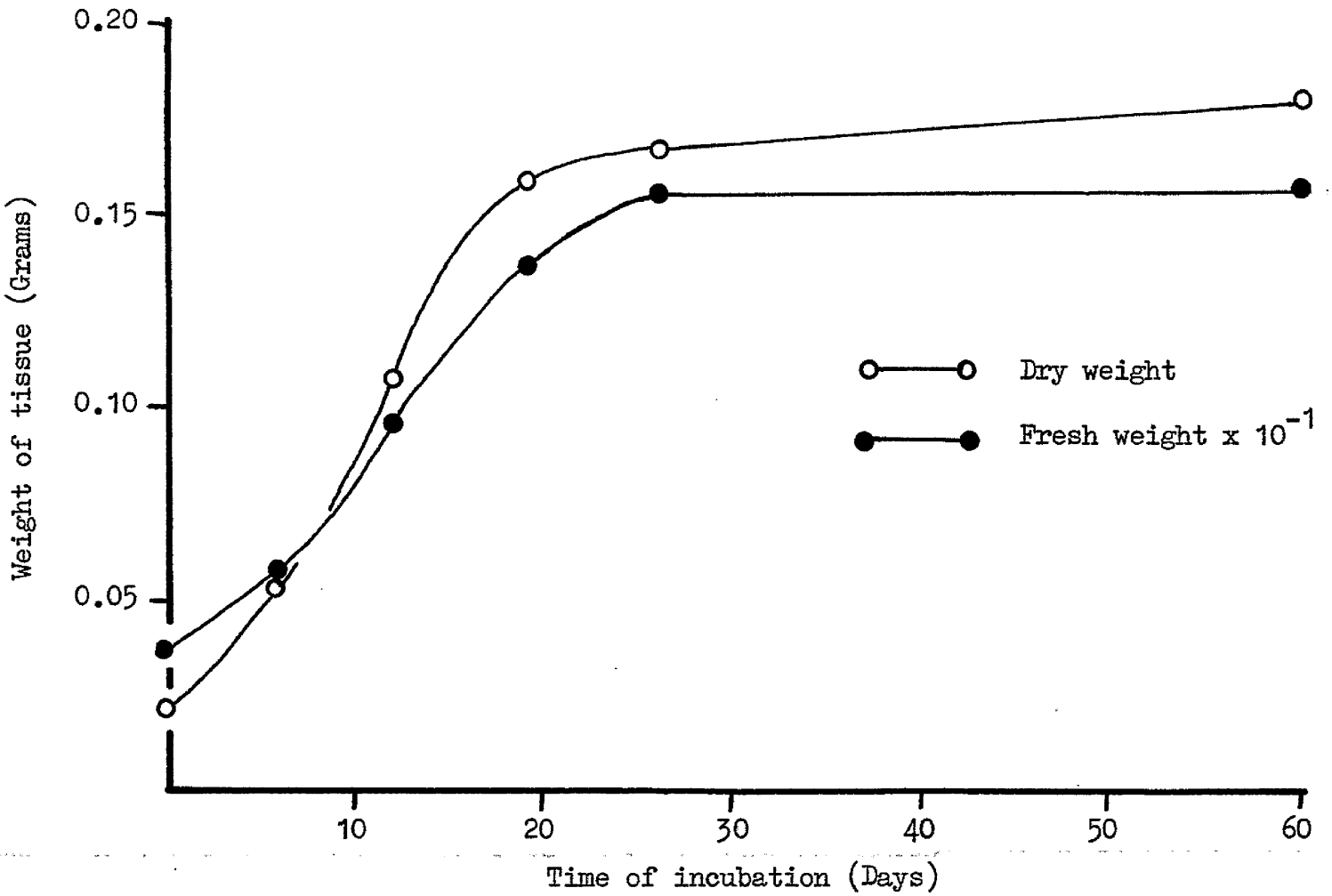


Fig. 4.3 Growth of young stem callus in liquid medium V.

Fig. 4.3 shows the expected sigmoid relationship obtained for growth. The lag period is short, four days, followed by a period of rapid growth for sixteen days, after which growth declines. The comparison of growth with pisatin was not achieved; no increase in pisatin production was demonstrated, and since this experiment was carried out the callus has consistently been producing only trace amounts of pisatin into the medium.

Experiment 3. The induced formation of pisatin by pea leaf discs in the presence of medium V.

The production of pisatin by pea callus tissues may possibly be due to the fact that the pea cells are surrounded by a medium which might itself induce the formation of pisatin. To investigate this, medium V was tested for the ability to induce pisatin formation in pea leaf discs. Three replicates of medium V and one control using sterile distilled water were prepared. 4 ml. of diffusate were obtained from each treatment and assayed for pisatin content. The results are shown in Table 4.4.

Table 4.4 The concentration of pisatin in diffusates of medium V obtained from pea leaf discs after 72 hours

Treatment	Pisatin Concentration	
	O.D. 309 mμ.	μg./ml.
Medium V		
Replicate 1	0.64	34.9
Replicate 2	0.62	33.8
Replicate 3	0.60	32.8
Sterile distilled water	0.12	6.5

The active constituents present in medium V were investigated in a similar manner. 5ml. of diffusate were obtained from each test solution. The concentrations of pisatin in the diffusates are shown in Table 4.5.

Table 4.5 The concentration of pisatin in the diffusates obtained from pea leaf discs after 48 hours

Treatment	Pisatin concentration	
	O.D. 309 mμ.	μg./ml.
Medium V	0.66	28.8
15% Coconut milk	0.56	24.5
6 mg./l. 2,4-D	0.21	9.2
10^{-3} Vitamin solution	0.21	9.2
2% Sucrose	0.22	9.2
10^{-1} Inorganic stock solution	0.18	7.8
Distilled water	0.18	7.8

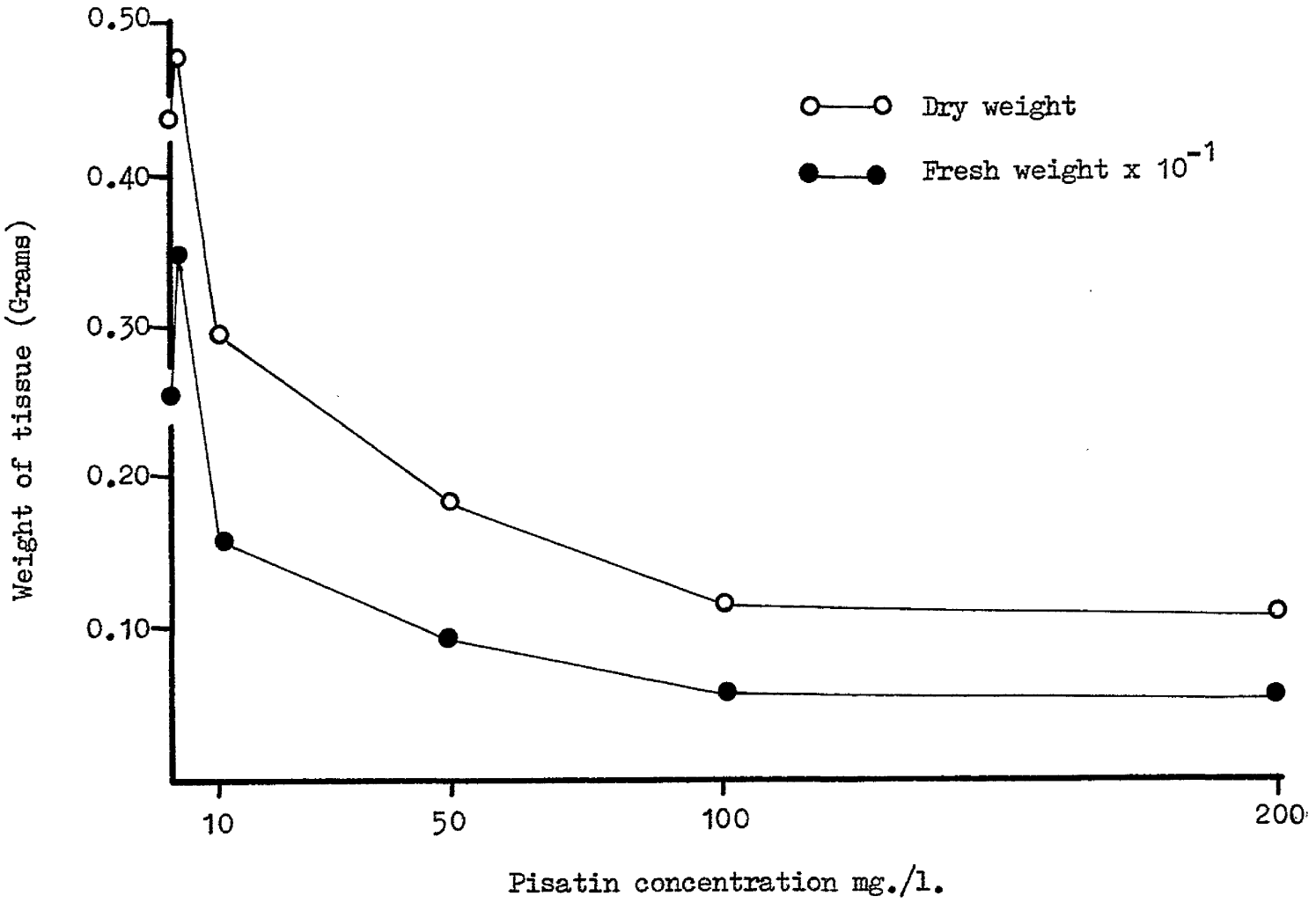
The most active ingredient which induced pisatin formation by pea leaf discs present in medium V was coconut milk. The remainder did not produce pisatin at levels above those formed in the presence of distilled water.

Experiment 4. The effect of pisatin on the growth of young stem callus in liquid medium

It had been observed that recently isolated callus tissues produce more pisatin than callus of the same culture line after it had been established in culture for a longer period, and that the rate of growth of recently established callus was slow but increased the longer the callus had been in culture. Although the attempt to correlate pisatin production with growth had failed, the possibility that one controls the other cannot be ignored. Cruickshank and Perrin (1961) have shown that pisatin is slightly phytotoxic to wheat roots and hence this experiment was designed to investigate whether pisatin affects the growth of young stem callus tissues. At the time of this experiment the young stem callus was producing very small amounts of pisatin.

Pisatin was prepared and purified from pea pods as described in Chapter 2. 60 mg. of pisatin were dissolved in 60 ml. of redistilled ether. This ether solution was distributed between 100 ml. Erlenmeyer flasks so that a series of flasks contained 6 mg., 3 mg., 0.3 mg., 0.03 mg. and 0.00 mg. Five replicates of each treatment were prepared. The final volume of ether was adjusted to 6.0 ml. in all these flasks. The ether was allowed to evaporate and 30 ml. of liquid medium V were dispensed into each flask to yield pisatin concentrations of 200 mg./l., 100 mg./l., 50 mg./l., 10 mg./l., 1 mg./l. and 0. mg./l. One further treatment of five replicates containing neither pisatin nor ether, was prepared. The flasks were sterilized at 115°C for 10 minutes. Young stem callus which had been grown on solid medium V for 28 days, was transferred to

Fig. 4.4 The effect of pisatin on the growth of young stem callus.



each flask which were maintained in a shaking incubator at 150 revs./min. for 20 days.

The first replicate was used to determine the amount of inoculum used and also to measure the initital concentration of pisatin in each flask. Three replicates were used to determine the amount of tissue produced after 20 days. The final replicate was used to measure the concentration of pisatin in the supernatant and also in the callus cells, after 20 days. The total concentration of pisatin present in the supernatant was measured after filtering the tissue growth and thoroughly washing the tissue and the flask with distilled water. To check the solubility of pisatin any residual pisatin, which was not removed in distilled water, was dissolved in ethanol and the O.D. 309 mμ. was measured. Only very small traces were detected even at the highest concentrations used (1.5% of the total pisatin concentration in the flask). It was concluded that pisatin was dissolved by medium V. The results are shown in Tables 4.6 and 4.7 and expressed graphically in Fig. 4.4.

The amount of pisatin measured in replicate one, i.e. after sterilization, was reduced by approximately 50% in all cases. This may indicate that pisatin was decomposed by autoclaving. However, the constant 50% loss in all flasks may be better explained in terms of an error during the preparation of the original concentrations.

The results indicate that pisatin at concentrations above 5 μg./ml. causes a marked inhibition of the growth of young stem callus. In addition, the appearance of the inhibited tissues is different: normal healthy callus when grown in liquid medium produced a finely fragmented "suspension" of small light-brown tissue aggregates, whereas, in the presence of pisatin

Table 4.6 The effect of pisatin on the growth of young stem callus

Treatment	Wt. of Tissue (gms)	Increase in wt. after 20 days (gms)	% Inhibitio of control
(a) Dry Weight			
Inoculum	0.00375 ± 0.00066	-	-
Medium V	0.03553 ± 0.00314	0.03178	-
Medium V Control*	0.004576± 0.00417	0.04217	100
1 µg./ml.Pisatin	0.05166 ± 0.00286	0.04791	114
10 µg./ml. "	0.03382 ± 0.00100	0.02907	69
50 µg./ml. "	0.02313 ± 0.00200	0.01938	46
100 µg./ml. "	0.01582 ± 0.00451	0.01207	28
200 µg./ml. "	0.01614 ± 0.00110	0.01239	29
(b) Fresh Weight			
Inoculum	0.087 ± 0.007	-	-
Medium V	0.266 ± 0.032	0.179	-
Medium V Control*	0.341 ± 0.029	0.254	100
1 µg./ml.Pisatin	0.430 ± 0.079	0.343	135
10 µg./ml. "	0.242 ± 0.013	0.155	61
50 µg./ml. "	0.183 ± 0.015	0.096	38
100 µg./ml. "	0.141 ± 0.022	0.054	21
200 µg./ml. "	0.142 ± 0.008	0.055	22

* The control flasks received 6 ml. of ether prior to sterilization.

Table 4.7 The weight of pisatin in the supernatants of medium V.

Treatment	Initial Wt. of pisatin in medium mg.	Wt. of pisatin in supernatants after 20 days mg.	Wt. of pisatin in tissue after 20 days mg.
Medium V	0.033	0.017	Trace
Medium V Control*	0.009	0.032	Trace
1 µg./ml. Pisatin	0.017	0.008	Trace
10 µg./ml. "	0.135	-	Trace
50 µg./ml. "	0.753	0.184	0.366
100 µg./ml. "	1.37	0.744	0.559
200 µg./ml. "	2.9	2.151	1.704

* The control flasks received 6 ml. of ether prior to sterilization.

at concentrations above 5 µg./ml., the callus failed to fragment and larger dark-brown tissue aggregates are formed. These inhibited aggregates closely resemble the appearance of recently isolated callus growing in liquid medium. The inhibition of growth by pisatin was not accompanied by any evidence which might indicate that the callus was capable of metabolising the pisatin (Table 4.7).

Experiment 5. To examine the relationship between pisatin production and the age of the culture line.

As described earlier, during the establishment of actively growing young stem callus it was observed that, as the number of passages increased, the production of pisatin decreased. Measurements of pisatin production by newly established callus cultures were not made using this culture line. However, supernatants were found containing over 9 µg./ml. of pisatin, when young stem callus, which had been established for 5 months, i.e. 6 passages, was grown in liquid medium V. Experiment 1 was carried out with callus 7 months old, i.e. 8 passages, and Experiments 3 and 4 with callus 8 and 9 months old respectively. As demonstrated in these experiments, very little pisatin was produced under these conditions.

The mode of establishing this fast growing strain is relevant. Initially, the callus was hard and dark brown. However, faster growing light-brown sectors were formed and it was these that were used as the source of fast growing callus.

The aim of this experiment was to initiate new culture lines of callus from young pea stem and to follow pisatin production by the succeeding callus cultures. The isolation procedure was similar to that described in Chapter 2. All cultures were initiated and maintained on

Table 4.8 Pisatin production by young stem callus

Culture line	Pisatin concentration µg./ml.						
	Number of passages						
	2	3	4	5	6	7	8
1	7.6*	9.2*	17.2*	9.8*	7.0	2.8	7.0
2	8.5	8.7	C ø	17.0	14.6	13.9	14.2
3	6.3*	5.7*	7.8*	17.2*	3.5	C	4.1
4	14.8	13.3	6.9	2.2	7.2	8.7	9.4
5	6.8*	7.4*	7.4*	17.8*	13.7	3.1	1.7
6	13.5	12.4	15.3	3.5	7.6	3.1	3.9
7	9.6*	15.7*	13.7*	7.4*	7.2	9.6	13.1
8	6.8*	5.2*	7.4	3.3	12.8	C	10.9
9	7.6*	5.9*	20.1*	15.5*	C	3.9	1.7
10	22.9	13.1	13.5	7.6	10.9	8.7	9.8
11	13.5	C	C	2.6	4.6	3.1	3.3
12	9.4*	15.0*	19.2	10.5	10.2	C	6.1
13	18.3	18.7	15.3	13.1	15.7	7.8	C
14	6.8*	5.7*	5.9*	8.7*	7.8	6.3	2.4
15	9.2	10.0	10.0	8.5	7.4	7.4	6.5
16	C	C	12.6	9.6	6.8	6.5	6.3
35	14.4	12.6	12.6	7.8	8.9	6.5	6.5

ø C indicates that the flask was contaminated.

* indicates that measurements were made after 20 days: those unmarked were made after 35 days.

medium V. Sections of young pea stem were placed on solid medium and callus cells were formed on their surfaces. When sufficient callus tissue had been produced the sections were divided into two; one half was transferred to fresh medium and incubated for 35 days; the other half was placed in a 100 ml. Erlenmeyer flask containing 40 ml. of liquid medium and incubated in a shaking incubator for either 20 or 35 days. The supernatant was then assayed for pisatin content. After 35 days the callus on the solid medium was again divided and the procedure repeated. Eight passages had been achieved at the time of reporting this work.

The results are shown in Table 4.8.

The results indicate that an isolate loses the ability to produce pisatin during the establishment of fast growing callus tissues. Although this experiment was only continued through eight passages, several of the culture lines show this effect clearly, e.g. numbers 3, 5, 6, 9, 11, 12 and 14. The newly produced callus tissue yielded supernatants containing up to 13 μg./ml. of pisatin. This value remained steady, or, in some culture lines, increased for a few passages and this was followed by a reduction in pisatin formation. Finally, callus tissues were produced which formed relatively low concentrations of pisatin, 1-4 μg./ml. This situation did not occur for all the culture lines. Numbers 2 and 10, although they grew quite readily, maintained a high level of pisatin production throughout the duration of the experiment. The general trend of the isolates to produce less pisatin is shown by the average pisatin content of the supernatants after 35 days.

Table 4.9 Pisatin production by four culture lines of pea tissue cultures

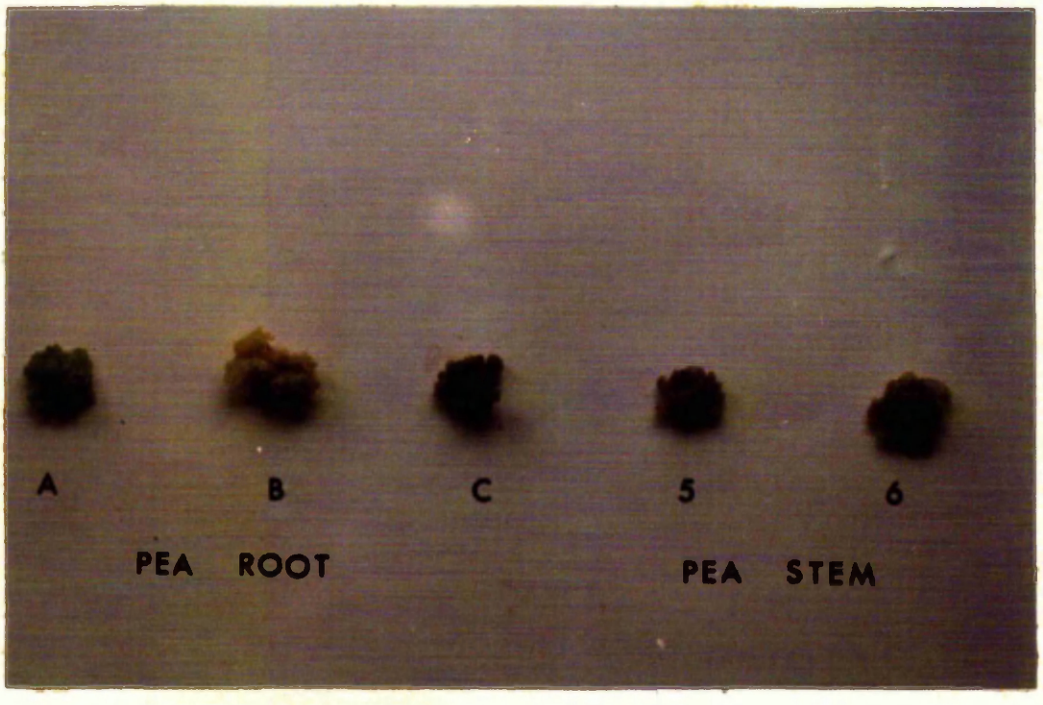
Culture line	Medium	Fresh wt. grams	Dry wt. grams	Pisatin concentration		
				O.D. 309 mμ.	μg./ml.	mg./l./mg. dry wt. callus
Pea Root A	VI					
Replicate 1		0.901	0.09086	0.14	3.0	0.033
2		0.916	0.07833	0.12	3.0	0.038
3		0.863	0.07442	0.18	5.0	0.067
4		0.853	0.06932	0.14	3.0	0.043
Average		0.883	0.07823	0.15	3.0	0.038
			SE=0.0039			
Pea Root B	VI					
Replicate 1		1.100	0.11776	0.40	8.8	0.075
2		1.346	0.11863	0.28	6.1	0.052
3		1.084	0.10271	0.36	7.8	0.076
4		1.301	0.10606	0.47	10.3	0.097
Average		1.207	0.11130	0.38	8.3	0.075
			SE=0.0035			
Pea Root C	VI					
Replicate 1		0.952	0.10740	0.94	20.6	0.193
2		0.790	0.08478	1.07	23.4	0.277
3		0.807	0.09652	1.21	26.4	0.274
4		0.784	0.08966	1.18	25.8	0.288
Average		0.833	0.09459	1.10	24.0	0.254
			SE=0.0042			
Pea Stem	VI					
Replicate 1		-	-	0.21	4.6	-
2		1.960	0.13868	0.23	5.0	0.036
3		1.185	0.11875	0.15	3.3	0.028
4		1.092	0.13651	0.19	4.1	0.030
Average		1.412	0.13131	0.19	4.1	0.031
			SE=0.0049			
Pea Stem	V					
Replicate 1		1.182	0.09354	-	-	-
2		1.271	0.08990	0.14	3.1	0.034
3		1.119	0.08435	-	-	-
4		1.436	0.12268	0.17	3.7	0.030
Average		1.252	0.09762	0.16	3.4	0.035
			SE=0.00735			

Experiment 6. Pisatin production by various culture lines of *Pisum sativum*

The production of pisatin by the young stem culture line and three root culture lines was investigated. All the culture lines had produced actively growing callus and visually stable strains were maintained by careful selection when they were subcultured on to fresh medium. The root cultures were grown as callus on medium VI for 28 days; the stem culture on medium V for 28 days. The calluses were then transferred to 100 ml. Erlenmeyer flasks containing 40 ml. of either medium V or VI. These flasks were incubated in a shaking incubator for 38 days. The concentration of pisatin present in the supernatant and the amount of tissue growth were measured.

The results are shown in Table 4.9.

The visual appearance of the callus tissues used is shown below.



Pisatin was shown to be produced by all the callus cultures. The different culture lines produced different amounts of pisatin. All the culture lines had been established for fifteen months and, as indicated by the fresh and dry weight measurements, grew quite readily in the media provided. Pea root culture C produced large quantities of pisatin (> 20 μg./ml.). However, the growth of the callus was not appreciably less than that of the other culture lines which only produced very small amounts of pisatin. Thus it has been shown that, by selection of dissimilar sectors of young callus, callus which differs visually can also be shown to differ biochemically, i.e. the ability to produce pisatin.

Discussion

The main conclusions which can be drawn from these results are as follows:

1. Pisatin is synthesised by callus cells under sterile conditions. Fungal inoculation is not required for the induction of this synthesis. The formation of pisatin by these tissues would seem to be explained by the presence of coconut milk which was shown to induce pisatin formation when applied to pea leaf discs in the nutrient media. The kinetics of pisatin formation by these callus tissues differed from the formation of pisatin by leaf discs. Using leaf discs (or pea pod endocarps), pisatin formation occurred at a very high level during the first 48 hours and then decreased. Whereas with callus there was a lag of eight days before pisatin formation occurred. This lag may be due to a possible "shock mechanism" caused by subculturing. This suggests that pisatin formation by callus tissues may be dependent on the active growth of the callus cells which similarly showed a slight lag when the callus was subcultured.

As stated, the formation of pisatin by the callus cultures is readily explained. However, it is not possible to explain why their ability to produce pisatin varies, nor why cultures can be established which, in the presence of the same nutrient media, produce little or no pisatin.

2. The production of pisatin by callus tissues is very variable. Generally, pisatin production is greatest in newly established callus and declines as the callus is maintained in culture for an extended period. Eventually a callus can be established which produces small amounts of pisatin. Although no measurements were made, the high production of

pisatin by newly established callus appeared to be associated with a slow growth rate. The faster growing sectors, which were used for subculturing, yielded callus which produced less pisatin. However, it has also been shown, on one occasion, that pisatin formation by the calluses can be maintained at a high level. Pea root culture C produced high amounts of pisatin and, at the same time, growth of this tissue was not markedly reduced when compared with root culture lines which had been shown to produce very small amounts of pisatin.

3. Pisatin has been shown to inhibit the growth of young stem callus, a culture line which produced only small amounts of pisatin. The concentration of pisatin required for inhibiting growth is very low, $E.D._{50}$ 25 µg./ml.

It is suggested that pisatin may have a marked effect on the establishment of pea callus tissues. Newly established callus is dark brown and slow growing, i.e. very similar in appearance to established callus growing in the presence of additional pisatin. Pisatin production by the newly established callus is very high, 10-25 µg./ml. This concentration of pisatin has been shown to be sufficient to inhibit the growth of established callus tissues. It thus seems probable that newly established pea stem callus grows slowly due to the presence of pisatin in both the nutrient medium and the cells themselves. While the callus is growing on solid medium sectors of faster growing cells are formed. When these sectors are subcultured on to fresh medium, callus is produced which grows more rapidly and produces less pisatin. By repeated subculturing of these sectors, a strain of callus is produced which grows quickly and produces only trace amounts of pisatin.

The difference in behaviour of pea callus in relation to pisatin production and growth rate, emphasizes that major problems encountered when working with tissue cultures are, firstly, the great variations, both visual and biochemical, shown by the callus and, secondly, that it is very difficult to determine whether the tissue cultures which have been established can be used as representative of the situation occurring in the natural plant tissues.

Although Cruickshank and Perrin (1961) have concluded that pisatin plays no role in producing the necrotic reaction associated with fungal infection and although it has been observed in this work that pisatin can be produced under conditions when necrosis does not occur, i.e. in the presence of a fungal culture filtrate, the effect of pisatin on callus cells suggests otherwise. At relatively low concentrations pisatin was shown to inhibit growth and also to cause these inhibited calluses to be dark coloured. During the course of a fungal infection, pisatin will come into close contact with healthy and injured cells.

It would appear likely that, under these conditions, pisatin, which may occur at concentrations well above those shown to affect callus cells, may cause the cells of the infected areas to become necrotic and dark pigmented. Under conditions when no tissue damage has occurred pisatin is unable to show this effect.

It has been reported that when virus-infected rhubarb meristem tips were grown on artificial medium, the plants which were obtained were usually free from virus, i.e. culturing the infected tip caused changes to occur which resulted in the eradication of the virus, (Tomlinson and Walkey 1967).

In the work described in this section and in Chapter 3 it has been shown that the metabolism of plant tissues were altered in such a way that artificial compounds, which were not detectable in healthy tissues, were produced when either apple or pea tissues were grown on artificial medium. Similar changes in the metabolism of the rhubarb cells may explain the eradication of the viruses from the meristem tips.

In conclusion, it is suggested that pisatin may be responsible for two major effects. Firstly, that it prevented rapid growth of newly established callus, and secondly, that the necrotic cells found associated with fungal infection are the result of the action of pisatin on cells which have been exposed or damaged by the infecting fungus.

Section II The role of fungal spore suspensions and their possible metabolites in the induced formation of pisatin by Pisum sativum.

Experiment 7. To investigate pisatin production by pea pod endocarps inoculated with spore suspensions of various fungi

Spore suspensions of eleven fungal species were prepared in distilled water. Using the "drop diffusate technique" described in Chapter 2, the formation of pisatin by the pod tissues was investigated. The pods were incubated in the presence of dense spore suspensions for 48 hours. The diffusate was collected and 5 ml. from each diffusate were assayed for pisatin concentration. The results are shown in Table 4.10.

Table 4.10 Concentration of pisatin produced by pea pod endocarps inoculated with spore suspensions of various fungi

Fungal species	Pisatin concentration µg./ml.
Botrytis allii	62.2
Botrytis cinerea	60.4
Fusarium solani var. martii*	62.6
Ascochyta pisi*	79.7
Cladosporium herbarum	75.3
Penicillium digitatum	48.2
Penicillium expansum	96.4
Myrothecium roridum*	42.9
Sclerotinia fructicola	56.1
Pleospora herbarum	54.7
Phoma ellipticum	42.9
Distilled water	4.1

* indicates fungal species which are pathogenic to Pisum sativum.

Table 4.11. Concentration of pisatin in diffusates obtained from pea pod endocarps after 48 hours.

Treatment	Pisatin concentration µg./ml.	
	Experiment I	Experiment II
Culture filtrate	131.4	53.4
Autoclaved culture filtrate	79.7	19.7
Medium I	3.4	2.4
Distilled water	2.7	2.1
P. expansum in medium I	122.6	-

The results are in agreement with Cruickshank, i.e. that all the fungi tested induced pisatin concentrations much greater than the levels of the water control. It will be seen that, under these conditions, Penicillium expansum, a non-pathogen of pea, was most active in inducing pisatin formation.

Experiment 8. To investigate the formation of pisatin by pea pod endocarps in the presence of sterile culture filtrate.

Penicillium expansum was grown in liquid medium I for four days at 24°C. The flasks were incubated in the dark and were only shaken very occasionally by hand. After four days the fungal growth was removed by passing through muslin and the filtrate was sterilized by filtration through standard Oxoid Membranes. The sterile culture filtrate was divided into two parts; one part was applied directly to the pea pods; the other was autoclaved at 121°C for 15 minutes and, after cooling, was then applied to the pea pod surface. The original medium, distilled water and a spore suspension of P. expansum in medium I, were also set up to act as controls. The pods were incubated for 48 hours. 5 ml. of each diffusate were assayed for pisatin concentration. The results of two experiments are shown in Table 4.11.

The culture filtrate of P. expansum was very active in producing pisatin when applied to pea pod endocarp surfaces. This activity was greatly, but not completely, destroyed after the filtrate had been heated at 121°C for 15 minutes.

Table 4.12 Concentration of pisatin in diffusates obtained from pea pod endocarps after 48 hours

Treatment	Pisatin concentration µg./ml.
Culture filtrate	120.5
Eluate from Zeo Carb 225	97.2
Eluate from Amberlite IR4B	7.9
"Acidic fraction"	65.3
Medium I	1.0

Table 4.13 Concentration of pisatin in diffusates obtained from pea pod endocarps after 48 hours

Treatment	pH	Pisatin concentration µg./ml.
Culture filtrate	3.3	104.3
Culture filtrate	5.0	64.8
Eluate from Zeo Carb 225	2.2	65.3
Eluate from Zeo Carb 225	5.0	19.7
Eluate from Amberlite IR4B	8.9	3.0
Eluate from Amberlite IR4B	5.0	6.1
Eluate from Amberlite IR4B	3.0	10.5
"Acidic fraction"	2.1	48.2
Medium I	5.5	1.0

An investigation into the identification of constituents present in the culture filtrate which may be responsible for the induced formation of pisatin by pea tissues

Experiment 9. To investigate the activity of various extracts to induce pisatin formation following fractionation of the culture filtrate by ion-exchange chromatography

Penicillium expansum was grown stationary in liquid medium I for six days at 22°C, and the culture filtrate was prepared as detailed in Experiment 8. The culture filtrate was fractionated through ion-exchange columns and the induction of pisatin by the various fractions was tested. The ion-exchange resins used were Zeo Carb 225 and Amberlite IR4B, which yield eluates containing acidic and neutral fractions, and basic and neutral fractions respectively. The acids retained on the Amberlite IR4B were removed by washing the columns with 2N ammonium hydroxide. The free acids, ("acidic fraction"), were obtained firstly by removing the excess ammonia under vacuum, and finally by passing the residual solution through Zeo Carb 225 (Ranson, 1955). The activity of the various fractions is shown in Table 4.12.

The pH of these various fractions differed greatly. In order to determine the importance of these differences, the pHs of the various fractions were adjusted before applying them to the pea pod endocarps. The pHs were adjusted using N.HCl and N.NaOH in order to minimize the dilution effect. The results are shown in Table 4.13.

The results confirm that the active constituents were retained by Amberlite IR4B, i.e. they are acidic. It was also demonstrated that

pisatin production was greatest at low pH. Weakly acidic substances tend to be less dissociated at low pH; it is common experience that undissociated molecules penetrate membranes more readily than ions, and this may explain the greater effect at low pH.

Experiment 10. The induced formation of pisatin by pea pod endocarps in the presence of organic acids

The high activity of the "acidic fraction" obtained from the culture filtrate suggested that organic acids, which may be present in this fraction, could be responsible for the stimulation of pisatin synthesis. To investigate this, 1% solutions of malic, succinic and fumaric acids were tested for their ability to produce pisatin when applied to pea pod endocarp surfaces. The results are shown in Table 4.14.

Table 4.14. Concentration of pisatin in diffusates obtained from pea pod endocarps after 48 hours

Treatment	Concentration of Organic acid	Pisatin concentration µg./ml.
Malic acid	8.3×10^{-3} M	51.7
Succinic acid	8.9×10^{-3} M	40.7
Fumaric acid	9×10^{-3} M	17.1
Distilled water		1.0

It was shown that organic acids of the Krebs cycle induced the formation of pisatin. In this experiment, malic acid was more active in inducing pisatin formation than the other acids tested.

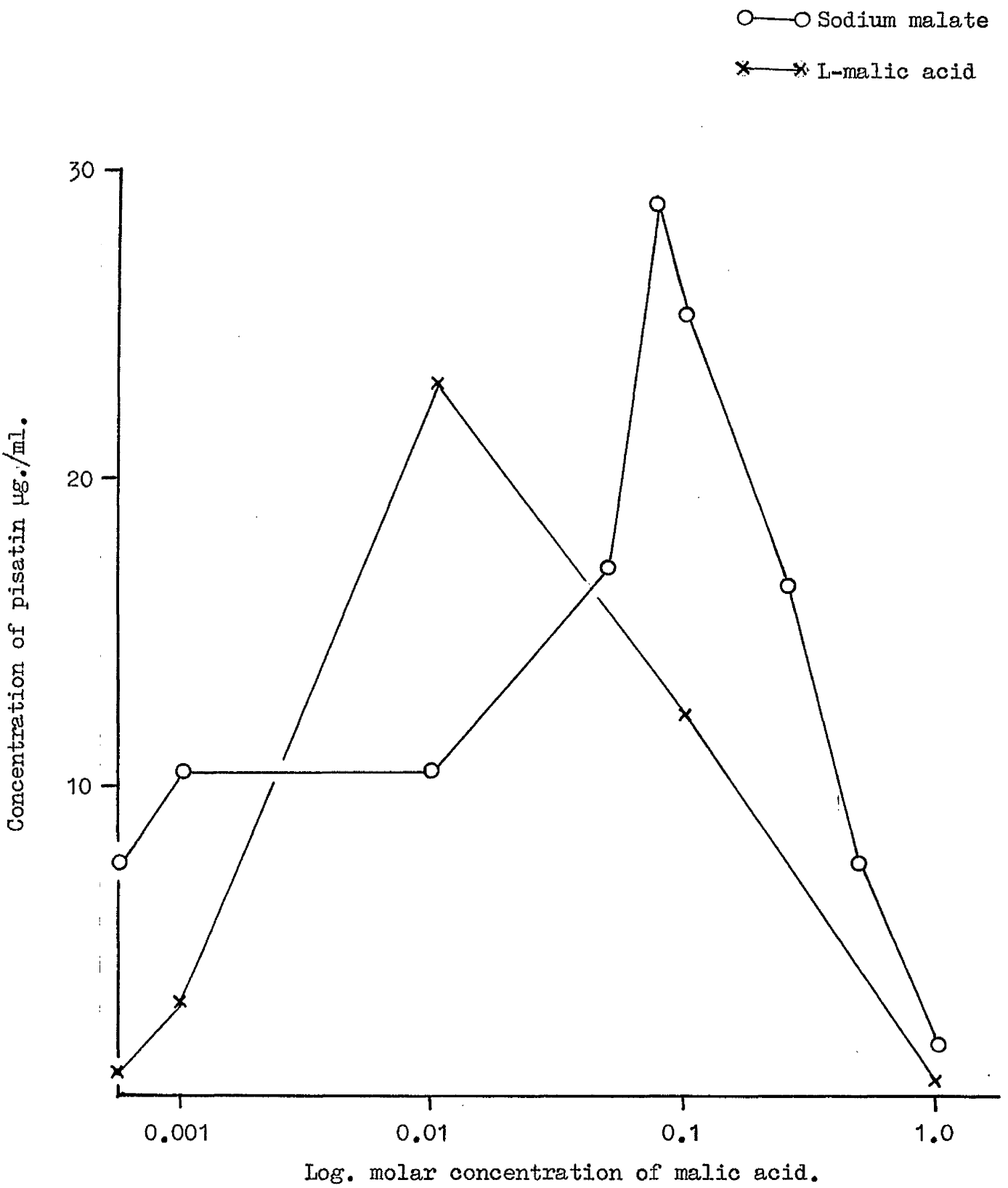


Fig. 4.5. The induced formation of pisatin by pea leaf discs in the presence of L-malic acid and sodium malate.

Experiment 11. The induced formation of pisatin by pea leaf discs in the presence of malic acid

To observe more accurately the effects of malic acid, several experiments were carried out in order to determine the concentrations of malic acid which would induce the formation of the highest amounts of pisatin, and also the effect of pH on this induction. Leaf discs, as described in Chapter 2, were used in this experiment. Molar solutions of malic acid were prepared by dissolving the acid in distilled water and molar solutions of sodium malate were prepared by dissolving the acid in 2N. sodium hydroxide. The dilutions were prepared from these solutions. The pH of the malic acid solutions varied between 2.2 and 3.5. The pH of the sodium malate solutions was 7.0. The results are shown in Tables 4.15 and 4.16, and expressed graphically in Fig. 4.5.

Table 4.15. Concentration of pisatin in diffusates obtained from pea leaf discs after 48 hours

Treatment	Pisatin concentration µg./ml.			
	Experiment I		Experiment II	
	Sodium salt	Free acid	Sodium salt	Free acid
Molar Malic acid	2.1	1.0	-	-
10^{-1} M Malic acid	24.1	14.1	18.4	11.4
10^{-2} M Malic acid	6.2	28.0	6.2	18.4
10^{-3} M Malic acid	-	-	6.4	3.1
Distilled water	1.0	1.0	1.0	1.0

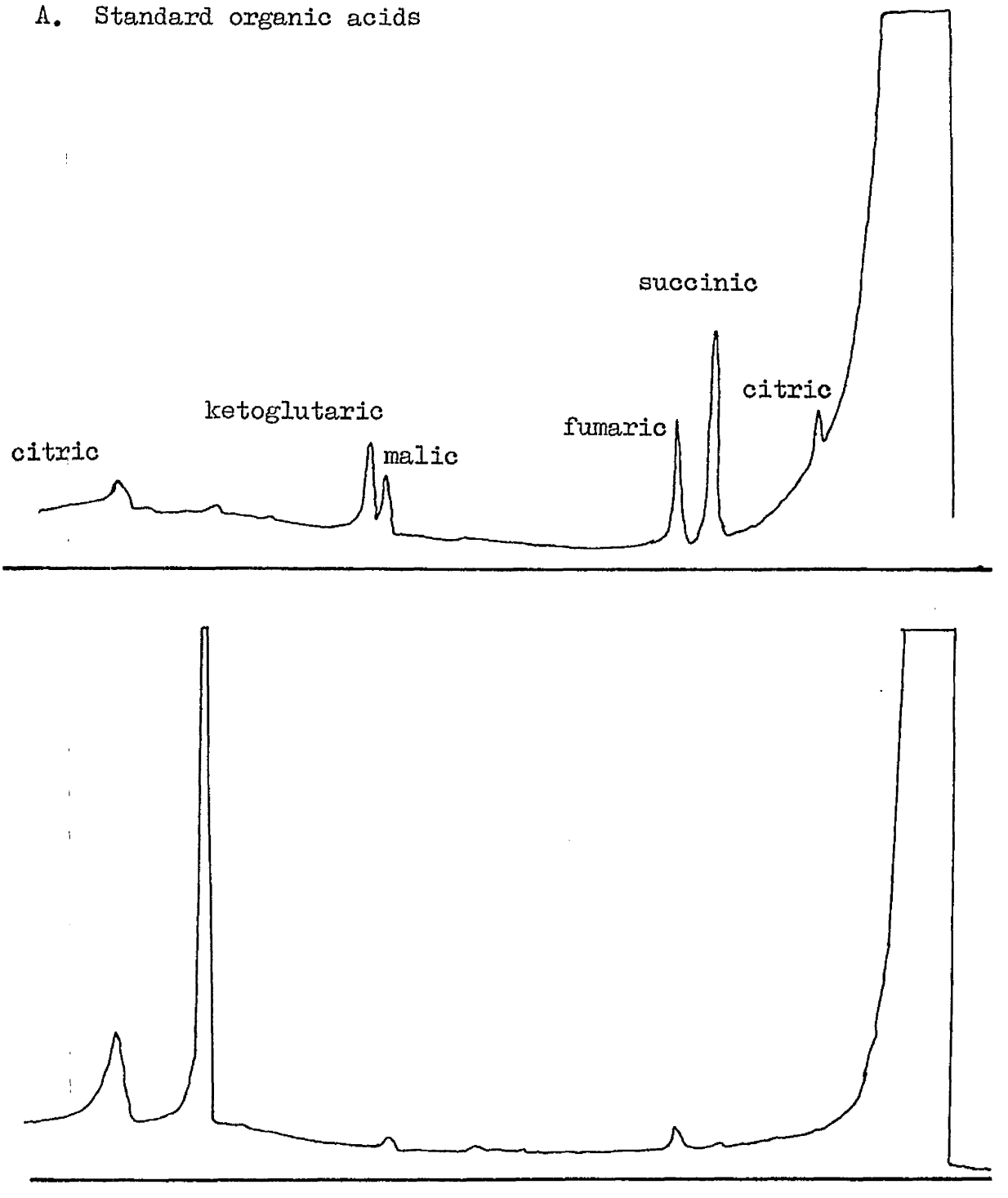
Malic acid and sodium malate were both shown to induce pisatin

Table 4.16. Concentration of pisatin in diffusates obtained from pea leaf discs after 48 hours

Treatment	Concentration of pisatin µg./ml.			
	Experiment I		Experiment II	
	Replicates	Mean	Replicates	Mean
M Sodium Malate		1.7		-
5×10^{-1} M Malate		7.9		7.4
2.5×10^{-1} M Malate		-	16.6 17.5	17.0
10^{-1} M Malate	24.1 25.8	25.0	25.8 26.2	26.0
7.5×10^{-2} M Malate		-	28.9 29.0	29.0
5×10^{-2} M Malate	16.2 18.0	17.1		17.5
10^{-2} M Malate		10.9		-
10^{-3} M Malate		10.5		-
Distilled water		7.0		8.1

formation. Table 4.15 indicates that the amount of pisatin produced was similar, but that the concentrations of malic acid and sodium malate which produced these amounts differed. 10^{-2} M malic acid produced amounts of pisatin which were formed by sodium malate only at a concentration of 10^{-1} M. This would again suggest that the undissociated molecules are more effective in producing pisatin than the dissociated ones.

Fig. 4.6. GLC traces of the acetate methyl esters of organic acids.



Experiment 12. The identification of the organic acids present in culture filtrates of Penicillium expansum

As a result of the earlier work which had shown that the culture filtrate of Penicillium expansum, the acidic fraction of this filtrate and several organic acids were all capable of inducing the formation of pisatin, the culture filtrate was analysed for the presence of carboxylic acids. The acid fraction from the ion-exchange columns was taken to dryness at 40°C under vacuum. The acids were converted to the methyl esters using boron trifluoride dissolved in methanol, and after removing some of the excess solvent under nitrogen, the mixture was reacted with acetic anhydride to convert the hydroxy-acids to the corresponding acetates. The derivatives obtained were analysed by gas-liquid chromatography using 5% SE-30 and programming the temperature from 50°C to 220°C at a rate of 3°C per minute. Traces of acids with retention times corresponding to fumarate, succinate and malate were detected, but the only major component corresponded to the derivatives formed from tartaric acid. By this method, tartaric acid produced two peaks (approximate retention times 18 and 25 minutes when analysed isothermally at 190°C) in the ratio 2.7 : 1. The natural extract produced an identical result. These results are shown in Figs. 4.6 and 4.7.

Experiment 13. The induced formation of pisatin by pea leaf discs in the presence of tartaric acid

As demonstrated above, the major carboxylic acid present in the culture filtrate was tartaric acid. This was not unexpected since medium I contains 0.1% ammonium tartrate. However, it is worth pointing

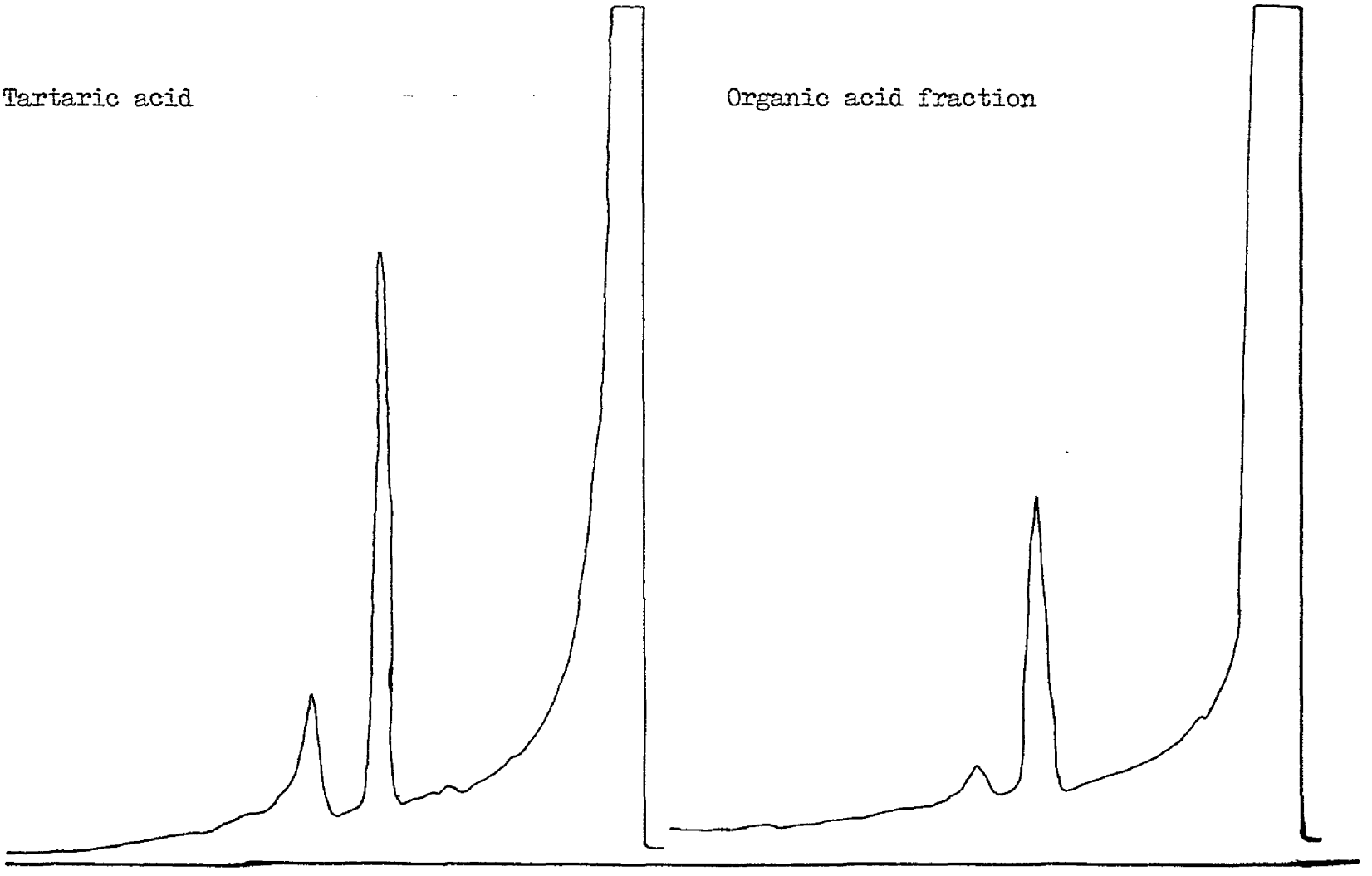


Fig. 4.7. GLC traces of the acetate methyl esters of organic acids.

out that the amounts of the other carboxylic acids present in the "acidic fraction" are extremely low compared with the amount of tartaric acid. Thus it could be assumed that these other carboxylic acids are not present in the culture filtrates at sufficient concentrations to stimulate pisatin production. This experiment was designed to investigate whether tartaric acid could induce the formation of pisatin in pea leaf discs. Only the free acid was tested and the results are shown in Table 4.17.

Table 4.17. Concentration of pisatin in diffusates obtained from pea leaf discs after 42 hours.

Treatment	Pisatin concentration µg./ml.
10^{-1} Molar Tartaric acid	1.3
10^{-2} " " "	14.5
10^{-3} " " "	12.3
10^{-4} " " "	10.5
10^{-2} Molar Malic acid	18.4
Distilled water	8.3

The amount of pisatin produced in the presence of tartaric acid was low. At a concentration of 10^{-2} Molar, malic acid was more efficient than tartaric acid. Neither, however, even if present at the optimum concentrations, could contribute to any great extent to the ability of the culture filtrate to induce pisatin formation.

Experiment 14. The induced formation of pisatin by pea leaf discs in the presence of patulin

These experiments indicated that the carboxylic acids, although present in the culture filtrate, could not explain the amounts of pisa produced by the pea tissues. It has been reported (Brian, Elson and Lowe, 1956) that Penicillium expansum produced the antibiotic patulin grown in artificial media. In view of this, Patulin, though not a ca boxylic acid, is acidic in nature and likely to appear in the acid fra of a culture filtrate, was therefore tested for its ability to induce formation of pisatin in pea leaf discs. The effect of patulin by its and also in the presence of the culture filtrate was investigated. T results are shown in Table 4.18 and 4.19 and expressed graphically in Fig. 4.8.

Table 4.18. Concentration of pisatin in diffusates obtained from pea leaf discs after 42 hours

Treatment	Pisatin concentration µg./ml.
Distilled water	8.7
1 mg./l. Patulin in distilled water	8.7
10 mg./l. " " " "	37.7
100 mg./l. " " " "	47.7
1000 mg./l. " " " "	12.3
Culture filtrate	115.6
1 mg./l. Patulin in culture filtrate	108.6
10 mg./l. " " " "	113.8
100 mg./l. " " " "	74.5
1000 mg./l. " " " "	2.6

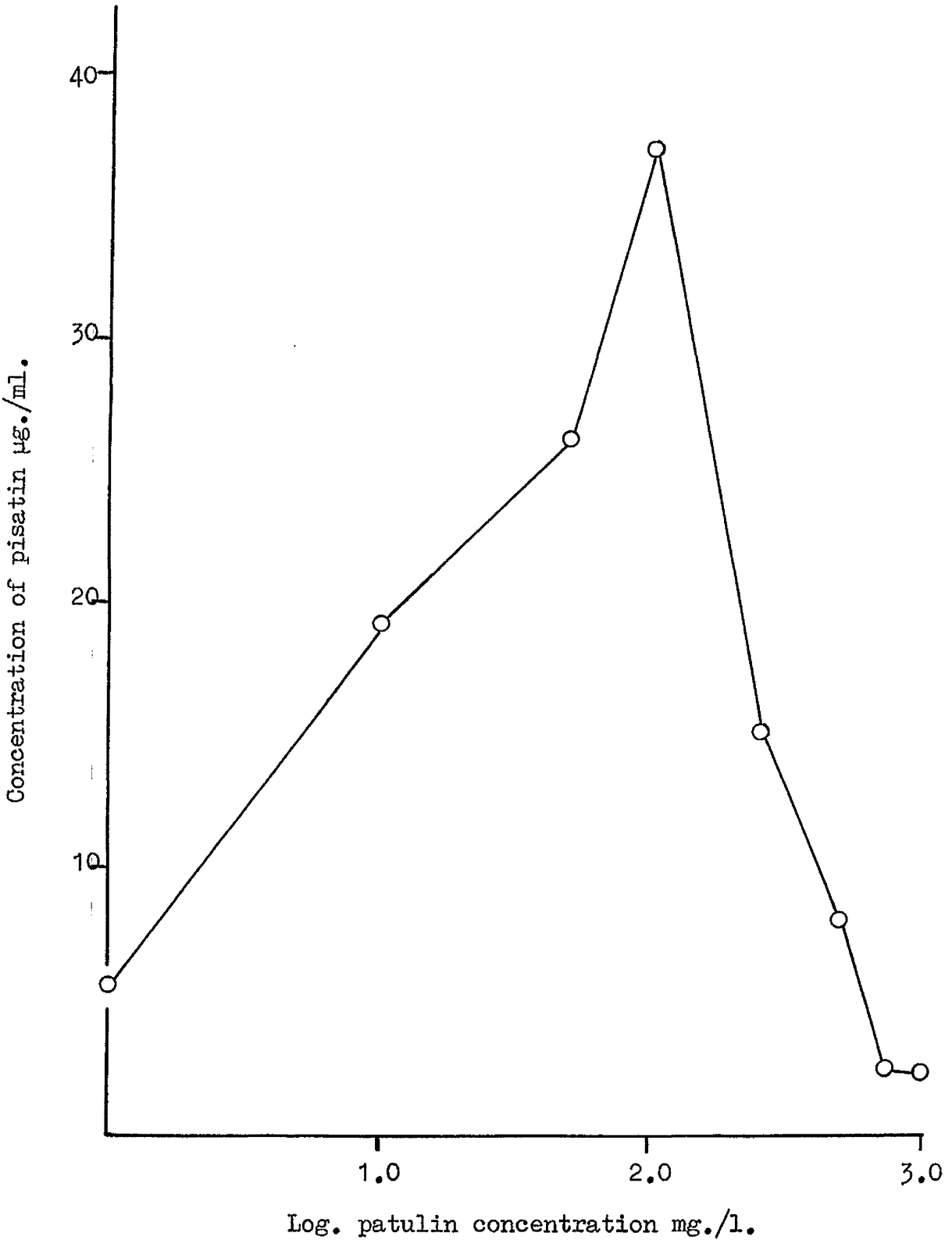


Fig. 4.8. The induced formation of pisatin by pea leaf discs in the presence of patulin.

Table 4.19 Concentration of pisatin in diffusates obtained from pea leaf discs after 42 hours

Treatment	Pisatin concentration µg./ml.
Distilled water	5.7
10 mg./l. Patulin in distilled water	19.3
50 mg./l. " " " "	26.3
100 mg./l. " " " "	37.2
250 mg./l. " " " "	17.8
500 mg./l. " " " "	8.3
750 mg./l. " " " "	2.6
1000 mg./l. " " " "	2.5
Culture filtrate	57.8

The results indicate that patulin induced the formation of large amounts of pisatin. The amounts produced were not as great as those obtained in the presence of the culture filtrate. When patulin was added to the culture filtrate the amount of pisatin formed was reduced.

Discussion

It has been reported that a wide variety of compounds are capable of inducing the formation of pisatin. Heavy metal ions, metabolic inhibitors, e.g. p-chloromercuribenzoate, sodium iodoacetate, sodium fluoride, sodium azide, sodium cyanide and thioglycollic acid, sodium selanate and sodium selanite, and certain amino acids have been shown to stimulate the production of pisatin (Cruickshank, 1965). The production of ipomeamarone was stimulated by mercuric chloride, trichloracetic acid, monoiodoacetic acid and 2,4-dinitrophenol (Uritani, Uritani and Yamada, 1960). Discs of carrot produced small amounts of the isocoumarin after immersion in a simple medium. Other chemicals e.g. potassium cyanide, p-chloromercuribenzoate, sodium arsenate, iodoacetate and malonate stimulated the production of the isocoumarin in larger quantities (Condon, Kúc and Draudt, 1963).

In the experiments reported in this thesis it was confirmed that the culture filtrate of a fungus, in this case P. expansum, was capable of inducing large amounts of pisatin. Cruickshank and Perrin (1963) had shown that if Sclerotinia fructicola was grown in a nutrient medium, i.e. a simple diffusate from pea pod endocarp surfaces, the filtrate obtained from this was able to stimulate pisatin production. More recently Cruickshank has reported that a culture filtrate obtained after growing S. fructicola in a simple culture medium also stimulated phaseollin production. From this filtrate he isolated a chemical, Monilicolin A, which proved to be very active in inducing phaseollin production (Cruickshank, 1968).

It has been possible to demonstrate that several compounds which may

be present in the culture filtrate of Penicillium expansum are capable of inducing the formation of pisatin. As a result of the ion-exchange chromatography, it can be concluded that the great majority of the active constituents were acidic in behaviour; Tables 4.12 and 4.13 indicate that the eluates from Amberlite IR4B had very little activity. The presence of carboxylic acids in the filtrate was investigated by means of gas-liquid chromatography. Tartaric acid was the major constituent, the other acids being present in only trace amounts. At the low pH of the culture filtrate tartaric acid would be present in its undissociated form, i.e. when it is most active. Despite this, the presence of carboxylic acids in the culture filtrate is unlikely to contribute directly to the amount of pisatin formed.

The induction of pisatin formation by sodium malate shows the same concentration dependence as demonstrated for the other chemical inducing agents (Cruickshank, 1965), i.e. at low concentrations of sodium malate, ($< 10^{-2}$ M, there was no pisatin produced beyond the usual low water control values; with greater concentrations, (10^{-2} - 7.5×10^{-2} M), pisatin production increased to a maximum; but at still greater concentrations, ($> 10^{-1}$ M), pisatin production fell and this was associated with visible phytotoxicity. As with the induction of pisatin formation by amino acids, the optimum effects occurred using physiologically abnormal concentrations.

Further identification of the active constituents was not achieved in this work. A proven metabolite of Penicillium expansum, patulin, which was quite possibly present in the culture filtrate, was tested for its ability to stimulate pisatin formation. An antibiotic, ~~pisatin~~ patulin has been reported to be produced by strains of P. expansum when grown in artificial medium and also to be detected in rotted apple tissue which was infected

with P. expansum (Brian, Elson and Lowe, 1956). Table 4.19 shows that patulin induced the synthesis of relatively large amounts of pisatin. A similar concentration dependence was demonstrated with the optimum effect shown by a concentration of 100 mg./l. The amounts of pisatin produced by patulin were less than those produced by a culture filtrate. The optimum concentrations of pisatin produced in the presence of patulin were only 41% and 61% as great as produced in the presence of the culture filtrate. No experiments were carried out to establish whether patulin was present in the culture filtrates of P. expansum used in this work. Nevertheless, the presence of patulin in the filtrate could explain to a large extent the ability of the filtrate to induce pisatin formation.

In these experiments it was shown that by lowering the pH of the test solutions, greater amounts of pisatin were produced. During growth of the culture medium the pH falls. This could be due to either the production of acidic materials or to the preferential absorption of cations (e.g. NH_4^+) by the fungus. Whatever the cause of the fall in pH, it follows that weak acids (e.g. carboxylic acids, phenols or patulin) will tend to be less dissociated than they would be at the higher pH values. It is known that the majority of such substances penetrate more rapidly into cells in the undissociated form and, as a result, appear to be more active at low pH. The production of pisatin in the presence of the culture filtrate was reduced by 40% when the pH was adjusted from 3.3 to 5.0. It can be seen from Table 4.15 that 10^{-2} malic acid (pH 3.0) was active in producing pisatin. However, 10^{-2} M sodium malate (pH 7.0) induced the formation of only very small amounts of pisatin. Thus, although the carboxylic acids present in the filtrate cannot be expected to contribute directly to the formation

of large amounts of pisatin, the decrease in pH which occurs after growth of the fungus could increase the activity of other inducing agents present in the culture filtrate. In view of this, and of the fact that the patulin solutions tested were at a high pH (5.5 - 6.0), it can be speculated that the amount of pisatin produced by patulin would be increased at lower pHs. It is apparent from Table 4.18 that the addition of 100 mg./l. of patulin to the culture filtrate caused a reduction in the amount of pisatin produced. This could be explained if patulin, or a similar compound, was already present and active in the medium, but due to the addition of patulin, the concentration of patulin was increased so that a toxic level was produced. This toxic effect was shown in Table 4.19 by concentrations of patulin above 100 mg./l.

Section III. The effect of antimetabolites on the ability of a culture filtrate of Penicillium expansum to stimulate the formation of pisatin

In addition to the question "which metabolites of the fungus are involved in stimulating pisatin synthesis?", there is also the question "how do these metabolites achieve their effect?". One possible mechanism which could be involved is the induced synthesis of a new enzyme(s) which is not present in the healthy tissue but which is essential for pisatin synthesis. The synthesis of new enzymes in higher plants, as a result of an external stimulus, has been reported on several occasions, e.g. nitrate and nitrite reductase in the presence of their respective substrates (Ingle, Joy and Hagemann, 1966), and β-amylase in the presence of gibberellic acid (Varner, 1964). Many more reports describe an effect which is mediated through new protein synthesis e.g. the phytochrome mediated synthesis of anthocyanin (Lange and Mohr, 1965), the red light-induced gibberellin synthesis (Reid and Clements, 1968) and the many auxin mediated effects discussed in the introduction.

Much of this work has relied on the results obtained using inhibitors of protein synthesis and direct evidence of de novo protein synthesis is usually lacking.

The following compounds were tested for their ability to affect pisatin production by pea tissues stimulated by the presence of a culture filtrate of Penicillium expansum, threo-chloramphenicol, p-fluorophenylalanine, puromycin hydrochloride and cycloheximide. The culture filtrate was used as a natural inducing system, in preference, for example, to a solution of a metallic ion. The use of an inducing solution, rather

than a spore suspension, was essential so that there was no interaction between the inhibitors and the fungal spores. Any variations which might be attributable to differences in spore germination and growth or to possible variations in spore concentration are also avoided. A final important factor, in addition to the latter ones described above, which is particularly relevant to the work on pisatin production by senescing leaf discs to be reported in section IV, is that the sterile culture filtrate can be stored for several days. I have assumed, although not demonstrated, that the ability of the filtrate to induce pisatin formation is not impaired by storage at low temperatures in the dark.

The solutions of the inhibitors were prepared both in the culture filtrate and in distilled water. The concentration of pisatin produced by pea leaf discs in the presence of these solutions was determined using the original "drop-diffusate technique". The results of the experiments with these inhibitors are shown in Tables 4.20, 4.21, 4.22 and 4.23 and are interpreted later.

Experiment 15. <u>The effect of chloramphenicol on the production of pisatin by pea leaf discs.</u>

Chloramphenicol (CA) was tested at 2×10^{-2} M., 2×10^{-3} M. and 2×10^{-4} M. The highest concentration was achieved by dissolving it in 30% ethanol and testing the resulting solution. The lower concentrations were obtained by dissolving 0.323 g. of CA. in 2.5 ml. of ethanol, placing 0.5 ml. of this solution into an Erlenmeyer flask, removing the ethanol under vacuum and dissolving the residue in 50 ml. of distilled water or culture filtrate to produce a final concentration of 2×10^{-3} M. 0.1 ml.

of the ethanolic solution was placed in another Erlenmeyer flask, the ethanol removed under vacuum and redissolved in 100 ml. of distilled water or culture filtrate to produce a final concentration of 2×10^{-4} M.

The results of two such experiments are shown in Table 4.20.

Table 4.20 Concentration of pisatin in diffusates from pea leaf discs

Treatment	Pisatin concentration µg./ml.	
	Experiment I (20 hours)	Experiment II (26 hours)
Culture filtrate	59.1	30.7
2×10^{-4} M CA in culture filtrate	52.1	28.5
2×10^{-3} M " " " "	39.8	19.3
2×10^{-2} M " " " "	< 1.0	-
Distilled water	< 1.0	-
2×10^{-4} M CA in distilled water	< 1.0	-
2×10^{-3} M " " " "	2.4	-
2×10^{-2} M " " " "	< 1.0	-

Experiment 16. The effect of p-fluorophenylalanine on the production of pisatin by pea leaf discs

p-Fluorophenylalanine was tested at 4 x 10^{-2}M., 4 x 10^{-3} M. and 4 x 10^{-4}M. All the solutions were prepared by diluting a stock solution of p-fluorophenylalanine at a concentration of 4 x 10^{-2} M which had been prepared by dissolving 0.165 g. in 25 ml. of either distilled water or culture filtrate.

The results are shown in Table 4.21.

Table 4.21 Concentration of pisatin in diffusates from pea leaf discs

Treatment	Pisatin concentration µg./ml.	
	Experiment I (26 hours)	Experiment II (26 hours)
Culture filtrate	27.2	30.7
4 x 10^{-4} M FA in culture filtrate	37.6	18.4
4 x 10^{-3} M FA " " "	17.5	16.2
4 x 10^{-2} M FA " " "	<1.0	7.9
Distilled water	<1.0	-
4 x 10^{-4} M FA in distilled water	<1.0	-
4 x 10^{-3} M FA " " "	<1.0	-
4 x 10^{-2} M FA " " "	<1.0	-

Experiment 17. The effect of puromycin on the production of pisatin by pea leaf discs

Puromycin was tested at 10^{-3} M and 10^{-4} M. It was dissolved directly in both distilled water and in the culture filtrate.

The results are shown in Table 4.22.

Table 4.22 Concentration of pisatin in diffusates from pea leaf discs

Treatment	Pisatin concentration µg./ml.	
	Experiment I (28 hours)	Experiment II (26 hours)
Culture filtrate	52.6	30.7
10^{-4} M Pu. in culture filtrate	65.3	34.2
10^{-3} M Pu. in culture filtrate	70.5	51.7
Distilled water	< 1.0	-
10^{-4} M Pu. in distilled water	16.6	-
10^{-3} M Pu. " " "	21.5	-

Experiment 18. The effect of cycloheximide on the production of pisatin by pea leaf discs

Cycloheximide was tested at 1, 10 and 100 mg./l. Stock solutions of cycloheximide in both distilled water and in the culture filtrate were prepared at 100 mg./l. by dissolving 10 mg. of cycloheximide directly in 100 ml. of either distilled water or the culture filtrate. The lower concentrations were prepared by diluting the stock solution.

The results are shown in Table 4.23.

Table 4.23 Concentration of pisatin in diffusates obtained from pea leaf discs

Treatment	Pisatin concentration µg./ml.	
	Experiment I (48 hours)	Experiment II (44 hours)
Culture filtrate	96.4	62.2
1 mg./l. Cycloheximide in culture filtrate	69.2	43.4
10 mg./l. " " " " "	14.5	4.8
100 mg./l. " " " " "	1.8	<1.0
Distilled water	12.7	11.8
1 mg./l. Cycloheximide in distilled water	29.8	28.9
10 mg./l. " " " " "	31.5	14.5
100 mg./l. " " " " "	2.2	<1.0

Discussion

It has been demonstrated that these inhibitors of protein synthesis show differing effects on the formation of pisatin.

Chloramphenicol. Chloramphenicol slightly reduced the amount of pisatin formed when added to the culture filtrate at relatively low concentrations. (The complete inhibition obtained using 2×10^{-2} M chloramphenicol cannot be attributed to an effect of the inhibitor. The concentration of ethanol required to maintain the chloramphenicol in solution also completely inhibited the synthesis of pisatin). Chloramphenicol is believed to inhibit protein synthesis of bacterial cells by preventing the function of the messenger RNA by successfully competing for ribosomal binding sites, and thus preventing the attachment of RNA to ribosomes (Weisberg and Wolfe, 1964). In plant tissues it has been shown to inhibit protein synthesis in the chloroplasts (Spencer, 1965), but protein synthesis in the cytoplasm of photosynthetic cells seems comparatively insensitive (Eisenstadt and Brawerman, 1964). Schrader, Beevers and Hagemann (1967) have reported that chloramphenicol inhibited the induced synthesis of nitrite reductase which occurred in the chloroplast, but slightly stimulated the synthesis of nitrate reductase which occurred in the cytoplasm. At high concentrations (> 1000 mg./l.) chloramphenicol was shown to inhibit energy-linked processes in maize mitochondria by acting in a similar way to 2,4-dinitrophenol, i.e. as an uncoupling agent.

p-Fluorophenylalanine. p-Fluorophenylalanine was shown to inhibit markedly the production of pisatin. p-Fluorophenylalanine is a synthetic amino acid analogue which has been shown to inhibit the synthesis of

functional enzymes. It is an analogue of phenylalanine and is believed to be incorporated, in place of phenylalanine, into the protein formed, with the result that an abnormal inactive molecule is produced. Bacillus subtilis was shown to incorporate p-fluorophenylalanine into α-amylase (Yoshida, 1960). p-Fluorophenylalanine has been reported to inhibit several plant processes. The growth of mung bean seeds was inhibited by p-fluorophenylalanine, but it was not possible to demonstrate its incorporation into the bean tissues (Fowden, 1963).

In common with the other amino acid analogues, there is the possibility that p-fluorophenylalanine may replace phenylalanine in other systems apart from protein synthesis. This is particularly relevant in this case as it is believed that phenylalanine plays a major role in the synthesis of pisatin. Hadwiger (1966) has shown that phenylalanine is readily incorporated into pisatin. Thus the inhibitory effect of p-fluorophenylalanine on pisatin formation may well be the result of its being incorporated in place of phenylalanine, but whether this is an effect on the synthesis of new enzymes or on the actual synthesis of pisatin is not known, but is worthy of further investigation.

Puromycin. In contrast to the other compounds tested, puromycin increased the amount of pisatin formed when applied in the presence of a culture filtrate. It also induced the formation of small amounts of pisatin when applied in distilled water. Puromycin is believed to inhibit protein synthesis by acting as a structural analogue of aminoacyl s-RNA, which has been shown to be the activated intermediate of protein synthesis (Yarmolinsky, 1959). By substituting for the amino acid end of amino acyl s-RNA, polypeptide formation is prevented. A secondary effect

which has been observed to occur is the release of amino acids and polypeptides.

Cycloheximide. Cycloheximide was shown, like patulin, to have two opposing effects. Firstly, in the presence of the culture filtrate, the production of pisatin was markedly decreased. Secondly, in distilled water low concentrations induced the formation of small amounts of pisatin, although the highest concentration tested was inhibitory as in the culture filtrate.

Cycloheximide has been shown to inhibit protein synthesis. The mechanisms involved seem various; Fiala and Davis (1965) indicated that the synthesis and methylation of ribosomal RNA was inhibited; Siegel and Sisler (1965) suggested that it inhibits the transfer of amino acids from s-RNA to proteins; Bennett, Smithers and Ward (1964) concluded that cycloheximide inhibited both DNA and protein synthesis. Cycloheximide has been shown to inhibit the growth of Chlorella. Although DNA, RNA and protein synthesis were all inhibited to some degree, it was concluded that the primary effect of cycloheximide was to inhibit protein synthesis (Morris, 1967).

Conclusions

Any precise conclusions from this work are impossible. The effects of the various inhibitors of protein synthesis on pisatin formation were complex and different from one another. Chloramphenicol had only a slight inhibitory effect. p-Fluorophenylalanine inhibited the formation of pisatin, but this could be explained in terms of incorporation into the pisatin molecule. Puromycin was shown not to inhibit pisatin formation, but in fact to increase the amount of pisatin formed by the culture filtrate and to induce the formation of pisatin by itself. Cycloheximide reduced the amount of pisatin produced by the culture filtrate, though by itself it also had inducing activity. These results are partly, but only partly, consistant with the view that de novo protein synthesis is required for the production of pisatin.

Pisatin formation was stimulated by both puromycin and cycloheximide when dissolved in distilled water. This suggests, paradoxically, that the inhibition of enzyme synthesis causes the stimulation of pisatin synthesis. Hadwiger (1967) has suggested that pisatin is formed as a result of the stimulation of an auxiliary pathway which is not operative under normal conditions. The stimulation of this pathway causes the accumulation of pisatin, the end-product, to occur. In healthy tissue, the rapid turnover of an enzyme may be essential for normal metabolism, i.e. no pisatin formation. If, however, the synthesis of this enzyme is prevented, the substrate of the enzyme could accumulate and this may then activate the pathway required for pisatin synthesis.

Section IV. The effect of senescence on the pisatin production of pea leaves

As indicated in the introduction, various treatments when applied to plants can be shown to affect their resistance to fungal invasion. In some cases this was correlated with phytoalexin production. The natural resistance of a host to fungal invasion varies greatly. During senescence, for example, host resistance declines progressively with the gradual approach of the death of the tissue. This is well shown by the existence of fungi which, although quite unable to invade the young vigorously growing tissues of a plant, are well able to invade senescing tissues of the same plant.

Botrytis cinerea is highly efficient at invading senescent tissues; Brooks (1908) found that spores of B. cinerea were unable to infect when sown on healthy green lettuce leaves. Nevertheless, if these leaves were maintained with the spores in situ until the leaves turned yellow, invasion by B. cinerea occurred. It has been shown that inoculations with a pathogenic species of Botrytis on to older and moribund leaves of Vicia faba produced vigorous lesions more readily than inoculations on to young leaves (Wilson, 1937). More recently, Leach (1955) has shown that, although infection of leaves of V. faba occurred during the winter, these infected regions only sporulated when the leaves senesced the following summer. The spores of B. fabae formed on the senescing leaves were able to produce aggressive lesions only on similarly senescent leaves. Moore and Leach (1968) have obtained more evidence of the close association between the state of senescence of a leaf and its susceptibility to aggressive infection by B. fabae.

It has been reported that, although young leaves of pea are readily invested with propagules of several saprophytic fungi, they do not become invaded at this early stage. However, with the onset of senescence and death, these fungi become very active and play a large part in the breakdown of pea tissues (Dickinson, 1967).

The fact that the onset of senescence of a tissue is often associated with an increase in susceptibility to facultative pathogens would indicate that changes occur in the leaf which are responsible for this alteration in resistance to fungal invasion.

The aim of the work to be described here was to investigate the senescence of pea leaf discs and to investigate whether this could be controlled; and, if so, to determine how pisatin production was affected.

Experiment 19. The effect of various plant growth hormones on the senescence of pea leaf discs

The leaf discs from leaves of various ages were found to senesce at different rates. Discs cut from fully expanded leaves produced at the 3rd - 7th node underwent senescence within 10 days. Discs from leaves produced at nodes above this region did not always senesce. Such discs were shown to maintain their turgidity and chlorophyll content after they had been floated on distilled water for more than fourteen days. It will be noticed in the experiments to be reported later that the rate of senescence varied greatly from one experiment to the next. It was thus found to be impossible to compare directly the various experiments.

Senescence of the leaf discs was achieved by floating twenty discs on 20 ml. of distilled water in 9 cm. petri-dishes. After a suitable time, the discs were removed and their chlorophyll content was measured.

Table 4.24. The effect of plant growth hormones on the chlorophyll content of pea leaf discs

Treatment	Chlorophyll content O.D.665 mμ.	't'	'p'	Chlorophyll content % of original value
Indolylacetic acid (IAA)				
Original value	0.41	-	-	100
Distilled water	0.065	-	-	15.8
2.5 mg./l. IAA	0.064	0.08	0.9	15.6
25 mg./l. IAA	0.069	0.35	0.7	16.8
50 mg./l. IAA	0.059	0.78	0.4	14.4
Benzyladenine (BA)				
Original value	0.36	-	-	100
Distilled water	0.069	-	-	18.9
2.5 mg./l. BA	0.19	0.76	0.001	51.6
25 mg./l. BA	0.17	0.74	0.001	46.4
50 mg./l. BA	0.10	3.50	0.005	27.5
Gibberellic acid (GA_3)				
Original value	0.40	-	-	100
Distilled water	0.10	-	-	23.9
2.5 mg./l. GA_3	0.09	0.35	0.7	23.8
25 mg./l. GA_3	0.12 *	1.73	0.5	30.5
50 mg./l. GA_3	0.16 *	2.99	0.005	39.6
Kinetin (K)				
Original value	0.33	-	-	100
Distilled water	0.063	-	-	20.1
5 mg./l. K	0.16	6.52	0.001	51.5
10 mg./l. K	0.14	4.90	0.001	43.8
50 mg./l. K	0.11	5.19	0.001	34.6
Benzimidazole (Bz)				
Original value	0.33	-	-	100
Distilled water	0.081	-	-	24.8
5 mg./l. Bz	0.077	0.27	0.7	23.6
50 mg./l. Bz	0.082	0.33	0.7	25.0
100 mg./l. Bz	0.094	0.37	0.7	28.7

* indicates a loss of turgidity.

The effect of various plant growth hormones was tested by floating similar discs on a solution of the required hormone and by comparing the chlorophyll content of the discs floated on distilled water with that of those floated on the growth hormone.

When designing the experiments, if the number of treatments was low, one disc from each leaf was placed on each solution, i.e. when the effect of benzyladenine was tested at three concentrations, leaves were chosen as described in Chapter 2 so that five discs could be cut from each leaf. One disc was used to measure the chlorophyll content of the original sample; another was floated on water to allow normal senescence to occur and the remaining three discs were floated on the three different concentrations of benzyladenine. Twenty such discs were used for each treatment. The leaf discs were incubated for 100 hours. The effects of benzyladenine, benzimidazole, kinetin, indolyl-acetic acid and gibberellic acid are shown in Table 4.24.

Experiment 20. The interaction of gibberellic acid with indolylacetic acid and 2,4-dichlorophenoxy acetic acid on the senescence of pea leaf discs

The effect of gibberellic acid, 10 mg./l., with or without indolylacetic acid, 1 or 10 mg./l., or 2,4-dichlorophenoxyacetic acid, 1 or 10 mg./l. on the senescence of pea leaf discs was investigated. The results are shown in Table 4.25.

Table 4.25. Chlorophyll content of pea leaf discs after being treated with various hormones for 96 hours

Growth hormone	Time Hours	Chlorophyll content Mean O.D. 665 mμ.	
		Distilled water	Gibberellic acid
Original value	0	0.62 ± 0.012	-
1 mg./l. IAA	96	0.17 ± 0.0079	0.18 ± 0.014
10 mg./l. IAA	96	0.19 ± 0.0089	0.17 ± 0.0089
1 mg./l. 2,4-D	96	0.21 ± 0.010	0.17 ± 0.0090
10 mg./l. 2,4-D	96	0.21 ± 0.0097	0.24 ± 0.0095
Distilled water	96	0.18 ± 0.012	0.22 ± 0.018

No major effect on the senescence of leaf discs was demonstrated. In a similar experiment no effect on senescence was observed using gibberellic acid at concentrations from 1000 - 0.01 mg./l.

Table 4.26. Chlorophyll content of pea leaf discs treated with benzyladenine or benzimidazole for 144 hours

Treatment	Chlorophyll content Mean O.D. 665 mμ.	Chlorophyll content % of original value
Original value	0.48 ± 0.010	100
Distilled water	0.21 ± 0.003	44
0.1 mg./l. BA	0.21 ± 0.0072	44
1.0 mg./l. BA	0.26 ± 0.0097 *	54
20 mg./l. BA	0.38 ± 0.0092 *	79
10 mg./l. BA	0.18 ± 0.0060 *	38
Distilled water	0.23 ± 0.0087	48
1 mg./l. Bz	0.25 ± 0.0148	52
10 mg./l. Bz	0.19 ± 0.0062	39
100 mg./l. Bz	0.24 ± 0.010 ϕ	50

* significant difference ($P < 0.001$) when compared with chlorophyll contents of discs floated on distilled water.

ϕ indicates loss of turgidity.

Experiment 21. To determine the effect of benzyladenine on the rate of senescence of pea leaf discs

It has been shown in Experiment 19 that the senescence of pea leaf discs was only delayed by treatment with the cytokinins, benzyladenine and kinetin. Both kinins are sparingly soluble in water and thus it was necessary to determine how solutions of these compounds could be obtained. Kinetin is very soluble in small volumes of concentrated hydrochloric acid and this can be neutralised with sodium hydroxide and diluted with distilled water to yield the concentrations required. However, due to the lack of a buffer, it was found difficult to standardize the amounts of acid and alkali required to produce a neutral solution. As a result, benzyladenine, which has been shown by Leopold and Kawase (1964) to be soluble in propylene glycol, was used in the following experiments. Benzyladenine was dissolved by warming in a minimum of propylene glycol and diluted to the required concentrations with distilled water. Propylene glycol, at the concentrations used, was shown to have no effect on the senescence of pea leaf discs. The effect of benzyladenine and benzimidazole, which is readily soluble in water, on the senescence of pea leaf discs is shown in Table 4.26.

In this experiment, as in Experiment 19, 2 mg./l. of benzyladenine was most efficient in delaying the senescence of pea leaf discs. Above a concentration of 2 mg./l. the benzyladenine is less able to delay senescence. It was demonstrated in this experiment that 10 mg./l. benzyladenine actually hastened the senescence of the leaf discs. It was confirmed that benzimidazole is unable to delay the senescence of pea leaf discs.

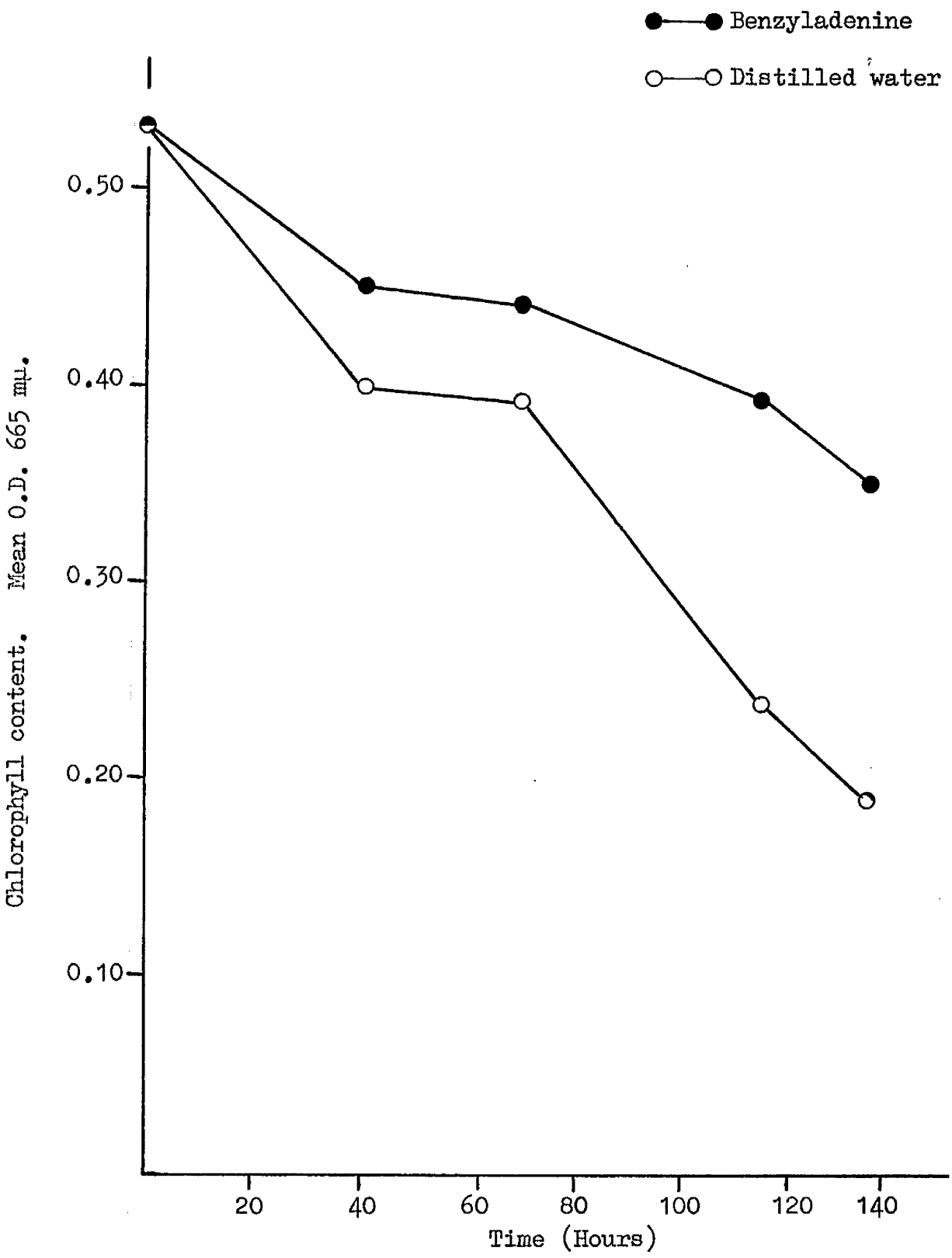


Fig. 4.9. The effect of benzyladenine on the senescence of pea leaf discs.

Experiment 22. The effect of benzyladenine on the rate of senescence of pea leaf discs

As a result of the information gained from the previous experiments, benzyladenine, 2 mg./l., was used to delay the senescence of pea leaf discs. (2 mg. of benzyladenine were dissolved in 1 ml. of propylene glycol and this solution was diluted to 1000 ml. with distilled water. Propylene glycol, 0.1% in distilled water, was shown to have no effect on the senescence of pea leaf discs.). The data shown in Table 4.27 are the results of experiments in which leaf discs were placed at random on either distilled water or benzyladenine. After various periods of time the chlorophyll content of the discs was measured. For example, in Experiment 1 five petri-dishes, each containing 20 discs, were prepared. The chlorophyll contents of the discs from one dish were measured immediately. The other dishes contained either distilled water or benzyladenine. The chlorophyll content of these discs was measured after 48 hours or 96 hours. The results are shown in Table 4.27 and expressed graphically in Fig. 4.9.

In all three experiments the discs which had been floated on benzyladenine senesced at a slower rate. Benzyladenine was not shown to prevent senescence.

Table 4.27 Chlorophyll content of pea leaf discs floated on distilled water or on 2 mg./l. benzyladenine

Age of discs	Experiment I		Experiment II		Experiment III	
Hours	BA	Dist. water	BA	Dist. water	BA	Dist. water
0	-	0.65		0.42 ± 0.012	0.53 ± 0.0098	0.53 ± 0.0073
24	-	-	0.38 ± 0.013	0.29 ± 0.012		
41	-	-			0.45 ± 0.0057	0.40 ± 0.0076
48	0.51	0.37				
69	-	-	0.29 ± 0.012	0.18 ± 0.014	0.44 ± 0.0086	0.39 ± 0.0063
96	0.31	0.20				
114	-	-			0.39 ± 0.0075	0.24 ± 0.0096
135	-	-			0.35 ± 0.0089	0.19 ± 0.0049

The mode of action of benzyladenine

As was discussed in the introduction, it has been suggested that compounds which delay senescence, do so as a result of their ability to stimulate protein synthesis. One would expect that compounds which inhibit protein synthesis would prevent the stimulation of protein synthesis and as a result senescence would not be delayed. The effect of p-fluorophenylalanine, chloramphenicol, puromycin and cycloheximide on the ability of benzyladenine to delay senescence of pea leaf discs was investigated. The results are shown in Tables 4.28, 4.29, 4.30 and 4.31.

Experiment 23. The effect of p-fluorophenylalanine on the ability of benzyladenine to delay the senescence of pea leaf discs

Table 4.28 Chlorophyll content of pea leaf discs after 96 hours

Treatment	Chlorophyll content Mean O.D. 665 mμ.
Original value	0.63 ± 0.0072
Distilled water	0.24 ± 0.0055
2 mg./l. BA	0.37 ± 0.0142
2 mg./l. BA + 2 x 10^{-3} M FPA	0.39 ± 0.0133
2 mg./l. BA + 2 x 10^{-2} M FPA	0.43 ± 0.0080
Distilled water + 2 x 10^{-3} M FPA	0.29 ± 0.0078
Distilled water + 2 x 10^{-2} M FPA	0.36 ± 0.0073

Experiment 24. The effect of chloramphenicol on the ability of benzyladenine to delay the senescence of pea leaf discs

Table 4.29 Chlorophyll content of pea leaf discs after 120 hours

Treatment	Chlorophyll content Mean O.D. 665 mμ.
Original value	0.62 ± 0.0078
Distilled water	0.27 ± 0.0061
2 mg./l. BA	0.48 ± 0.0115
2 mg./l. BA + 30 mg./l. CA	0.45 ± 0.0081
2 mg./l. BA + 300 mg./l. CA	0.44 ± 0.0096
Distilled water + 30 mg./l. CA	0.16 ± 0.0037
Distilled water + 300 mg./l. CA	0.24 ± 0.0085

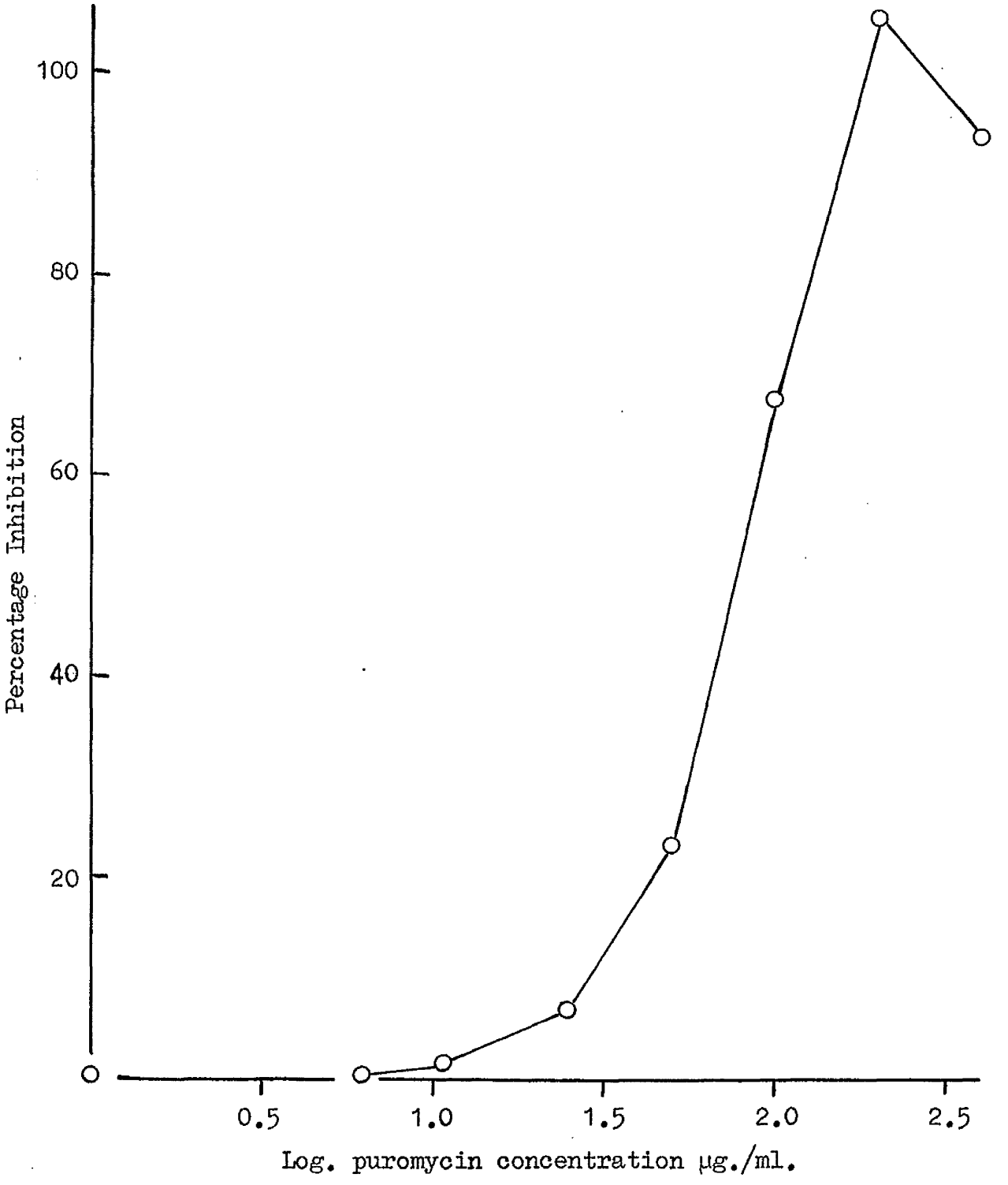


Fig. 4.10 The effect of puromycin on the ability of benzyladenine to delay the senescence of pea leaf discs.

Experiment 25. The effect of puromycin on the ability of benzyladenine to delay the senescence of pea leaf discs

The results are expressed graphically in Fig. 4.10. ø

Table 4.30. Chlorophyll content of pea leaf discs

Treatment	Chlorophyll content Experiment I (96 hrs)	Mean O.D. 665 mμ. Experiment II (120 hrs)
Original value	0.56 ± 0.0067	0.56 ± 0.0091
2 mg./l. BA	0.37 ± 0.0052	0.34 ± 0.0079
Distilled water	0.22 ± 0.0037	0.19 ± 0.0038
Distilled water + 50 mg./l. Pu.	0.21 ± 0.0043	
2 mg./l. BA + 6.25 mg./l. Pu.		0.34 ± 0.0076
2 mg./l. BA + 12.5 mg./l. Pu.		0.34 ± 0.0099
2 mg./l. BA + 25 mg./l. Pu.		0.33 ± 0.0101
2 mg./l. BA + 50 mg./l. Pu.	0.33 ± 0.0051 *	0.31 ± 0.0092 *
2 mg./l. BA + 100 mg./l. Pu.		0.24 ± 0.0074 *
2 mg./l. BA + 200 mg./l. Pu.		0.18 ± 0.0051 *
2 mg./l. BA + 400 mg./l. Pu.		0.20 ± 0.0077 *

* significant difference ($P < 0.001$) when compared with chlorophyll contents of leaf discs on benzyladenine alone.

ø Percentage inhibition was calculated:-

$$\frac{[\text{Increase in chlorophyll content due to BA}] - [\text{Increase due to BA + Pu.}]}{\text{Increase in chlorophyll content due to BA}} \times 100$$

Experiment 26. The effect of cycloheximide on the ability of benzyladenine to delay the senescence of pea leaf discs

Table 4.31 Chlorophyll content of pea leaf discs after 120 hours

Treatment	Chlorophyll content Mean O.D. 665 mμ.
Original value	0.46 ± 0.0079
2 mg./l. BA	0.44 ± 0.0063
Distilled water	0.33 ± 0.0084
2 mg./l. BA + 1 mg./l. cycloheximide	0.34 ± 0.0073 *
Distilled water + 1 mg./l. cycloheximide	0.24 ± 0.0038

* significant difference ($P < 0.001$) when compared with the chlorophyll content of leaf discs on BA alone.

Conclusions

p-Fluorophenylalanine and chloramphenicol were shown to have little effect on the ability of benzyladenine to delay the senescence of pea leaf discs. Both puromycin and cycloheximide were found to inhibit markedly the action of benzyladenine. The effects of puromycin and cycloheximide would support the view that benzyladenine delays senescence by stimulating protein synthesis and that, if protein synthesis is prevented, i.e. in the presence of these inhibitors, benzyladenine is unable to have any effect.

It has been shown that pea leaf discs floating on water senesce. The rate of senescence can be considerably reduced, but not prevented, by floating these discs on benzyladenine. The following experiments are an attempt to study the ability of leaves which have been allowed to senesce, to produce pisatin and to investigate the differences, if any, between the amount of pisatin produced by leaves which have been floated on water and those which were floated on benzyladenine.

The production of pisatin by leaf discs was induced by a culture filtrate of Penicillium expansum (See Section 2). The filtrate was maintained frozen at -2°C under sterile conditions throughout the time of each experiment. Benzyladenine was used at a concentration of 2 mg./l., fresh solutions being prepared on each occasion. The total number of leaf discs which were required for each experiment, were cut and bulked together. A series of the different treatments, (one petri-dish per treatment), was set up at the same time. Discs were placed individually into each petri-dish of the series until there were twenty discs in each petri-dish. The procedure was repeated for the remaining series of treatments until enough discs were available to ensure that sufficient diffusate could be obtained. The number of series required differed, depending on which "drop diffusate technique" was used. The original technique required twelve series (i.e. 240 discs) for each treatment to ensure that sufficient diffusate was produced, whereas the modified technique, which was used in all but a few experiments, necessitated the use of only six dishes (i.e. 120 discs). (See Chapter 2). An additional series was set up in exactly the same way and was used to

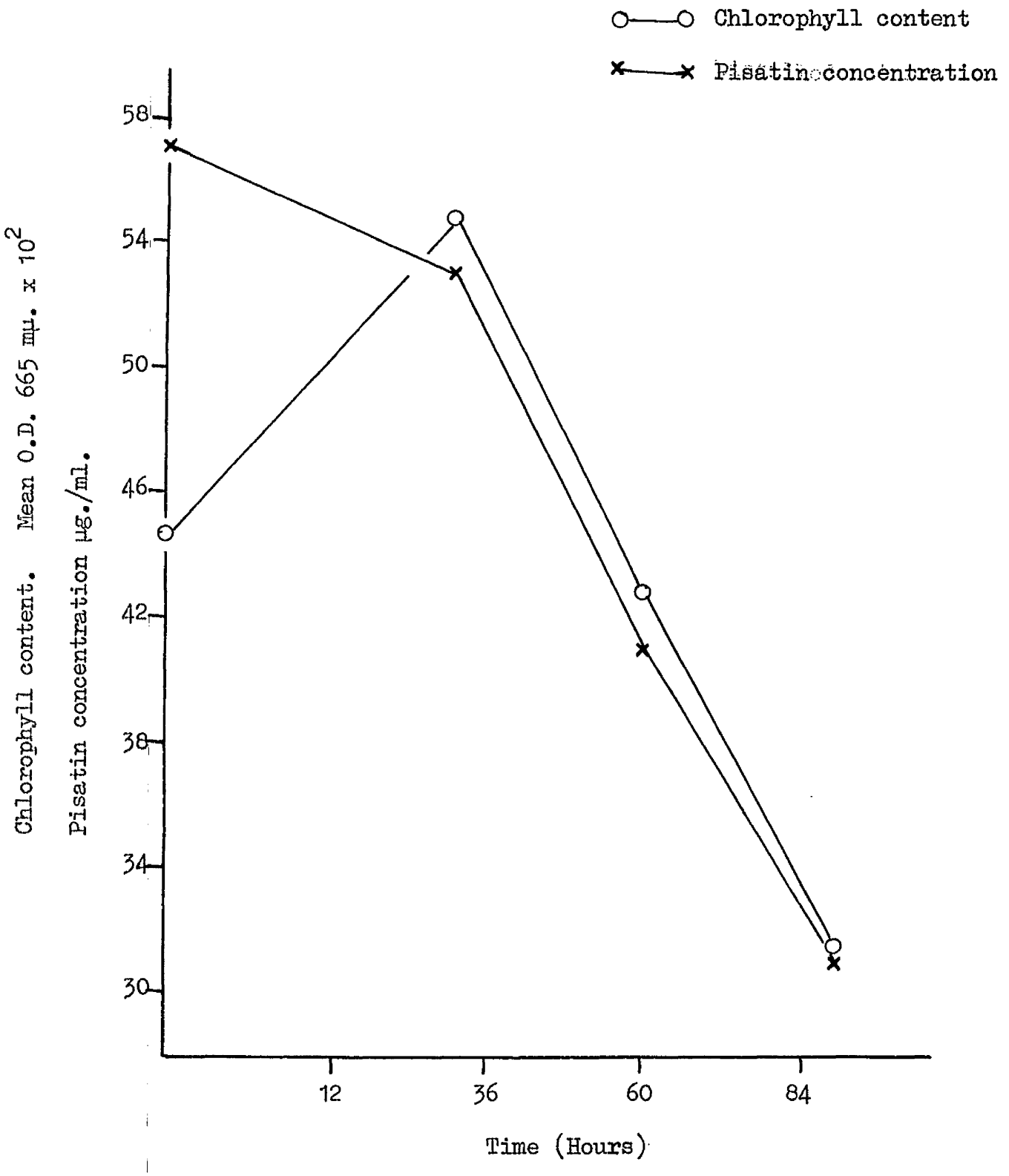


Fig. 4.11. Pisatin production by senescing pea leaves.

determine the chlorophyll content of the leaf discs at the time when they were also assayed for pisatin production.

Experiment 27. The production of pisatin by leaf discs cut from pea leaves which had been allowed to senesce with their petioles in water

Leaves of pea were removed from the sixth node of plants grown outside. 40 such leaves, each with two leaflets, were selected visually for uniformity of size and healthiness. They were placed with their petioles in 20 ml. of distilled water in 25 ml. Erlenmeyer flasks and divided into four samples of ten leaves each. One sample was assayed for chlorophyll content and pisatin production, and the remainder were incubated in the dark at 24°C. They were assayed after 36 hours, 60 hours and 84 hours. The design of the experiment was as follows: from each leaflet of a sample, six discs were removed; five were used to assay pisatin production and the other for chlorophyll content. This was repeated for the ten leaves, i.e. twenty leaflets, thus yielding 100 discs for the assay of pisatin production and twenty discs for the assay of chlorophyll content. The concentration of pisatin in the diffusates was measured after 48 hours. The results are shown in Table 4.32, and expressed graphically in Fig. 4.11.

The results clearly show that when the leaves senesce, as shown by the decrease in chlorophyll content, the production of pisatin by discs from these leaves also decreases.

Table 4.32. Chlorophyll content of pea leaf discs and the pisatin concentration in diffusates obtained from similar discs

Treatment	Pisatin concentration µg./ml.	Chlorophyll content Mean O.D. 665 mµ.
Original value	44.7	0.57 ± 0.0105
Incubated for 36 hours	54.8	0.53 ± 0.0076
Incubated for 60 hours	42.9	0.41 ± 0.0166
Incubated for 84 hours	31.5	0.31 ± 0.0198

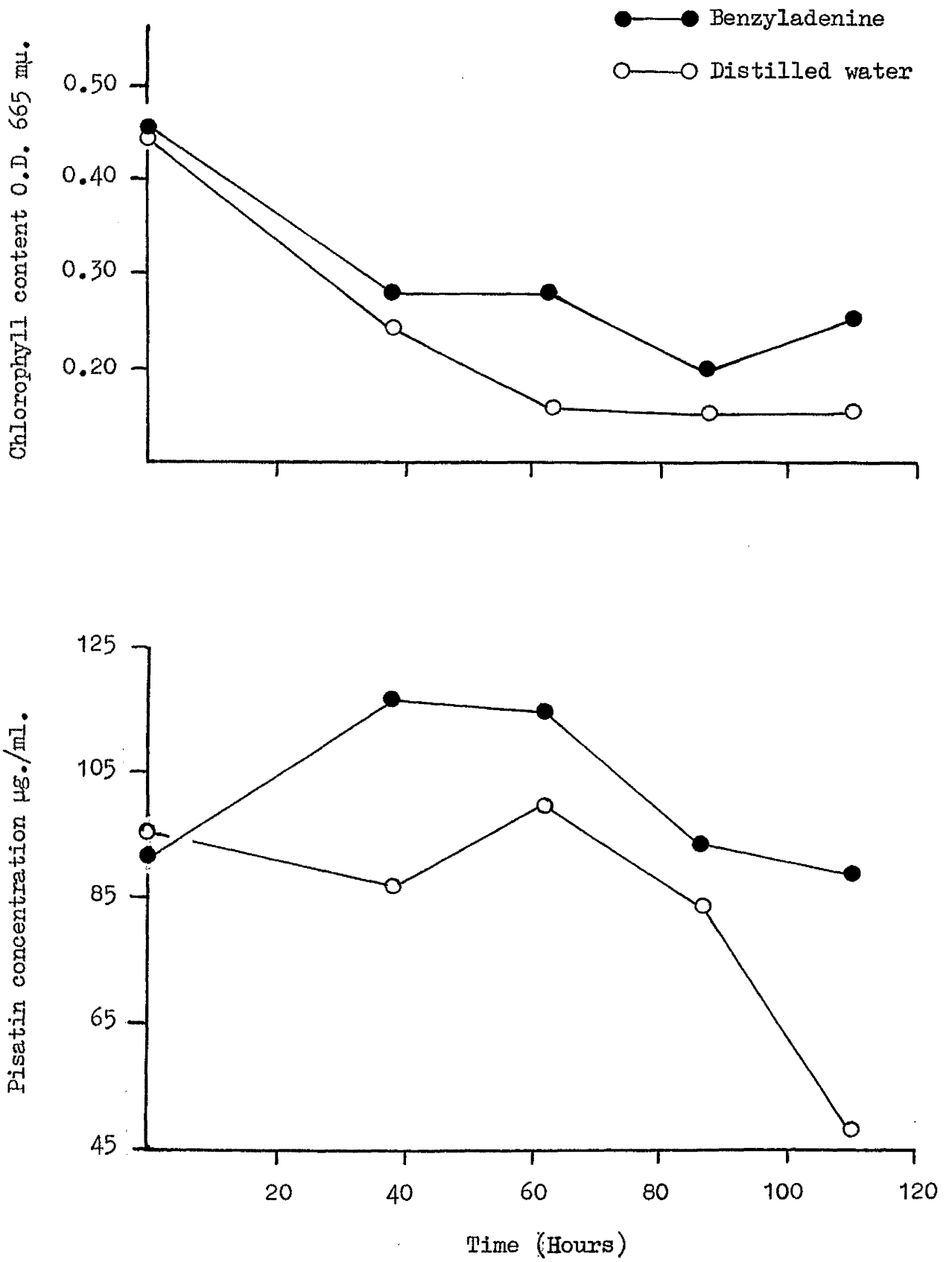


Fig. 4.12. The effect of benzyladenine on senescence and pisatin production of pea leaf discs.

Experiment 28. The production of pisatin by pea leaf discs which have been floated on distilled water or on benzyladenine

It has been shown in the previous experiment that the pisatin production of pea leaf discs cut from entire leaves which had been allowed to senesce, declined as the leaves became increasingly senescent. Experiment 22 has shown that leaf discs cut from healthy leaves will senesce when they are floated on water, and that this senescence can be delayed by floating them on benzyladenine. The following experiments were designed to investigate changes in the chlorophyll content and the pisatin production of leaf discs which were treated in these ways.

Experiment 28a.

The leaf discs were cut from leaves removed from the fourth node of plants grown outside. The discs were cut and dispensed into the various treatments at random. After the discs had been floating on either distilled water or benzyladenine for 0, 38, 62, 86 and 110 hours, they were removed, any excess liquid was absorbed on to filter paper and then they were assayed for chlorophyll content and pisatin production. Pisatin production was assayed using the original "drop diffusate technique" with a culture filtrate of Penicillium expansum. The pisatin concentration in the diffusate was measured after 42 hours. The results are shown in Table 4.33 and expressed graphically in Fig. 4.12.

Table 4.33. Chlorophyll content of pea leaf discs and the pisatin concentration in diffusates obtained from similar discs

Treatment	Pisatin concentration			Chlorophyll content O.D. 665 mμ.
	Volume of diffusate (ml)	O.D. 309 mμ.	μg./ml.	
0 hrs on 2 mg./l. BA	4.0	1.67	91.5	0.46 ± 0.0177
0 hrs on distilled water	4.0	1.76	96.4	0.45 ± 0.0178
38 hrs on 2 mg./l. BA	4.0	2.14	117.0	0.28 ± 0.0147
38 hrs on distilled water	4.0	1.60	87.7	0.24 ± 0.0183
62 hrs on 2 mg./l. BA	4.0	2.10	115.0	0.28 ± 0.0186
62 hrs on distilled water	2.5	1.15	100.8	0.16 ± 0.0155
86 hrs on 2 mg./l. BA	4.0	1.71	93.7	0.20 ± 0.0118
86 hrs on distilled water	2.0	0.72	78.9	0.14 ± 0.0182
110 hrs on 2 mg./l. BA	4.0	1.62	88.8	0.25 ± 0.0232
110 hrs on distilled water	1.0	0.22	48.2	0.15 ± 0.0127

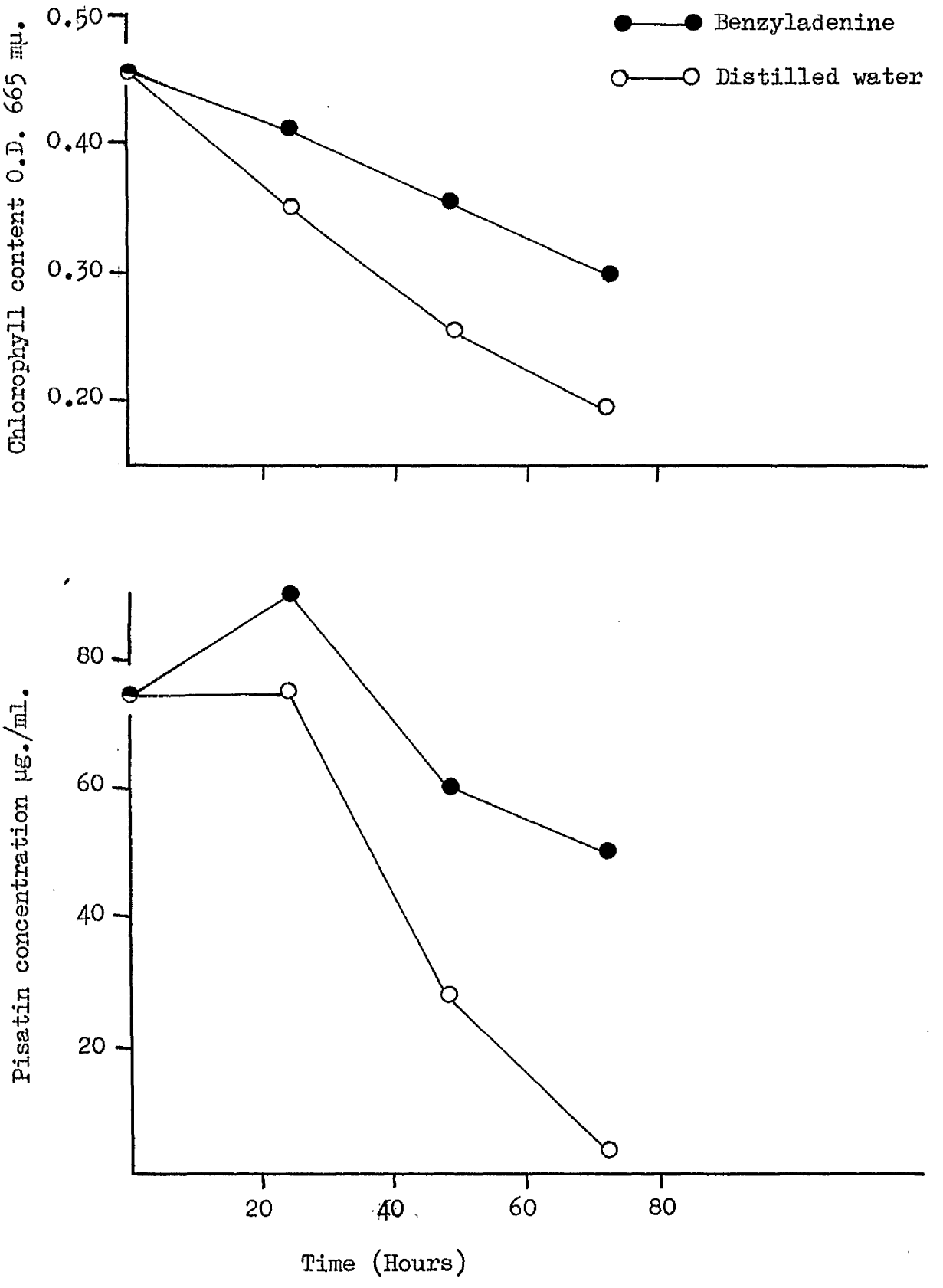


Fig. 4.13. The effect of benzyladenine on senescence and pisatin production of pea leaf discs.

Experiment 28b.

Measurements were made after 0, 24, 48 and 72 hours. The results are shown in Table 4.34 and expressed graphically in Fig. 4.13.

Table 4.34. Chlorophyll content of pea leaf discs and the concentration of pisatin in diffusates obtained from similar discs

Treatment	Pisatin concentration			Chlorophyll content O.D. 665 mμ.
	Volume of diffusate (ml)	O.D. 309 mμ.	μg./ml.	
0 hrs on distilled water	2.5	0.85	74.5	0.46 ± 0.0148
24 hrs on 2 mg./l. BA	2.0	0.83	90.8	0.42 ± 0.0160
24 hrs on distilled water	2.0	0.69	75.5	0.35 ± 0.0179
48 hrs on 2 mg./l. BA	2.5	0.69	60.4	0.36 ± 0.0144
48 hrs on distilled water	1.0	0.13	28.5	0.26 ± 0.0116
72 hrs on 2 mg./l. BA	2.0	0.46	50.4	0.30 ± 0.0171
72 hrs on distilled water	1.0	0.02	4.4	0.19 ± 0.0160

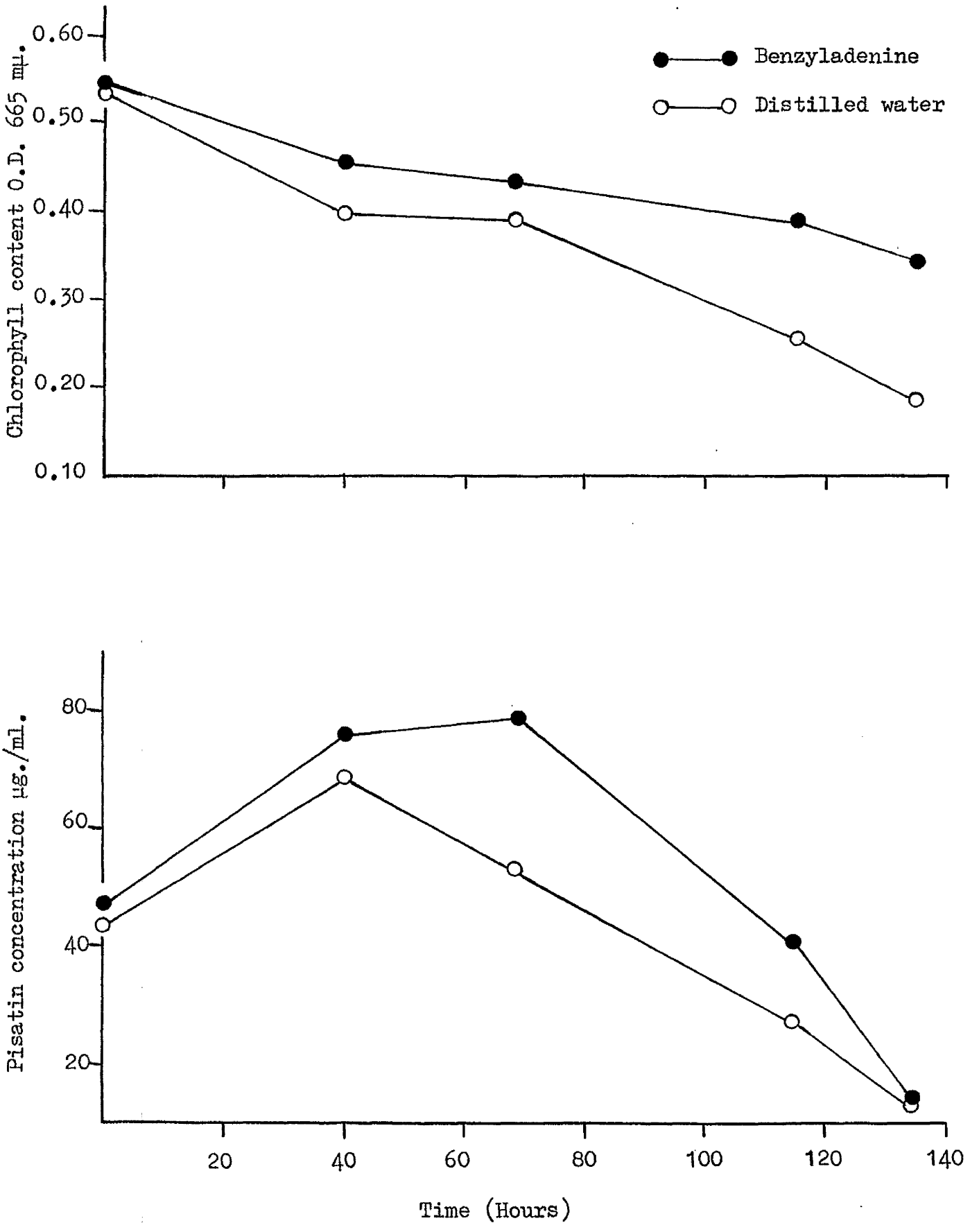


Fig. 4.14. The effect of benzyladenine on senescence and pisatin production of pea leaf discs.

A critical investigation of the effect of benzyladenine on the senescence of pea leaf discs and on the production of pisatin by these discs

The modified "drop-diffusate technique" was used in these experiments. Leaf discs were cut from leaves removed from the fifth mode of pea plants. The drop size was maintained constant throughout the experiment by using the same pasteur pipette. The volume of the drops was estimated to be approximately 0.08 ml. In the first experiment 200 discs were used to assay pisatin production. These discs yielded nearly 15 ml. of diffusate and, as a result, the number of discs used in the later experiments was reduced to 120. The pisatin concentration of diffusates was measured after 42 hours.

Experiment 29a. The production of pisatin by pea leaf discs which have been floated on distilled water or on benzyladenine

The chlorophyll content and pisatin production of the discs were measured after 0, 41, 69, 114 and 135 hours (Table 4.27). 5 ml. of diffusate were obtained from each treatment. The results are shown in Table 4.35, and expressed graphically in Fig. 4.14.

Table 4.35. Chlorophyll content of pea leaf discs and the concentration of pisatin in diffusates obtained from similar discs

Treatment	Pisatin concentration µg./ml.	Chlorophyll content Mean O.D. 665 mµ.
0 hrs on 2 mg./l. BA	47.3	0.54 ± 0.0102
0 hrs on distilled water	43.8	0.53 ± 0.0073
41 hrs on 2 mg./l. BA	76.2	0.45 ± 0.0057
41 hrs on distilled water	69.6	0.40 ± 0.0076
69 hrs on 2 mg./l. BA	79.7	0.44 ± 0.0086
69 hrs on distilled water	53.0	0.39 ± 0.0063
14 hrs on 2 mg./l. BA	41.2	0.39 ± 0.0075
14 hrs on distilled water	27.1	0.25 ± 0.0097
35 hrs on 2 mg./l. BA	14.0	0.35 ± 0.0089
35 hrs on distilled water	13.0	0.19 ± 0.0050

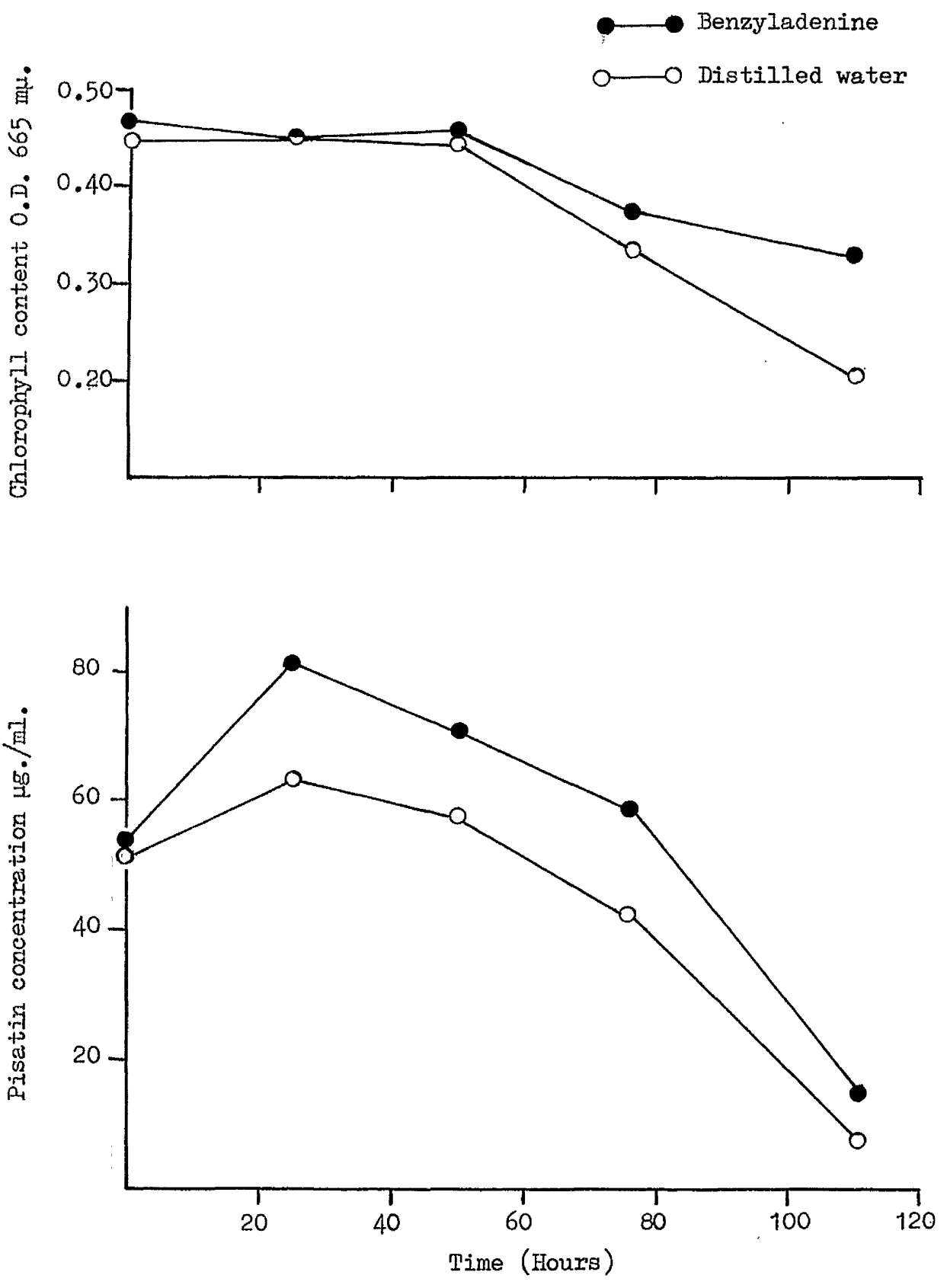


Fig. 4.15. The effect of benzyladenine on senescence and pisatin production of pea leaf discs.

Experiment 29b.

Measurements were made after 0, 25, 50, 76 and 111 hours. The results are shown in Table 4.36 and expressed graphically in Fig. 4.15.

Table 4.36. Chlorophyll content of pea leaf discs and the concentration of pisatin in diffusates obtained from similar discs

Treatment	Pisatin concentration µg./ml.	Chlorophyll content Mean O.D. 665 mµ.
0 hrs on 2 mg./l. BA	51.7	0.46 ± 0.0075
0 hrs on dist. water	53.4	0.44 ± 0.0074
25 hrs on 2 mg./l. BA	81.9	0.44 ± 0.0075
25 hrs on dist. water	63.9	0.44 ± 0.0079
50 hrs on 2 mg./l. BA	71.4	0.45 ± 0.0089
50 hrs on dist. water	58.3	0.43 ± 0.0066
76 hrs on 2 mg./l. BA	59.1	0.37 ± 0.0076
76 hrs on dist. water	43.4	0.34 ± 0.0091
111 hrs on 2 mg./l. BA	14.9	0.33 ± 0.0093
111 hrs on dist. water	8.3	0.21 ± 0.0058

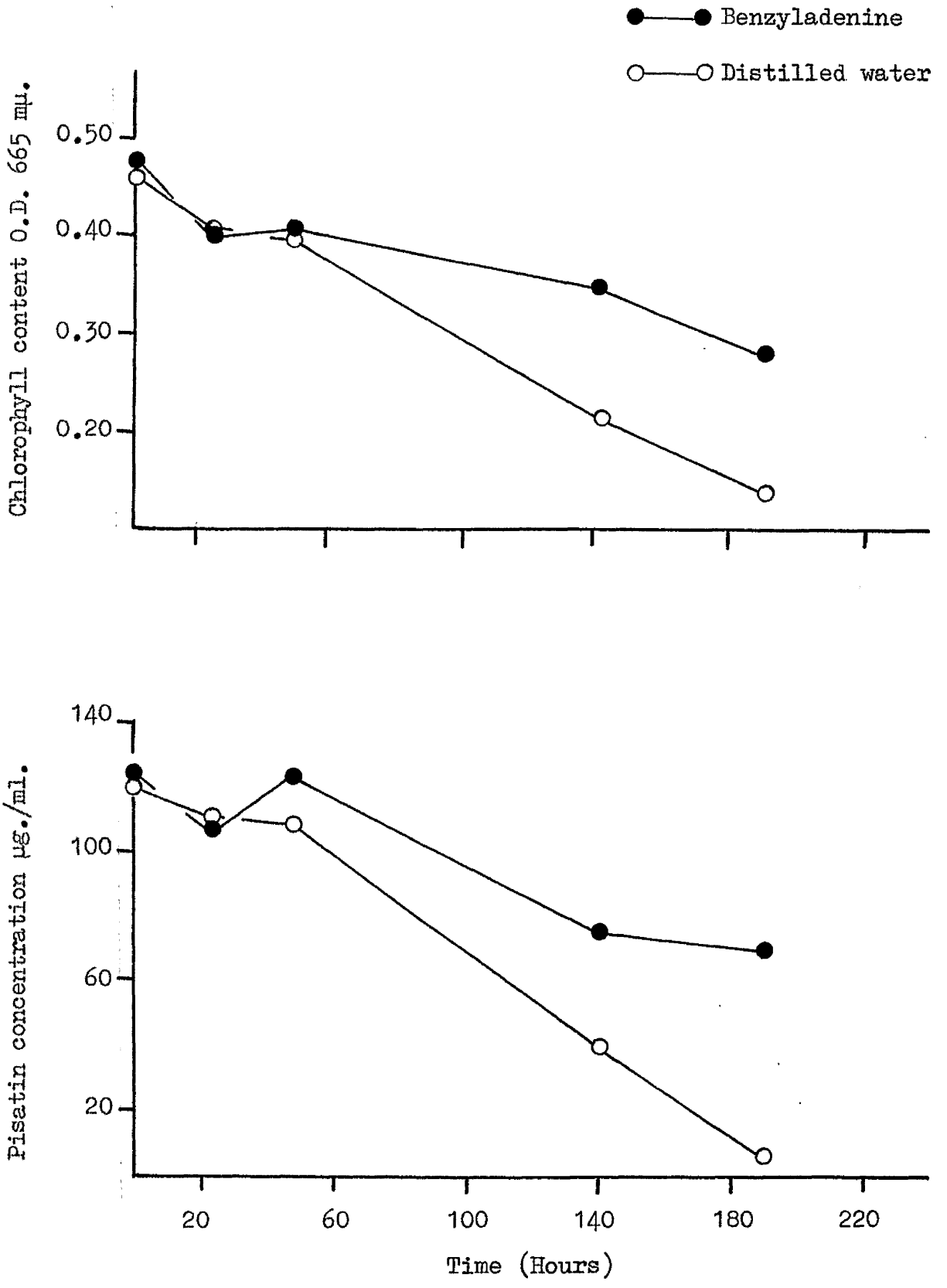


Fig. 4.16. The effect of benzyladenine on senescence and pisatin production of pea leaf discs.

Experiment 29c.

Leaf discs were obtained from plants grown under cloches during the early summer. Measurements were made after 0, 24, 48, 140 and 190 hours. The results are shown in Table 4.37 and expressed graphically in Fig. 4.16.

Table 4.37. Chlorophyll content of pea leaf discs and the pisatin concentration in diffusates obtained from similar discs

Treatment	Pisatin concentration µg./ml.	Chlorophyll content Mean O.D. 665 mµ.
0 hrs on 2 mg./l. BA	122.6	0.47 ± 0.0048
0 hrs on distilled water	120.0	0.45 ± 0.0060
24 hrs on 2 mg./l. BA	106.9	0.40 ± 0.0045
24 hrs on distilled water	111.3	0.41 ± 0.0032
48 hrs on 2 mg./l. BA	123.0	0.41 ± 0.0073
48 hrs on distilled water	109.5	0.40 ± 0.0084
140 hrs on 2 mg./l. BA	76.2	0.35 ± 0.0058
140 hrs on distilled water	41.2	0.21 ± 0.0078
190 hrs on 2 mg./l. BA	70.9	0.27 ± 0.0095
190 hrs on distilled water	7.1	0.13 ± 0.0048

Conclusions

Two major effects have been demonstrated. Firstly, that when the senescence of leaf discs was delayed using benzyladenine, the production of pisatin by these discs was maintained at a higher level than by discs which had senesced in the absence of benzyladenine. Secondly, when discs were floated on either distilled water or benzyladenine for only a short period (1 to 2 days) the amount of pisatin produced by these discs was greater than by discs which had not been treated in this way. Benzyladenine caused a greater increase in pisatin production than distilled water.

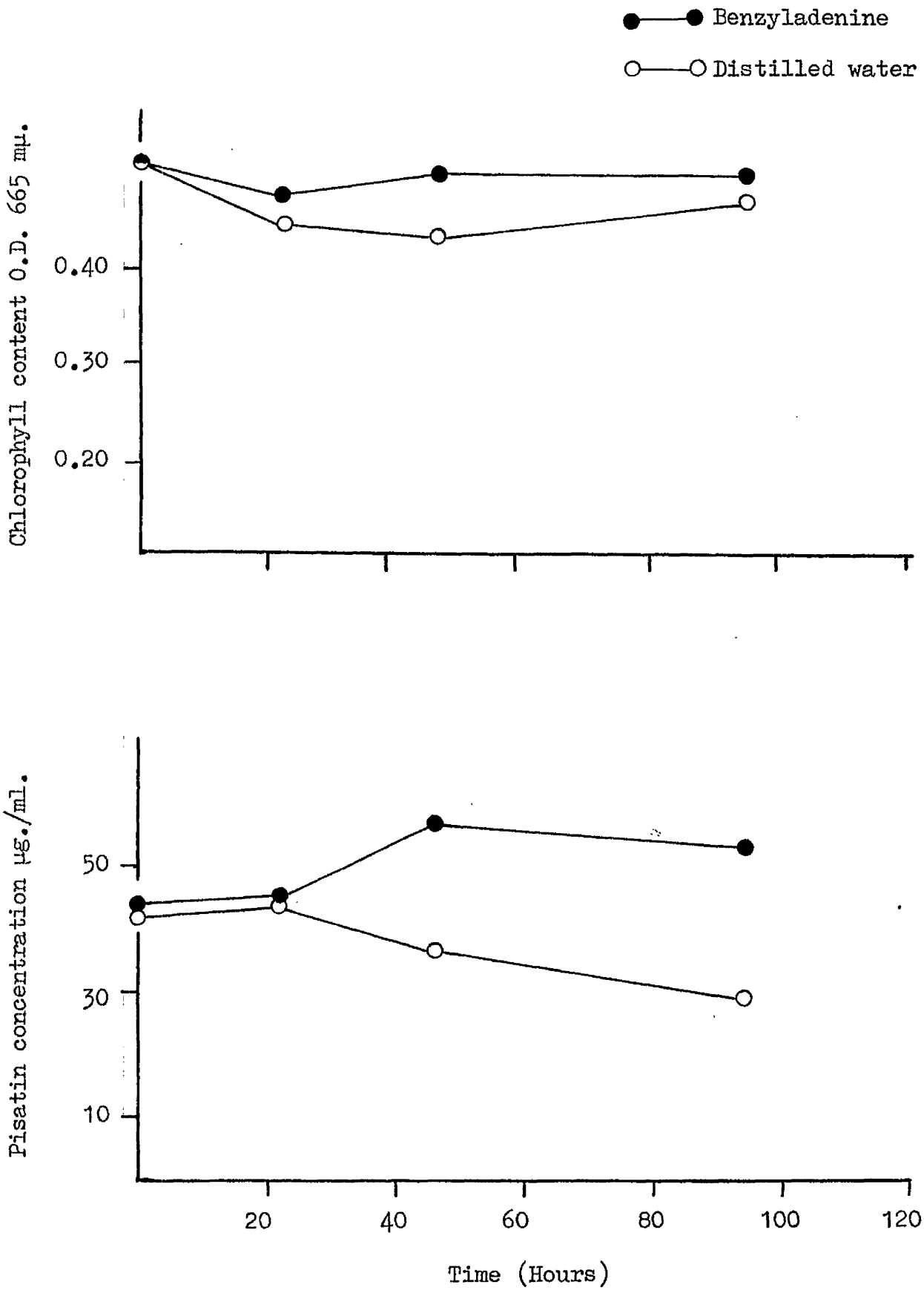


Fig. 4.17. The effect of benzyladenine on senescence and pisatin production of pea leaf discs maintained in the light.

Experiment 30. The effect of light on the senescence of pea leaf discs and on the production of pisatin by similar discs

The aim of this experiment was to investigate the effect of light on the ability of benzyladenine both to delay senescence and to maintain the pisatin production of pea leaf discs. The chlorophyll content of the leaf discs and the pisatin production of similar discs were measured after the discs had been floated on either distilled water or on benzyladenine in the light or in the dark. The discs in the light were placed on a bench in the laboratory near the window. Continuous supplementary light was provided by three 20 watt fluorescent tubes. Discs in the dark were maintained in an adjacent position enclosed in petri-dish cans. They were analysed for chlorophyll content and pisatin production after 0, 22, 46 and 94 hours. The pisatin concentration of the diffusates was measured after 40 hours. The results are shown in Table 4.38 and expressed graphically in Figs. 4.17 and 4.18.

Conclusions

Light delayed the senescence of pea leaf discs which were floated on distilled water. Leaf discs which were kept in the light also produced greater amounts of pisatin than similar discs which had been kept in the dark. This effect occurred with discs floated either on benzyladenine or on distilled water.

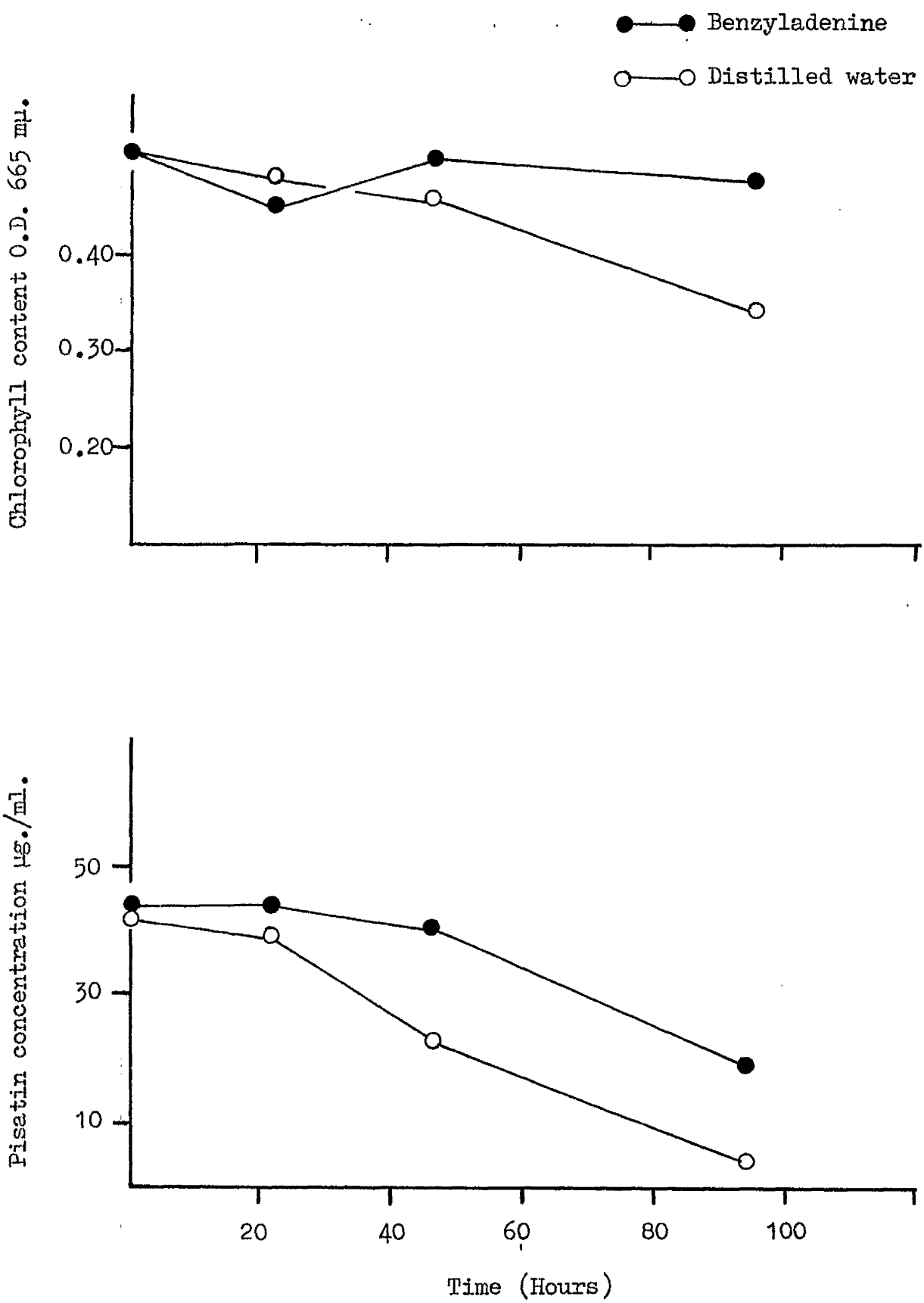


Fig. 4.18. The effect of benzyladenine on senescence and pisatin production of pea leaf discs maintained in the dark.

Table 4.38. Chlorophyll content of pea leaf discs and the pisatin concentration in diffusates obtained from similar discs

Treatment	Light		Dark	
	Pisatin µg./ml.	Chlorophyll cont. Mean O.D. 665 mµ.	Pisatin µg./ml.	Chlorophyll cont. Mean O.D. 665 mµ.
0 hrs on 2 mg./l. BA	43.8	0.51 ± 0.0095	-	-
0 hrs on dist. water	41.6	0.51 ± 0.0080	-	-
22 hrs on 2 mg./l. BA	45.5	0.47 ± 0.0090	43.8	0.45 ± 0.0088
22 hrs on dist. water	44.7	0.44 ± 0.0074	39.8	0.48 ± 0.0079
46 hrs on 2 mg./l. BA	57.4	0.50 ± 0.0080	40.3	0.50 ± 0.0082
46 hrs on dist. water	37.7	0.44 ± 0.0127	23.2	0.46 ± 0.0068
94 hrs on 2 mg./l. BA	53.0	0.50 ± 0.0097	19.7	0.48 ± 0.0072
94 hrs on dist. water	28.6	0.47 ± 0.010	4.38	0.34 ± 0.0148

Experiment 31. To investigate the effect of cycloheximide on the enhanced production of pisatin by pea leaf discs which have been treated with distilled water or with benzyladenine

It has been shown that benzyladenine caused the production of greater amounts of pisatin. In this experiment the effect of cycloheximide, an inhibitor of protein synthesis, on this enhanced production of pisatin was investigated.

The results are shown in Tables 4.39 and 4.40.

Table 4.39. Chlorophyll content of pea leaf discs and the pisatin concentration in diffusates obtained from similar discs after 46 hours

Treatment	Pisatin conc. µg./ml.	Chlorophyll cont. Mean O.D. 665 mµ.
Original value	74.0	0.50 ± 0.0105
Distilled water	74.0	0.38 ± 0.0107
1 mg./l. cycloheximide in dist. water	59.6	0.39 ± 0.0106
10 mg./l. cycloheximide in dist. water	39.4	0.39 ± 0.0739
2 mg./l. BA	95.5	0.41 ± 0.0112
1 mg./l. cycloheximide in 2 mg./l. BA	75.3	0.40 ± 0.0096
10 mg./l. cycloheximide in 2 mg./l. BA	47.3	0.41 ± 0.0083

able 4.40. Chlorophyll content of pea leaf discs and the pisatin concentration in diffusates obtained from similar discs after 44 hours

Treatment	Pisatin conc. µg./ml.	Chlorophyll cont. Mean O.D. 665 mµ.
)riginal value	0.70	0.55 ± 0.0065
Distilled water	0.96	0.43 ± 0.0041
1 mg./l. cycloheximide in dist. water	1.17	0.45 ± 0.0055
10 mg./l. cycloheximide in dist. water	0.78	0.46 ± 0.0077
2 mg./l. BA	1.23	0.47 ± 0.0060
1 mg./l. cycloheximide in 2 mg./l. BA	1.17	0.49 ± 0.0050
10 mg./l. cycloheximide in 2 mg./l. BA	0.79	0.49 ± 0.0052

Conclusions

It can be seen from Tables 4.39 and 4.40 that cycloheximide prevented the increase in pisatin production which occurred in leaf discs which had been floated on benzyladenine. A similar effect is shown in Table 4.39 for discs floated on distilled water, but in Table 4.40, 1 mg./l. cycloheximide caused a slight stimulation of pisatin production. It has been concluded earlier that cycloheximide inhibited protein synthesis in pea leaf discs and in this way it prevented benzyladenine delaying their senescence.

In this experiment, cycloheximide has been similarly shown to prevent benzyladenine enhancing pisatin production before any effect on senescence could be observed.

Although it has been shown that cycloheximide could be having a direct inhibitory effect on pisatin synthesis, it is more likely that cycloheximide prevented the increase in pisatin synthesis, by inhibiting the stimulation of protein synthesis which occurs in the presence of benzyladenine.

Discussion

The leaf discs of Pisum sativum were shown to senesce, i.e. lose chlorophyll, when floated on distilled water in the dark. The turgidity of the discs was maintained until the discs were extremely pale. At this stage, the discs became infiltrated with water and rotting occurred. It can be observed from the various experiments that the rate of senescence of pea leaf discs occurred in two main ways. Firstly, as shown by Figs. 4.12, 4.13, 4.14 and 4.16, the chlorophyll content began to fall immediately and continued until the discs were completely senescent. Secondly, as shown by Figs. 4.9, 4.15 and 4.19, following an initial small loss of chlorophyll, probably a result of leakage from the cut surfaces, the chlorophyll content remained level and only after 40 to 60 hours did it begin to fall. This continued until the discs were fully senescent. In the second case, the hormonal balance of the leaves was probably such that changes had to occur in the leaf discs before the discs would senesce. These changes had already occurred while the leaves were on the plant in those leaves which senesced immediately. Similar variations have been reported by other workers (Dennis, Stubbs and Coultate, 1967). Fletcher and Osborne (1966) reported that they kept the leaves of Taraxacum officinale in the laboratory under low light conditions in order to obtain leaf discs which would senesce uniformly.

The ability of various growth hormones to prevent senescence of leaf discs of pea was tested. Only the cytokinins, benzyladenine and kinetin, were effective. These compounds did not completely prevent

senescence but did considerably delay it. As indicated in the introduction, this specificity has been demonstrated on many occasions where growth hormones delay senescence. The results are in close agreement with Dennis et al. (1967) who showed that although benzyladenine and kinetin effectively delayed the senescence of mature leaf discs of Brussels sprouts, indolylacetic acid, benzimidazole and several other growth regulators failed to show any effect. Dedolph, Wittwer, Tuli and Gilbart (1962) showed similar specificity. Benzyladenine was used in this work in preference to kinetin because of its more suitable solubility. The concentration of benzyladenine which most effectively delayed senescence was again fairly specific. The concentration of benzyladenine, 2 mg./l., used in this work was less than that used by many other workers (Halevy, Diley and Wittwer, 1966; Tsugita and Andrew, 1967; and Dennis et al. 1967).

Substantial evidence that benzyladenine acts on leaf senescence in the same way as the other growth hormones, i.e. to stimulate protein synthesis, is lacking. Pozsár, Hammady and Király (1967) showed that nucleic acid and protein synthesis increased in bean leaves which had been treated with benzyladenine. In the experiments reported in this thesis, the inhibitory effects of puromycin and cycloheximide would indicate that protein synthesis is stimulated by benzyladenine.

It has been demonstrated that the production of pisatin by leaves decreased as they senesced. This was shown not only by leaf discs obtained from entire leaves which had been allowed to senesce with their petioles in water, but also by leaf discs which themselves had senesced floating on water. A progressive decline in pisatin production is shown

in Figs. 4.13, 4.16 and 4.18. However, it will be seen from Figs. 4.11, 4.14 and 4.15 that in these experiments this progressive decline in pisatin production occurred only after the discs had been floating for 1 to 2 days. The enhanced production of pisatin, which occurred during these 1 to 2 days, is similar to the effect reported by Cruickshank and Perrin (1963). Pisatin production by pea pods increased during the first three days of storage at 20°C but after this there was a progressive decline and none could be detected after six days.

It has been discussed above that senescent plant tissues are more susceptible to fungal invasion than young healthy tissues. It is suggested that this change to susceptibility in the case of pea leaves, may be due to the inability of the senescent tissues to produce sufficient pisatin to inhibit the invading fungus, rather than to any changes in the nutrient supply of the fungus or to the breakdown of mechanical barriers.

Benzyladenine was shown not only to delay senescence but also to maintain high levels of pisatin production by the leaf discs. (Figs. 4.12, 4.13, 4.16, 4.18). Benzyladenine was shown to have no direct effect on the amount of pisatin produced by a culture filtrate. In Experiment 29c. after 190 hours, the leaf discs which had been floated on distilled water produced only 7.1 μg./ml. pisatin in the diffusate, whilst leaf discs floated on benzyladenine for a similar period produced 70.9 μg./ml. pisatin.

In some cases, the pisatin production at the end of an experiment was not much greater from leaf discs which had been floating on benzyladenine than from leaf discs which had been floating on distilled water.

Nevertheless, the amount of pisatin produced by the leaf discs which had been floated on benzyladenine for only a few days was usually much greater than that produced by leaf discs on distilled water. It was a general effect that leaf discs which had been floating on benzyladenine produced greater amounts of pisatin. The enhanced production of pisatin by leaf discs floated on water for 1 to 2 days was increased if they were floated on benzyladenine.

In order to attempt to explain these phenomena it is necessary to discuss briefly the possible changes which occur when leaves are treated in these ways and also to mention the probable biochemical synthesis of pisatin.

A major effect which has been demonstrated to occur when tissues are damaged, is a marked increase in the rate of respiration (Click and Hackett, 1963). Cut potato slices showed a great increase in oxygen consumption. Click and Hackett were able to show that inhibitors of protein synthesis, e.g. puromycin and actinomycin D prevented this increase. By using ^{14}C-leucine they were able to demonstrate that protein synthesis took place within two hours of cutting, i.e. before any significant rise in the rate of respiration occurred.

The activity of many enzymes is known to increase in cut storage tissues. The increases in the activities of invertase (Edelman and Hall,1965) and peroxidases (Kanazawa, Schichi and Uritani, 1965) can be prevented by various inhibitors of protein and nucleic acid synthesis. Bagi and Farkas (1967) have demonstrated that ribonuclease activity increased in tobacco leaf discs maintained on moist filter paper. This

increase was more marked if the discs were illuminated. Farkas, Solymosy and Lovrekovitch (1965) have shown that pentoses accumulate in detached yellowing leaves, and that this is associated with a strong activation of the pentose phosphate pathway. They suggested that there was a correlation between the activation of 6-phosphogluconate dehydrogenase and this accumulation of pentoses. The activity of glucose-6-phosphate dehydrogenase has also been shown to increase in detached leaves, but no evidence was obtained to indicate that this was the result of new enzyme synthesis (Farkas, Dézsi, Horváth, Kisban and Udvardy, 1964).

Light was again shown to enhance the increase in activity of glucose-6-phosphate dehydrogenase (Horváth and Farkas, 1966). It would appear from these results that leaf discs may be expected to show an increase in the activity of the pentose phosphate pathway and especially in the enzymes involved in the conversion of glucose-6-phosphate to ribulose-5-phosphate. These increases could be enhanced if the leaf discs were maintained in the light.

The cytokinins, benzyladenine and kinetin, have been shown to stimulate protein synthesis (Osborne, 1962; Suguira, Umemura and Oota, 1962). They have also been shown to inhibit respiration (Dedolph, Wittwer and Tuli, 1961). It has been suggested that their effect on senescence may be the consequence of this decrease in respiration which is achieved as a result of inhibiting glycolytic kinases, e.g. hexokinases and pyruvic kinase (Tuli, Dilley and Wittwer, 1964). Suguira (1963) has demonstrated that kinetin inhibits the rise in respiration which occurred in tobacco leaf discs. He did not report whether there was any effect on the

activity of the pentose phosphate pathway in these discs.

The route of biosynthesis of pisatin is not fully established. It has been demonstrated that cinnamic acid-^{14}COOH and phenylalanine-^{14}C are incorporated into pisatin at a high rate and this has indicated that the normal shikimic acid pathway is operative in forming part of the pisatin molecule (Hadwiger, 1966). This pathway is known to involve erythrose-4-phosphate and phosphoenol pyrurate in the early stages, prior to the formation of shikimic acid itself.

It is suggested that the enhanced production of pisatin by pea leaf discs, which have been floated on either benzyladenine or water, is the result of an increase in the production of these pisatin precursors. Thus when an inducing agent, e.g. the culture filtrate, is placed on the leaf more pisatin is produced from such discs than from leaf discs in which this increase in precursors has not occurred.

It would seem likely that glucose-6-phosphate plays a major role in these effects. It would be expected to accumulate:-

(1) as the result of starch breakdown due to placing the discs in the dark,

(2) as the result of the inhibition of kinase enzymes by benzyladenine.

The utilization of the glucose-6-phosphate will be further increased by the stimulated activity of glucose-6-phosphate dehydrogenase which may occur as a result of cutting the leaf discs.

It has been shown by treating the leaf discs with benzyladenine that the production of pisatin is increased. Conversely, in Experiment 31, cycloheximide was shown to decrease the amount of pisatin

formed. It is not known whether cycloheximide acts in this way directly on pisatin synthesis or in an indirect way to prevent benzyladenine stimulating protein synthesis.

It thus appears that active protein synthesis is essential for the production of pisatin. Although it has not been proved that de novo protein synthesis is required for the formation of pisatin, large amounts of pisatin are only produced by leaves in which protein synthesis is active. Pisatin production increases when the discs are kept under conditions where protein synthesis is likely to be enhanced, e.g. in healthy leaves or in the presence of benzyladenine, and decreases when the discs are kept under conditions where protein synthesis is reduced, e.g. during senescence or in the presence of cycloheximide.

In 1963 Dekker indicated that cytokinins may delay senescence and thus prevent fungal invasion. Dekker showed that if leaf discs of cucumber were floated on water and dusted with spores of Erysiphe cichoracearum, infection occurred and the fungus was visible on the surface of the leaf. If, however, the leaves were floated on kinetin no infection occurred and the leaves remained dark green. This was shown for several powdery mildews but kinetin failed to prevent the development of Botrytis fabae on leaf discs of Vicia faba. Using detached leaves of V. faba, Moore and Leach (1968) have shown that benzyladenine delayed senescence and reduced the formation of aggressive lesions. In view of the experimental work described in this thesis, it would appear that these phenomena can be plausibly explained as a result of the production of phytoalexins.

Leaves treated with the cytokinin produced enough phytoalexin to

inhibit the fungi, but those leaves which were floated on water produced insufficient phytoalexin to prevent fungal invasion. Moore and Leach reported attempts to control Botrytis fabae on Vicia faba in the field. Although senescence of these plants was delayed by spraying the plants with benzyladenine, the amount of aggressive lesions which were formed was increased.

It is to be hoped that by maintaining the health of crop plants, either by better cultivation or by the application of external compounds, the ability of plants to produce phytoalexins and hence resist fungal invasion will be increased. It is not inconceivable that plants could be treated so that the precursors of phytoalexins are accumulated and in this way infection by fungi would cause the production of larger amounts of phytoalexin.

These effects may well be important in controlling diseases caused by weak facultative pathogens, e.g. B. cinerea, but it is unlikely that the more specialised pathogens will be inhibited by relatively small increases in the amount of pisatin produced.

Conclusions

Two aspects of pisatin production were studied. Firstly, it was shown that the production of pisatin by pea tissues could be stimulated by various agents, e.g. fungal spore suspensions, a culture filtrate, carboxylic acids, patulin and antibiotics.

The amount of pisatin produced in the presence of these compounds was shown to vary depending on their concentration and on their pH. The ability of these compounds to induce pisatin formation differed greatly. Secondly, it was shown that the production of pisatin by pea leaves depends on the state of the leaves at the time of infection.

This, of course, varies naturally and major decreases in the amounts of pisatin produced have been shown to occur during senescence. Benzyladenine was shown to delay senescence and to maintain pisatin production at a high level. Physiological injury, i.e. cutting the leaf discs and maintaining them in the dark increased the amount of pisatin produced by the leaf discs; this was further increased in the presence of benzyladenine. Light caused similar effects to those observed with benzyladenine; senescence was delayed and pisatin production was maintained at a high level. The increase in pisatin production resulting from physiological injury was enhanced if the leaf discs were illuminated.

GENERAL CONCLUSIONS

The work reported in this thesis has been concerned with an investigation into the possible formation of phytoalexins by apple fruit tissues, and into factors which affect the production of pisatin, the phytoalexin of Pisum sativum.

No inhibitory effects on fungal spore germination were demonstrated with juice extracted from either healthy or rotted apple tissues. Callus cultures of mature apple fruits were established, and it was shown that these cultures were unable to support the growth of Cylindrocarpon heteronema, i.e. they were resistant. C. heteronema was also found to be non-pathogenic to the apple fruits tested in these experiments. Spore germination of C. heteronema was inhibited by the callus and by juice extracted from both healthy and infected calluses. The inhibitory juice was highly unstable and further identification of the active constituents was not possible. In view of the fact that pisatin was also produced by healthy callus, the relevance of these results to the possible production of phytoalexins by apple fruit tissues was discussed.

Ideally, tissue cultures offer the plant pathologist an extremely valuable tool for investigating the post-infectional changes which occur in the host. Large volumes of actively growing cells can be exposed to the fungus. This is particularly relevant when dealing with non-pathogenic fungi, since when whole plants are used the number of cells affected by the non-pathogen is small and hence any effects are very localised; but using tissue cultures, the number of cells affected by a non-pathogen could be very large and similar to the number of affected by a pathogen. How-

ever, in this thesis a major defect in this argument has been demonstrated. If the above argument is valid, it is necessary to assume that the callus cells behave in an exactly similar manner to the original host cells. It has been shown, using callus from apple and pea tissues, that this is not always correct. These callus tissues produced inhibitors which could not be detected in healthy host plant tissues.

As a result of the experiments carried out with pea callus cultures, it was suggested that pisatin could be involved in two phenomena; firstly, that the growth rate of recently isolated pea callus was restricted by the presence of pisatin, since it was shown that pisatin is very phytotoxic to callus cells and that recently isolated callus produced large amounts of pisatin; secondly, that the necrotic regions which are formed on the plant after infection by fungi are the result of this phytotoxicity.

Various compounds, e.g. carboxylic acids, patulin, antibiotics and coconut milk were shown to induce pisatin formation. The amounts of pisatin produced by these compounds differed greatly and were found to be affected markedly by pH.

Antimetabolites have been used in this work to determine whether protein synthesis was required for pisatin synthesis, for benzyladenine to delay senescence, and for benzyladenine to enhance pisatin production. Cycloheximide, which was used most widely, inhibited all three effects, and it was concluded that benzyladenine caused a stimulation of protein synthesis in pea leaf discs. However, the incompatible effects of the other antimetabolites make any definite conclusions as to whether protein synthesis is required for pisatin synthesis impossible.

The amount of pisatin produced by pea leaves was shown to decrease as they senesced. When senescence was delayed by treating with benzyladenine, the decrease in pisatin production was also delayed. Leaf discs which were cut and maintained on either distilled water or on benzyladenine for 1 to 2 days, produced more pisatin than similar discs which had not been treated in this way. The increase in pisatin production is attributed to an increase in the concentration of pisatin precursors. The possible mechanisms which could cause this increase were discussed.

In view of these effects, it is suggested that such information may lead to new prophylactic measures based on increasing the potential of plants to produce phytoalexins after infection by a pathogenic fungus.

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