STUDIES ON PHYTOALEXINS

VI. PISATIN: THE EFFECT OF SOME FACTORS ON ITS FORMATION IN PISUM SATIVUM L., AND THE SIGNIFICANCE OF PISATIN IN DISEASE RESISTANCE

By I. A. M. CRUICKSHANK* and DAWN R. PERRIN*

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Summary

The effect of some biological, physical, and chemical factors on the formation of pisatin was studied by the chemical analysis of the extracellular fluids (diffusates) from the endocarp tissues of pea pods. The following points were demonstrated:

(1) Pisatin formation did not occur as a result of gross mechanical injury.

(2) There was an inverse relationship between pod maturity and concentration of pisatin formed as a result of inoculation.

(3) Pea pods retained their capacity to form pisatin at inhibitory concentration levels longest when stored under aerobic conditions at 4°C.

(4) Concentration of inoculum and postinoculation temperature affected both the total concentration of pisatin formed and its rate of formation.

(5) Pisatin formation was stimulated by all fungi tested (facultative and obligate), by spore-free germination fluids, and by certain chemical treatments, but not by bacteria.

(6) Non-pathogens of peas were associated with the formation of concentrations of pisatin in excess of the ED_{50} value of pisatin and pathogens of peas with concentrations less than the ED_{50} values of pisatin in relationship to the fungi in these two groups *in vitro*. Obligate pathogens could not be tested. The disease reaction of the endocarp of pea pods to fungi used in inoculation studies appeared to be primarily dependent on the sensitivity of the infecting fungus to the concentration of pisatin formed during the first few days of the incubation phase.

These results are discussed in relation to the phytoalexin theory of disease resistance in plants.

I. INTRODUCTION

Mizukami (1953), Kuć (1955, 1957), Müller (1956, 1958), and Uehara (1958, 1959) reported, on the basis of biological assays, the effect of some physiological factors on the formation of fungitoxic compounds subsequent to fungal inoculation in barley, corn, French beans, and soy beans respectively. Gäumann and Kern (1959) and Gäumann, Nüesch, and Rimpau (1960) in studies on orchids, and Akazawa and Wada (1961), in work on sweet potato roots, have isolated the toxins, namely orchinol and ipomeamarone respectively, and have studied some aspects of the conditions and rate at which these compounds were formed. In these two

* Division of Plant Industry, C.S.I.R.O., Canberra.

latter cases, the toxin situation in inoculated tissues appears complex and there still appears to be doubt as to the specific role of orchinol and ipomeamarone in the biological phenomena with which they are associated.

Pisatin, a new chromano-coumarane (Perrin and Bottomley 1962), has been isolated from laboratory-inoculated and naturally infected tissues of *Pisum sativum* L. (Cruickshank and Perrin 1960, 1961). The differential sensitivity of fungal pathogens and non-pathogens of *P. sativum* to pisatin *in vitro* has also been reported (Cruickshank 1962). The combined results presented in the above reports provide strong evidence in support of the hypothesis that pisatin plays a primary role in the natural resistance of the tissues of *P. sativum* to fungal infection.

The primary objective of the present studies was to provide evidence on the formation of pisatin by chemical analysis of the extracellular fluids (Cruickshank 1962) from endocarp tissues of pea pods. The effect of a range of biological, physical, and chemical factors on pisatin formation is described. The significance of the results are discussed in relation to the phytoalexin theory of disease resistance in plants.

II. Materials and Methods

Detached pea pods (class 2 and 3 host material, see Table 1) (chiefly cv. Greenfeast) were used as the host tissue. *Monilinia fructicola* (Wint.) Honey was used as the primary test fungus in all experiments except those specifically dealing with fungal species.

The standard conditions of fungal culture, preparation of spore suspensions, and inoculation of pod tissues were as follows: Cultures were maintained on potato dextrose agar at 20°C in the dark. Spore suspensions were prepared by flooding 7-day-old cultures with sterile distilled water, and gently rubbing the surface to dislodge the spores. The suspensions were then filtered through fine muslin and washed twice by centrifugation. The washed spores were finally suspended in sterile distilled water and the concentration adjusted to 4×10^5 spores per millilitre. Sterile water was used as control. Pod halves were set out on moist filter paper in covered containers suitable in size for the experiment involved. The endocarps of the pods were inoculated with 1 ml of spore suspension per half-pod and incubated at 20°C in the dark for 40 hr. Diffusate solutions were then removed. Incubation of the pods was continued for a further 5–7 days, when observations were made on the disease reaction symptoms.

Diffusate solutions from 10 half-pods were combined to form a single sample. Triplicate samples were taken from each treatment. Pisatin concentration in each sample was estimated by the chemical assay method described by Cruickshank and Perrin (1961). The data from the chemical analyses were statistically analysed after logarithmic transformation. The results given in the tables and figures are for the mean values. The curves are drawn with due allowance given to the statistical analysis of the data.

Only variations to the above standard experimental procedure are described in Section III.

III. Experimental and Results

(a) *The Host*

(i) *Uninfected Pea Tissue.*—To determine if pisatin occurred as a normal constituent of healthy tissues of the pea pod, endocarp was removed from uninfected freshly harvested pea pods and chemically assayed for pisatin (see endocarp analysis method, Cruickshank and Perrin 1961). No trace of pisatin was detected.

(ii) *Injury.*—A batch of pea pods was divided into three samples. The endocarp of sample (1) was scarified. Samples (2) and (3) were not damaged. Sterile water was immediately placed on the endocarp of samples (1) and (2). The third sample of pods was inoculated with *M. fructicola*. No pisatin was formed as a result of injury.

(iii) *Maturity of Tissues.*—Freshly harvested field-grown peas were divided into four classes representing the readily recognizable stages in pod development. For description of host material see Table 1.

Table 1

Relationship between maturity of pea pod tissues and their capacity to form pisatin following inoculation with Monilinia fructicola

Maturity Class	Description of Pea Pods	Pisatin Concentration (μg/ml)	
		Spore Suspension	Water
1	Green flat seeds, <2 mm diameter	110	9·6
2	Green slightly rounded seeds, 2–4 mm diameter	72·7	9·1
3	Green seeds, developed to full size, 8–10 mm diameter	28·9	<3
4	Pale green, slightly wrinkled mature seeds	14·6	<3

The results presented in Table 1 show that there is an inverse relationship between pod maturity and pisatin concentration. The most mature of the pea pods in class 4 which gave the lowest pisatin values were, in fact, susceptible as sporulation occurred during subsequent incubation of the pods.

(iv) *Storage.*—Detached pods were used in experiments associated with pisatin formation. The effect of duration, temperature, and condition of storage of whole pea pods, during the period from picking to inoculation, on the capacity of the endocarp tissues to form pisatin, was determined.

In the first experiment, whole pods were stored in loosely covered jars at 4 and 20°C. Samples were removed at 3-day intervals for inoculation over a 27-day period. The results given in Figure 1 show that, in the case of pods stored at 4°C, an increase in pisatin concentration occurred between the response on zero day and that on the third day of storage. Subsequent storage, under these conditions, did not significantly depress the capacity of the pea pods to form pisatin. The pods stored at 4°C remained resistant to *M. fructicola*. Pods stored at 20°C rapidly lost their capacity to form pisatin. Sporulation occurred on pods inoculated after 6 days storage under the latter conditions. No change occurred with time in the low level of pisatin (<3 μg/ml) formed in the control pods over the 27-day storage period.

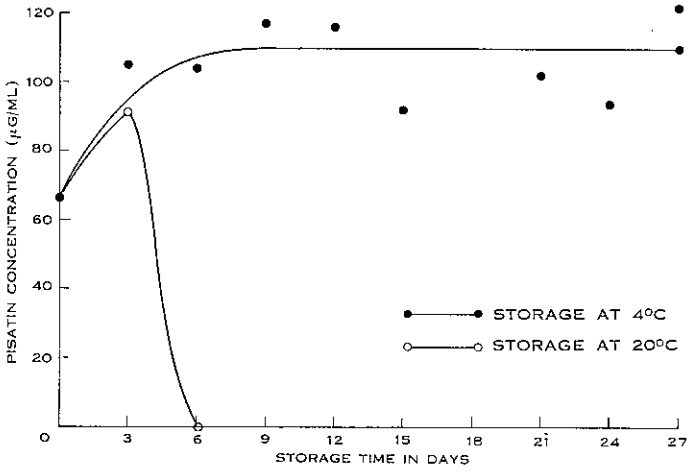

Fig. 1.—Effect of temperature of storage of whole pea pods on their subsequent capacity to form pisatin after inoculation with *Monilinia fructicola*.

In the second experiment, pea pods were stored in sealed and open containers at 4°C. Pods were sampled at 2-day intervals over a 12-day period. The results given in Figure 2, clearly show that the pods retain their capacity to form pisatin longest under conditions of aerobic storage. Under conditions of sealed storage, there was a temporary increase in pisatin concentration which rapidly decreased with further storage.

(*b*) *Bacterial Inoculum*

To ascertain if bacteria could induce the formation of pisatin, pods were inoculated with *Pseudomonas pisi*, *Ps. angulata*, *Xanthomonas campestris*, *X. phaseoli*, *Rhizobium leguminosarum* SU302, *Rh. leguminosarum* SU331, and *Rhizobium* sp. ex *Lotus uliginosis* CC806. Bacterial cell suspensions (10^6 cells/ml) were prepared in sterile physiological saline. Sterile physiological saline, sterile water, and a spore suspension of *M. fructicola* were included as controls. None of the bacteria tested were able to induce the formation of significant amounts of pisatin. Similar results

were obtained in other tests in which a mixed inoculum of bacteria isolated from normal diffusate solutions was used. Bacterial contamination could be avoided by careful selection of very immature pod material (class 1 host material, see Table 1) and by working under aseptic conditions without affecting the capacity of endocarp tissue to produce pisatin after fungal inoculation. It is considered that the results of the fungal studies in this paper were not significantly affected by the bacteria present in the diffusate solutions.

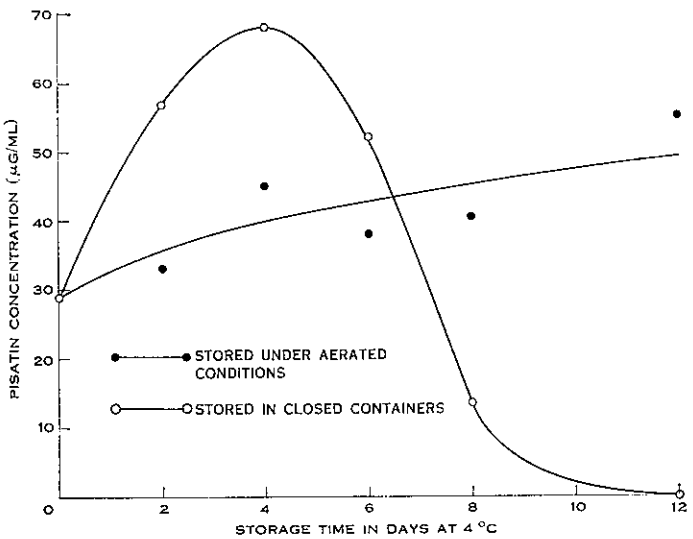

Fig. 2.—Effect of storage condition of whole pea pods on their subsequent capacity to form pisatin, following inoculation with *Monilinia fructicola*.

(c) *Fungal Inoculum*

(i) *Nutritional State of the Fluid in which Spores are Suspended at the Time of Inoculation.*—A spore suspension was prepared, in part, as described above. Prior to the washing stage, the suspension was divided into three equal samples which were treated as follows: Sample (1) received no further treatment. Samples (2) and (3) were centrifugally washed twice in sterile distilled water. Sample (2) was resuspended in 0·05% sterile sucrose solution. Sample (3) was resuspended in sterile distilled water. The rate of change of pisatin concentration in the diffusate solutions was studied by withdrawing samples for chemical analysis at 6-hourly intervals from the 6th to the 48th hour, and a final sample at 60 hr after inoculation. Results are presented in Figure 3. Percentage germination data on spore suspensions *in vitro* were obtained concurrently with the above experiment using the standard slide germination technique. The mean values for the three conditions were as follows: 6 hr—6·6, 3·3, 0%; 12 hr—43, 30, 1%; and 18 hr—93, 95, 3%.

(ii) *Effect of Spore Concentration of the Inoculum on Pisatin Formation.*—Spore suspensions of *M. fructicola* and *Ascochyta pisi* were prepared at concentrations

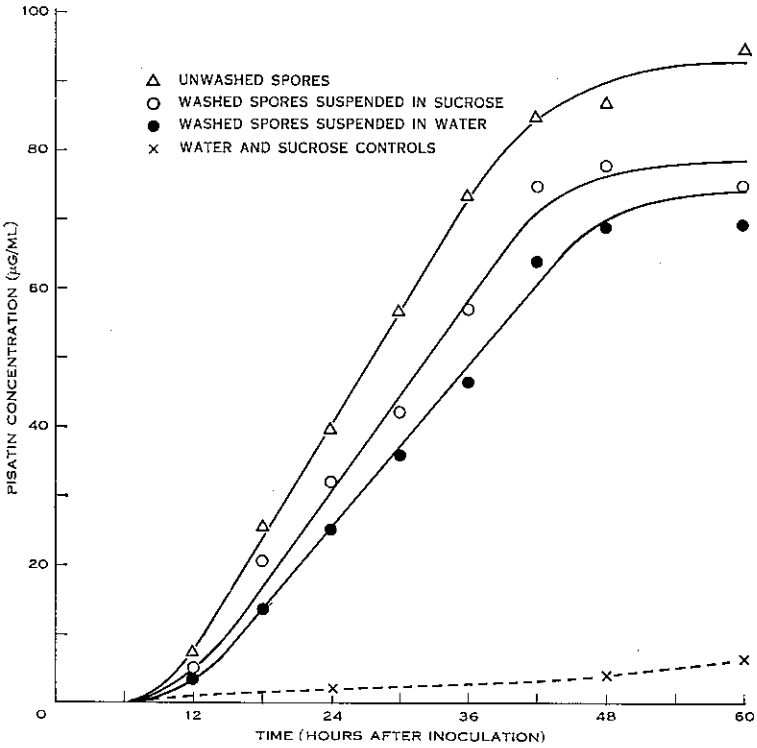

Fig. 3.—Relationship between initial nutritional state of spores of *Monilinia fructicola* and changes with time of pisatin concentration in diffusate solutions on pea pods.

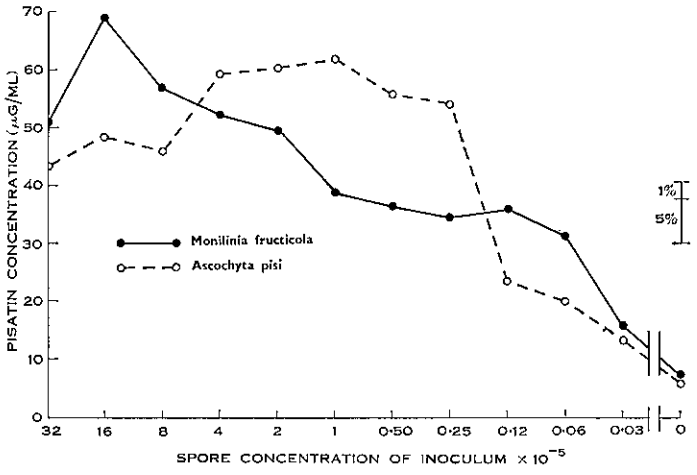

Fig. 4.—Relationship between spore concentration and pisatin concentration.

ranging from $0{\cdot}03\times10^5$ to 32×10^5 spores per millilitre of spore suspension. Pods were inoculated under standard conditions. The results are presented in Figure 4.

(iii) *Effect of Spore Concentration on the Rate of Change of Pisatin Concentration in Diffusate Solutions.*—Suspensions of *M. fructicola*, containing 10^5, 10^4, and 10^3 spores per millilitre of suspension, were used to inoculate endocarp tissues. Diffusate

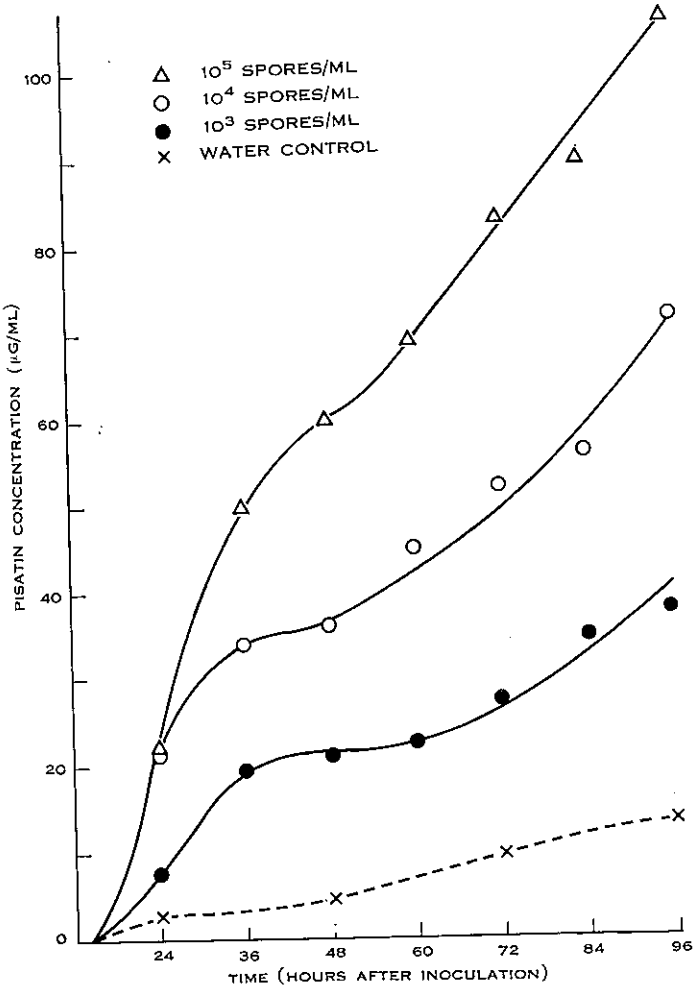

Fig. 5.—Comparison of the effect of spore concentration of *Monilinia fructicola* on the time course of change in pisatin concentration.

samples were withdrawn initially after 24 hr and then at 12-hr intervals over a 96-hr incubation period. The results are presented in Figure 5.

The results of these two experiments show a clear relationship between the spore concentration in the inoculum and the concentration of pisatin in the diffusate solutions. In Figure 4, it is seen that an optimal range of spore concentration exists for both fungi tested. In Figure 5, it is seen that the change with time in pisatin

concentration is directly related to the concentration of spores in the inoculum. In these experiments, the fungal spores germinate in fluid directly in contact with exposed host cells of the endocarp of pea pods. Under these conditions, infection of host cells is not essential to the formation of pisatin. Whereas *M. fructicola* does not infect the host cells, *A. pisi* does, but both initiate pisatin formation.

TABLE 2
CONCENTRATIONS OF PISATIN FORMED FOLLOWING INOCULATION OF THE ENDOCARP OF PEA PODS WITH A RANGE OF FUNGAL PHYTOPATHOGENS

Fungal Species	Pisatin Concentration (μg/ml) after 72 Hours
Facultative pathogens	
Ascochyta pisi Lib.	116
Ascochyta pinodella L. K. Jones	53
Botrytis allii Munn.	65
Botrytis cinerea Pers.	85
Colletotrichum lindemuthianum (Sacc. & Magn.) Bri. & Cav.	110
Fusarium solani var. *martii* f. *pisi* Appel & Wollenw.	90
Kabatiella caulivora (Kirch.) Karak.	62
Leptosphaeria maculans (Desm.) Ces. & de Not.	109
Monilinia fructicola (Wint.) Honey	81
Penicillium digitatum (Fr.) Sacc.	59
Penicillium gladioli McCull. & Thom.	51
Septoria apii Chester	88
Septoria pisi West.	10
Obligate pathogens	
Erysiphe sp. ex. Marrow	54
Puccinia coronata Corda	17
Peronospora tabacina Adam	26
Uromyces fabae Pers.	14
Uromyces phaseoli var. *typica* Arth.	27
Ustilago bullata Berk.	45
Controls	
Water	<3
"Tween 20" (1 : 10,000 in water)	<3

(iv) *Effect of a Range of Plant Pathogenic Fungi on the Formation of Pisatin.*—To determine if the formation of pisatin may be induced by a wide range of fungal species, some miscellaneous facultative and obligate species from the three main Classes of the Fungi and the Fungi Imperfecti were tested as inocula (see Table 2). With the exception of *Fusarium solani* var. *martii* f. *pisi*, *Leptosphaeria maculans*, *Septoria apii*, *S. pisi*, and *Ustilago bullata* the facultative species were cultured on oatmeal agar. *F. solani* var. *martii* f. *pisi* was grown on potato dextrose agar and *L. maculans* and *S. apii* were cultured on autoclaved pea seed. Spores of the obligate species and *U. bullata* and *S. pisi* were collected from infected tissues of their natural hosts, care being taken that all diseased plant tissue used was infected by one species only. In the case of *S. pisi*, leaf and stem lesions were used from young glasshouse-

grown pea plants, produced by plant inoculation with a washed mycelial macerate of *S. pisi* from broth culture.

The spore suspensions of the facultative group of species (excluding *U. bullata*) and *Erysiphe* sp. and *Peronospora tabacina* were prepared as described in Section II. Spore suspensions of *Puccinia coronata*, *Uromyces* spp., and *U. bullata* were prepared gravimetrically (5 mg of spores in 30 ml of liquid). "Tween 20"–water solution (1 : 10,000 v/v) was used for this group to assist in their uniform dispersion.

Spore suspensions of each species were incubated *in vitro* for the same period as those incubated on endocarp. Analyses of the suspensions failed to detect any pisatin.

The results presented in Table 2 show that all fungi tested induced the formation of pisatin at concentrations significantly above the levels of the water control. The values of pisatin obtained by testing a miscellaneous group of species under a given set of standard conditions, as described above, however, may in no degree represent the maximum concentrations for all the species tested as the conditions may not be optimal for the germination of each species. In spite of this, it does appear significant that, even if the values in Table 2 are taken as characteristic of the species listed, then, with the exception of the known pea pathogens in the group, the pisatin concentrations for all the facultative fungi tested exceeded the ED_{50} range value reported for these species *in vitro* (Cruickshank 1962). The corresponding situation in relation to the obligate species is at present not known on account of technical difficulties associated with their culture *in vitro*.

(v) *Comparison of the Change with Time of Pisatin Concentration following the Inoculation of Endocarp Tissues with Several Pathogens and Non-pathogens of Peas.—Ascochyta pisi*, *Fusarium solani* var. *martii* f. *pisi*, and *Septoria pisi* were chosen as representing three distinct genera of pea pathogens. *Monilinia fructicola*, *Colletotrichum lindemuthianum*, and *Botrytis allii* were selected as representatives of fungi non-pathogenic to peas. A seventh fungus, *B. cinerea*, was also included as an example of a wound pathogen with a wide host range, including peas. The preparation of spore suspensions and the conditions of incubation were as described in the previous subsection. Samples of the diffusate were withdrawn at 6-hourly intervals from the 12th to the 48th hr after inoculation and then at 12-hourly intervals until 72 hr after inoculation.

The results presented in Figures 6 and 7 show that the curves representing the change with time of pisatin concentration in the diffusate solutions are multi-inflectional. There was an initial lag period. Detailed sampling over the first 12 hr, not reported here, showed that the lag period between inoculation and first detection of pisatin was 6–8 hr. After the lag period, pisatin concentration rose almost linearly with time for a period of 12–30 hr. Over the subsequent 12–20 hr, the rate of change in pisatin concentration progressively fell to zero. A second cycle of increase in pisatin concentration commenced between 48 and 60 hr after inoculation.

The three non-pathogens stimulated levels of pisatin adequate to inhibit their growth. Two of the pea pathogens stimulated high levels of pisatin to be formed. The levels were not, however, adequate to inhibit their growth. This was shown

by subsequent growth of the pathogen on the pods after 7 days incubation. The third pathogen, *S. pisi*, also pathogenic on the basis of clinical symptoms on the pods, stimulated the formation of only very low concentrations of pisatin. The wound pathogen, *B. cinerea*, fell between these two groups. In this case, pisatin was formed at a rate similar to that of *A. pisi* over the first 24 hr; however, after this time, the rate of increase dropped rapidly and the secondary cycle was not

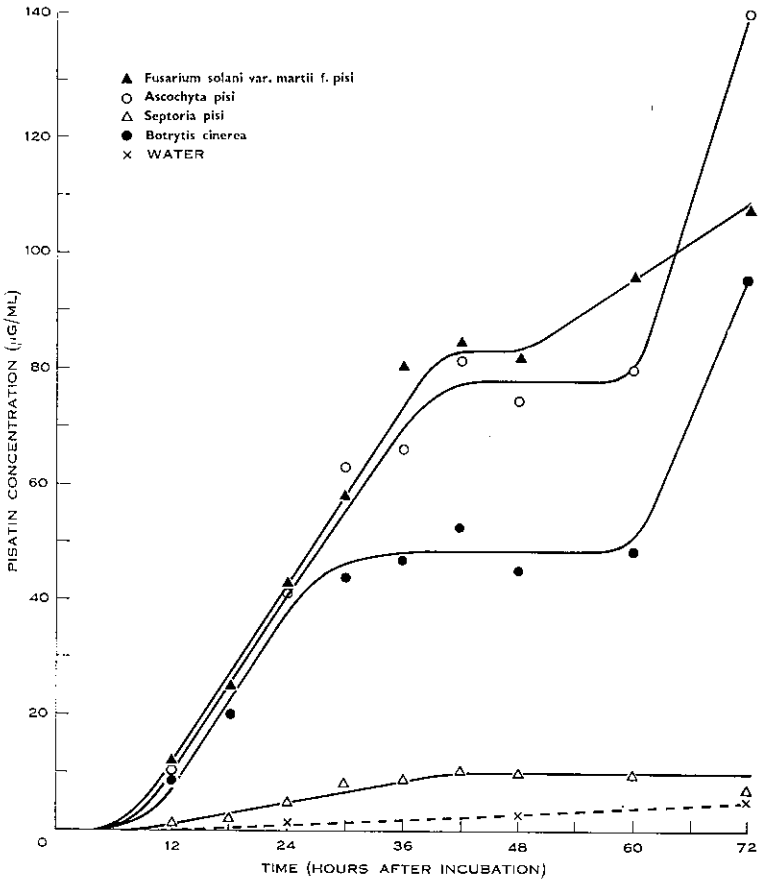

Fig. 6.—Comparison of rate of formation of pisatin in diffusate solution following inoculation of pea pod endocarp with three pathogens and a wound pathogen of *Pisum sativum*.

detected until after 60 hr. Over the period from 30 to 60 hr after inoculation, the pisatin concentration in this case did not exceed the ED_{50} range value for this species *in vitro* (Cruickshank, loc. cit.).

(*d*) *Conditions Affecting both the Fungus and the Host Plant Tissues*

(i) *Anaerobic Conditions.*—Pea pods were placed in a series of air-tight chambers connected in series to a source of oxygen-free nitrogen gas which was flushed through

the system for 30 min prior to inoculation. Following inoculation, the system was immediately reflushed with oxygen-free nitrogen and nitrogen was passed continuously (1 lb/sq. in.) through the system over the entire subsequent incubation period. Controls consisted of sterile water on pods under anaerobic conditions, as described above, and spore suspension and sterile water on pods under standard conditions in air.

No pisatin was formed in pods incubated in the oxygen-free atmosphere. It is concluded that aerobic conditions are required for the formation of pisatin.

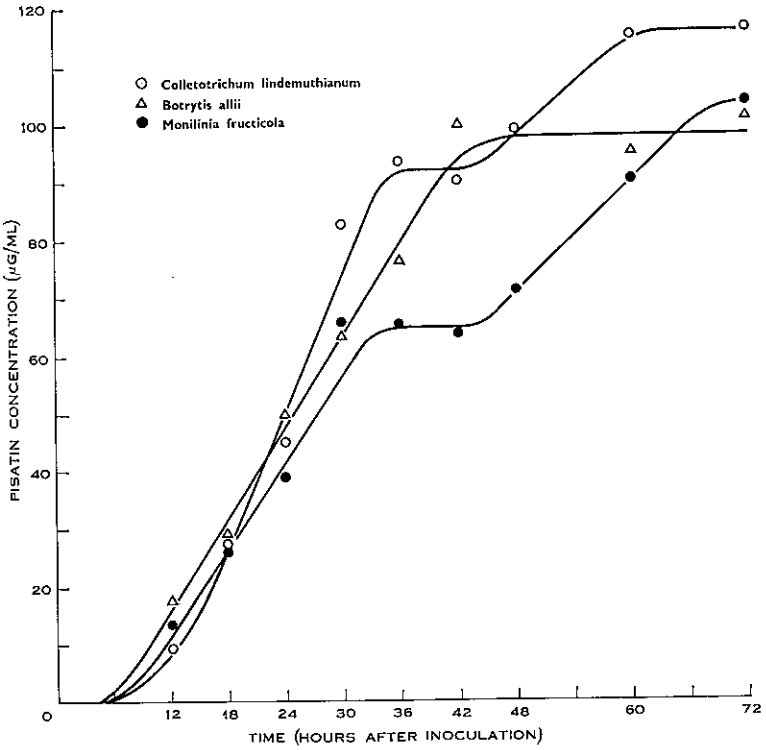

Fig. 7.—Comparison of rate of formation of pisatin in diffusate solution following inoculation of pea pod endocarp with three non-pathogens of *Pisum sativum*.

(ii) *Temperature.*—Pea pods were inoculated and placed in a series of incubators set at temperatures ranging from 2 to 35°C. *M. fructicola* and *A. pisi* were used as the test fungi. Sterile water was placed on the control pod groups at each temperature.

The results presented in Figure 8 show that pisatin was formed in considerable quantities over the temperature range 10–30°C. A cubic regression fitted to log $(x+5)$, where x is the pisatin concentration, failed to show any difference between the effect of the two organisms.

(e) Formation of Pisatin in the Absence of Microorganisms

(i) *Effect of Spore-free Spore Germination Exudate.*—Washed spores of *M. fructicola* were suspended in a sterile dilute nutrient solution* and in sterile water. Both spore suspensions were incubated with shaking at 20°C in the dark. Samples were taken for germination counts after 24 hr. The remainder of the spore suspension was centrifuged at 3200 *g* for 10 min to remove fungal material. Centrifugation was repeated on the supernatant.

Endocarp tissues of fresh pea pods were inoculated with the spore-free germination fluids prepared above and incubated under standard conditions. A standard spore suspension of *M. fructicola*, a sample of the sterile nutrient solution, and sterile water were used as control inocula.

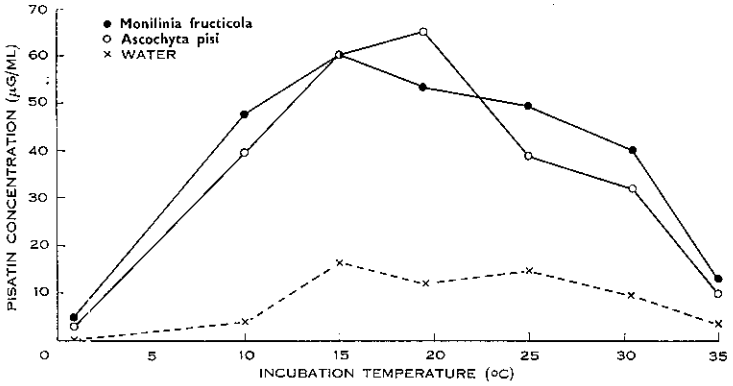

Fig. 8.—Relationship between incubation temperature and pisatin concentration.

The results (mean of six replicates) of the spore germination counts on the spore suspensions used in the preparative section of the experiment were 86% in the dilute nutrient solution and less than 1% in the water. The results of the second section of the experiment are presented in Table 3. They show that the presence of fungal spores is not essential to the stimulation of the formation of pisatin. They suggest that pisatin formation is initiated by a substance exuded from germinating spores into the germination fluid.

(ii) *Effect of Chemicals.*—Solutions of cupric chloride, mercuric chloride, calcium chloride, and sodium chloride were prepared to cover the concentration range 3×10^{-6}–3×10^{-1}M. Drops of these chemical solutions were placed on the endocarp tissue of pea pods and incubated under the standard conditions described above. The resulting fluids were tested for pisatin concentration by the standard chemical assay. The identity of pisatin was confirmed by bioassay for antifungal activity and by the conversion of it to anhydropisatin (Cruickshank and Perrin 1961).

* The dilute nutrient solution was prepared by placing drops of sterile distilled water on endocarp tissues of pea pods for 12 hr at 20°C, then withdrawing and bulking the drops of fluid remaining on the tissues. The bulk solution was centrifuged and the supernatant sterilized by filtration.

The results presented in Table 4 show, first, that pisatin formation may be initiated in the complete absence of microorganisms; secondly, that, of the four chloride solutions studied, only those of the heavy metal ions, such as copper and mercury, induced pisatin formation, and that the concentration of these chemical

TABLE 3
STIMULATION OF PISATIN FORMATION BY SPORE-FREE GERMINATION EXUDATES

Treatment	Pisatin Concentration (μg/ml)	Controls	Pisatin Concentration (μg/ml)
(i) Spore-free exudate (germination >80%)	38·9	(1) Standard spore suspension of *Monilinia fructicola*	87·8
(ii) Spore-free exudate (germination <1%)	9·9	(2) Nutrient solution for incubation of spores in treatment (i)	6·5
		(3) Water used for incubation of spores in treatment (ii)	6·9

solutions determined the concentration of pisatin arising in the diffusate. The optimum concentration depended on the particular ion used. The optimum concentration range of mercuric chloride was relatively wide, 3×10^{-5}–10^{-3}M, whereas, cupric chloride, on the other hand, was effective over a much more narrowly defined concentration range (*c.* 3×10^{-3}M).

TABLE 4
PISATIN FORMATION FOLLOWING APPLICATION OF CHLORIDE SOLUTIONS

Concentration of Chemical Solution (M)	Pisatin Concentration (μg/ml)			
	$CuCl_2$	$CaCl_2$	$HgCl_2$	NaCl
3×10^{-6}	<5	<5	<5	<5
3×10^{-5}	<5	<5	>100	<5
1×10^{-4}	11		>100	
3×10^{-4}	11	<5	>100	<5
1×10^{-3}	26		80	
3×10^{-3}	94	<5	20	<5
3×10^{-2}	<5	<5	<5	<5
3×10^{-1}	Nil	<5	Nil	<5

The melanization of the endocarp cells characteristic of fungal inoculation did not occur. The diffusate solutions were clear. In the case of mercuric chloride, the solutions were colourless. Symptoms of phytotoxicity were macroscopically visible only at concentrations of the applied chemicals exceeding those required for maximum pisatin formation. In fact, as visible phytotoxicity increased, pisatin concentration rapidly decreased.

(iii) *Time Course Study.*—The time course of pisatin formation was studied following the application to pod cavities of cupric chloride (3×10^{-3}M) solutions. The results shown in Figure 9 indicate that there was an initial lag period of 6–8 hr, (cf. Figs. 6 and 7). Following this initial lag phase, pisatin concentration rose almost linearly with time over the full incubation period. The concentration of pisatin in the solutions formed after the application of cupric chloride was of the same order as that

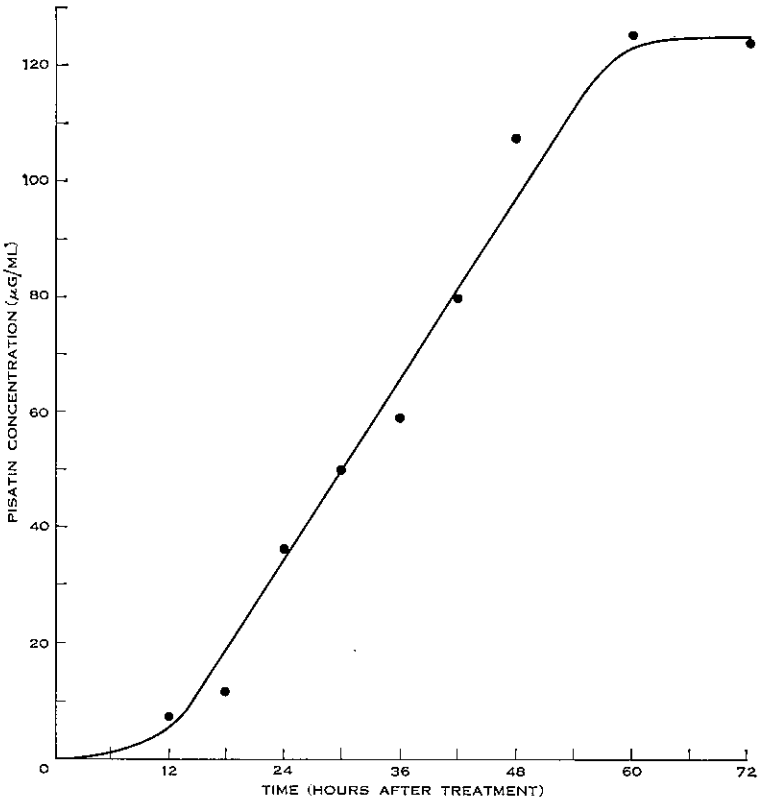

Fig. 9.—Rate of formation of pisatin in "diffusate" following endocarp treatment with cupric chloride solution.

of pisatin formed after fungal inoculation. The rates of formation, over the first 24–30 hr, were similar for both fungi and chemical solution; however, in the latter case, there was only one cycle of pisatin formation detected while a multi-inflectional curve was characteristic of most time course studies in which fungal spore suspensions were used as the inocula.

IV. Discussion

The primary objective of the present studies was to provide evidence on the formation of pisatin under a range of different conditions in an attempt to define the significance of pisatin in the disease resistance of tissues of *Pisum sativum.* It is clear

that the quantitative level of pisatin concentration is influenced by the physiological condition of the host, the type of inoculum, and the physical conditions both before and during incubation. Pisatin formation may also be induced by non-microbiological agents. The results of most interest to disease resistance are those concerned with the miscellaneous group of species of fungi including several pathogens of peas.

Kuć (1955, 1957) has reported the formation of inhibitory compounds resulting from the inoculation of potato tubers with several pathogens and non-pathogens of that host. Gäumann, Nüesch, and Rimpau (1960) have shown that orchinol formation in orchid tubers is provoked by a range of mycorrhizal fungi and some bacterial species but not by some 24 saprophytic or semi-parasitic soil fungi. Ipomeamarone formation has been stimulated in sweet potato roots by several fungi and poisonous chemicals (Suzuki 1957; Uritani, Uritani, and Yamoda 1960) and in roots injured by a weevil (*Cylas formicarius eleguantulus* Sum.) (Akazawa, Uritani, and Kubota 1960). The time course of detection of the fungitoxic compound(s) formed as a response to inoculation of French bean with *M. fructicola* has shown the presence of the inhibitory compound(s) 14 hr after inoculation (Müller 1958). This result has been confirmed by Uehara (1960). In similar studies, orchinol was detected between 24 and 48 hr after infection (Gäumann and Hohl 1960). The time course of ipomeamarone formation resulting from infection of sweet potato roots with *Ceratocystis fimbriata* (Ell. & Hals.) Elliott also showed a lag period of some 24 hr and then a rapid synthesis (Akazawa and Wada 1961).

Studies on the formation of pisatin have shown that the capacity to stimulate pisatin formation is not limited to any particular Class of fungi, or to obligates, facultatives, biotrophs, or necrotrophs (Table 2). It has, in fact, been formed in response to inoculation with all plant parasitic fungi tested, and treatments of pods with certain chemical solutions, but not by bacteria. Time course studies have shown that there is a lag phase of 6–8 hr followed by a rapid synthesis of pisatin over the next 12–30 hr. A second period of rapid increase in pisatin concentration in the diffusate solution occurred about 48–60 hr after inoculation. This could represent a second cycle of synthesis, or merely a release of the pisatin formed, from the host cells due to changes in cell wall permeability which would be expected to occur. This pattern of progressive change in pisatin concentration in diffusate solutions appears to be fairly general following the inoculation of the endocarp tissues with the various fungal species. For the species which conformed to the generalized pattern, however, the details of the curves varied. Quantitative differences occurred in the ability of the various species to induce the formation of pisatin, but no qualitative distinction existed.

It is of interest to examine the concentrations of pisatin (Table 2) and the time course of change in pisatin concentration (Figs. 6 and 7) in relation to pathogens and non-pathogens of peas. If the known pathogens are considered first, it is seen that *Ascochyta pisi* and *Fusarium solani* var. *martii* f. *pisi* induce the formation of high concentrations of pisatin and that the rate of formation compares favourably with that of any of the non-pathogens studied. *Septoria pisi*, on the other hand, induces the formation of only very low concentrations of pisatin. *Botrytis cinerea*, a wound pathogen, appears to occupy an intermediate position in terms of the rate of formation.

The final concentration after 72 hr, in the latter case, is similar, however, to that of the other two pathogens which readily induce pisatin formation. If the fungi non-pathogenic to peas are now considered, it is seen that, without exception, they induce the formation of pisatin at moderate to high concentrations, but that the concentration varies with the fungus tested. When, however, the quantitative levels of pisatin formed in each case (pathogens and non-pathogens) are considered in relation to their sensitivity* towards pisatin *in vitro* (Cruickshank 1962), it is seen that they are differentiated in the following way. Non-pathogens of peas induce the formation of pisatin at concentrations in excess of the ED_{50} values of pisatin towards them. On the other hand, pea pathogens, if they induce the formation of pisatin, induce it at concentrations less than that of the ED_{50} values of pisatin towards them.

Müller and Börger (1940) postulated that the basis of differentiation between resistant and susceptible hosts was primarily the speed of response of the host. If the rate of increase of pisatin concentration is assumed to be a measure of the speed of response, then this alone does not serve as a reliable basis of differentiation between resistant and susceptible reactions, as normally understood. On the other hand, examples may exist where this postulation is valid. The susceptibility of peas to *B. cinerea*, considered in relation to the curve for the rate of formation of pisatin after inoculation with this fungus (Fig. 6), may be interpreted on this basis. The potato–*Phytophthora infestans* interactions studied by Müller and Börger (1940) may represent a similar situation. The concept may also be valid in more obscure examples, if within the pathogen group self-limitation of lesion size is considered to be a form of resistance. *A. pisi* forms a small self-limiting type of lesion on pea leaves and pods. *S. pisi* appears to be unlimited in its capacity to form large spreading lesions on leaves, stems, and pods. These two characteristic lesion types may be a reflection of the ultimate build-up of high concentrations of pisatin which inhibit further spread of *A. pisi* where it is the infecting fungus, and of the low concentration of pisatin or any other inhibiting compound where *S. pisi* is the pathogen.

The formation of brown pigment in host cells of infected resistant tissues has been reported by Tomiyama (1955, 1956), Müller (1958), Akazawa and Uritani (1961), and several earlier authors. Similar pigmentation has been observed as part of the disease syndrome in the present studies. It does not, however, appear to be directly associated with pisatin formation as this compound was formed in the absence of pigmentation when chemical treatments were used. Further evidence of the relative unimportance of the brown pigments in the endocarp cells was provided by assays which failed to demonstrate any fungitoxicity associated with extracts [residues after pisatin extraction (Cruickshank and Perrin 1961)] of them. It thus appears that the brown pigments which have been observed in host cells associated with resistant reactions and broadly described as polyphenol oxidation products by earlier authors, are probably of secondary significance, if any, to the phenomenon of resistance. It is suggested that they are a result of infection, but have no causal relationship to resistance *per se*.

* For pea pathogens, with the exception of *S. pisi* the dosage–response curves must be extrapolated to determine the ED_{50} values as the solubility of pisatin in the system used limited the maximum test concentration to 100 μg/ml.

Insufficient examples of host–pathogen interactions have been studied for any broad generalizations to be made which would have no exceptions. It does, however, appear clear, when the results presented in this paper are considered together with those of the earlier workers referred to in the Introduction, that there is little doubt that phytoalexins, of which pisatin is an example, play an important primary role of pathological significance to the hosts in which they occur. The primary basis of differentiation between resistant and susceptible hosts was postulated by Müller and Börger (1940) and more recently by Müller (1958, 1961) to be the speed of response of the host tissue to the invading fungus. The differential sensitivity of pathogens and non-pathogens of peas to pisatin was suggested by Cruickshank (1962) as a basis for differential disease reaction in *P. sativum*. It is now clear that the situation is more complex than can be explained by either of these factors separately, and that both must be considered in the extension of the phytoalexin theory to account for the results of the host–pathogen combinations analysed in these investigations. It is concluded that the situation required for a resistant reaction is a host in which infection stimulates production of a phytoalexin at a concentration above the threshold which inhibits the fungus. Susceptibility may be due to the inability of the infecting fungus to stimulate the formation of the phytoalexin characteristic of the host or to the capacity of the fungal pathogen to be tolerant of the phytoalexin produced.

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