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A COMPARISON OF THE SITES OF PHYTOALEXIN ACCUMULATION AND OF BIOSYNTHETIC ACTIVITY IN POTATO TUBER TISSUE INOCULATED WITH BIOTIC ELICITORS

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Key Word Index—*Solanum tuberosum*; Solanaceae; *Phytophthora infestans*; elicitors; accumulation; biosynthesis; cell-free system; sesquiterpenoids; phytoalexins; rishitin; lubimin.

Abstract—Aged discs cut from Kennebec potato tubers were inoculated with one of the following: an elicitor preparation from mycelia of *Phytophthora infestans* race 4, zoospores from either race 4 or race TY complex of this fungus, or sodium arachidonate. At 24 hr intervals after inoculation, four successive 0.5 mm thick layers of tissue were cut from the discs. This tissue was analysed for accumulated phytoalexins and also used to prepare cell-free enzyme systems for lubimin biosynthesis. In tissue treated with either the elicitor preparation or race 4 zoospores, levels of phytoalexin accumulation were highest in the first layer of tissue. Surprisingly, however, cell-free lubimin biosynthesis from [1-^{14}C]isopentenyl pyrophosphate was also generally greater in preparations derived from the first 0.5 mm of tissue. Accumulation of phytoalexins in tissue inoculated with zoospores from race TY complex was very low, whereas cell-free biosynthetic activity was initially comparable to that seen in preparations from tissue treated with the elicitor preparation. By the end of the experimental period lower layers of tissue from discs treated with sodium arachidonate contained the highest levels of phytoalexins and yielded cell-free enzyme preparations with the greatest lubimin biosynthetic activity.

INTRODUCTION

The role phytoalexins have in disease resistance in potatoes is one of either toxicity to, or inhibition of normal growth of, the infective agent. Generally the rate of accumulation and final concentration of phytoalexins are greater in the incompatible interaction than in the compatible interaction [see 1, 2]. The production of phytoalexins in the compatible interaction [3] and the potent elicitor activity of extracts from compatible races of a fungus [4] raise questions as to the nature of the specificity of the interactions. It has been suggested that specificity results from the suppression of events including phytoalexin accumulation [1, 5, 6]. A number of studies have been performed in which the site of synthesis of phytoalexins has been compared to the site of accumulation. The evidence from a number of systems suggests that accumulation occurs at or close to the lesion, whereas synthesis occurs in the surrounding healthy tissue. This not only applies to carbocyclic sesquiterpenes [7–14] but also to isoflavanones [15, 16], acetylenes [17, 18] and furanosesquiterpenes (ipomeamarone) [19–21].

In the present study we have inoculated potato tuber discs with elicitor prepared from mycelia of *P. infestans* race 4, zoospores from *P. infestans* race 4 (incompatible) and TY complex (compatible) and with the biotic elicitor [22] sodium arachidonate. Using the cell-free enzyme system described previously [23] we have compared the sites of synthesis of lubimin from IPP with the sites of phytoalexin accumulation.

RESULTS

Tissue treated with elicitor from P. infestans *race* 4 *mycelia*

At all times after inoculation, phytoalexin levels were highest in layer 1 (Fig. 1A), although the amounts had significantly increased in layer 2 by 48 hr after treatment and in layers 3 and 4 by 72 hr. Rishitin was the major phytoalexin in all extracts, but the proportion of lubimin tended to increase as the period after inoculation lengthened. Thus in layer 1 rishitin comprised 90% of the total phytoalexin content at 24 hr but only 70% at both 48 and 72 hr and 45% after 96 hr after inoculation. The increase in rishitin content in layer 1 was considerably greater over the first 24 hr period compared to the second and third periods, while lubimin accumulation continued to increase at a rapid rate in the second 24 hr period. There was a slight fall in the phytoalexin content of layer 1 at 96 hr compared to 72 hr, but the total phytoalexin content in these two sets of discs were very similar. The total phytoalexin content of the discs was 5.7, 8.2, 11.7 and 12.4 $\mu g \cdot g$ fr. wt^{-1} at 24, 48, 72 and 96 hr after treatment respectively.

The biosynthetic activity of cell-free preparations from the respective layers (Fig. 1B) generally presented similar profiles to that of accumulation. The biosynthetic activity in layer 1 was generally higher than in layer 2 at all times after inoculation, particularly at 48 hr when it was observed to be almost double. Only low levels of incorpor-

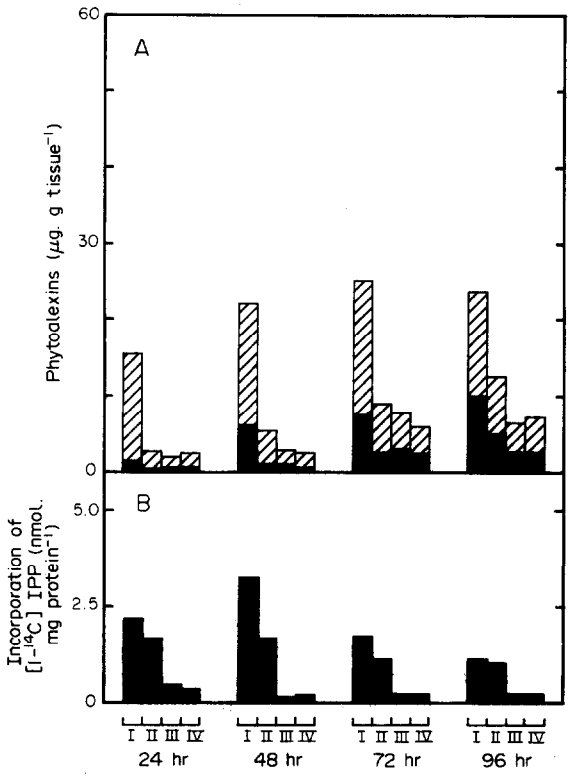

Fig. 1. Phytoalexin accumulation (A) and lubimin biosynthesis (B) in tissue from Kennebec potato tuber discs inoculated with elicitor prepared from mycelia of *P. infestans*, race 4, and then incubated for 24 to 96 hr. A. Rishitin (▨) and lubimin (■) levels in tissue from successive 0.5 mm layers cut from treated discs. B. Incorporation of [1-^{14}C]IPP into lubimin in 30 min incubations of cell-free enzyme preparations made from similar tissue. Layers are as follows, I: 0–0.05 mm; II: 0.5–1.0 mm; III: 1.0–1.5 mm; IV: 1.5–2.0 mm. Discs were inoculated for times shown.

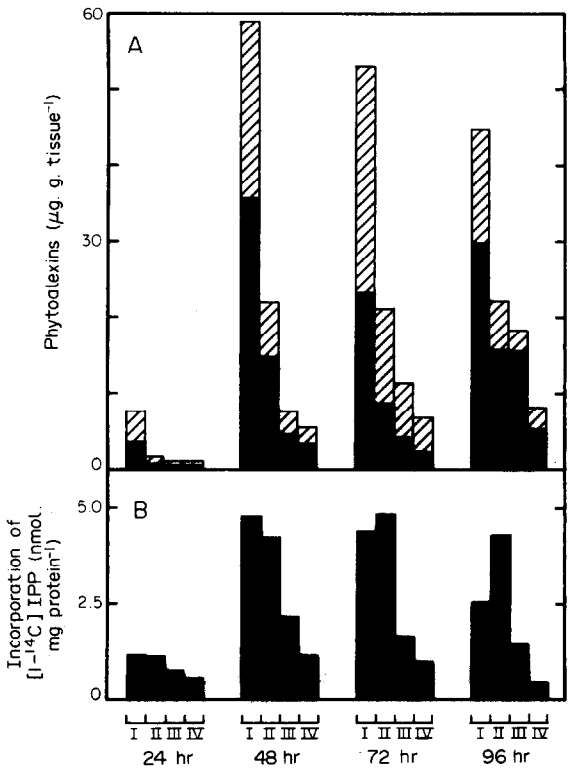

Fig. 2. Phytoalexin accumulation (A) and lubimin biosynthesis (B) in tissue from Kennebec potato tuber discs inoculated with zoospores of *P. infestans*, race 4, and then incubated for 24 to 96 hr. Key to figure as given in Fig. 1.

ation of [1-^{14}C]IPP into lubimin were apparent in layers 3 and 4 at all time points.

Tissue inoculated with race 4 zoospores

As with treatment with elicitor from race 4 mycelia, accumulation of phytoalexins in tissue inoculated with race 4 zoospores (Fig. 2A) occurred mainly in layer 1. At 24 hr after inoculation only low levels of phytoalexins were observed (7.5 $\mu g \cdot g$ fr. wt^{-1} in layer 1) compared to the levels found at subsequent times (e.g. 58.7 $\mu g \cdot g$ fr. wt^{-1} in layer 1 at 48 hr). The level of phytoalexins in layer 1 steadily decreased from the maximum at 48 hr over the remainder of the 96 hr period studied, while layer 2 levels were maintained and those in the deeper layers, especially layer 3, increased steadily. The total amount of phytoalexin in all the slices at 48 hr onwards remained fairly constant at just over 23 $\mu g \cdot g$ fr. wt^{-1}. It is noteworthy that generally lubimin was either the predominant phytoalexin present or at least equal to rishitin as regards concentration. At 96 hr after treatment, lubimin was greatly in excess of rishitin in all the layers.

Again, generally, incorporation of [1-^{14}C]IPP into lubimin by cell-free preparations made from the various slices (Fig. 2B) followed a similar pattern to that of phytoalexin accumulation. At 24 hr after inoculation, enzyme activity was lower than at any other time (except when compared to activity in layer 4 at 96 hr). At 24 hr, layers 1 and 2 had similar biosynthetic activity, layers 3 and 4 had progressively less. Biosynthetic activity at 48 hr after inoculation was at a maximum in all layers compared to subsequent times. At 72 and 96 hr it was apparent that the greater activity was present in cell-free enzyme preparations from layer 2. At 96 hr activity was 0.5 nmole IPP incorporated mg. protein^{-1}, layers 1, 3 and 4 containing 55, 30 and 10% of this activity respectively at this time point.

Tissue inoculated with TY complex zoospores

Phytoalexin accumulation in tissue from potato discs inoculated with zoospores from race TY complex (Fig. 3A) was found to be extremely low. Maximum levels were seen in layer 1, 96 hr after inoculation and were 8 $\mu g \cdot g$ fr. wt^{-1}. Generally, at all time points phytoalexin content was highest in the upper layers and decreased down through the disc. The ratio of lubimin to rishitin was approximately unity throughout.

The levels of enzyme activity with regard to [1-^{14}C]IPP incorporation into lubimin in cell-free preparations derived from TY complex zoospores inoculated tissue are presented in Fig. 3B. Enzyme activity at 24 hr after inoculation was higher than that seen at the same time point for race 4 elicitor preparation or zoospore in-

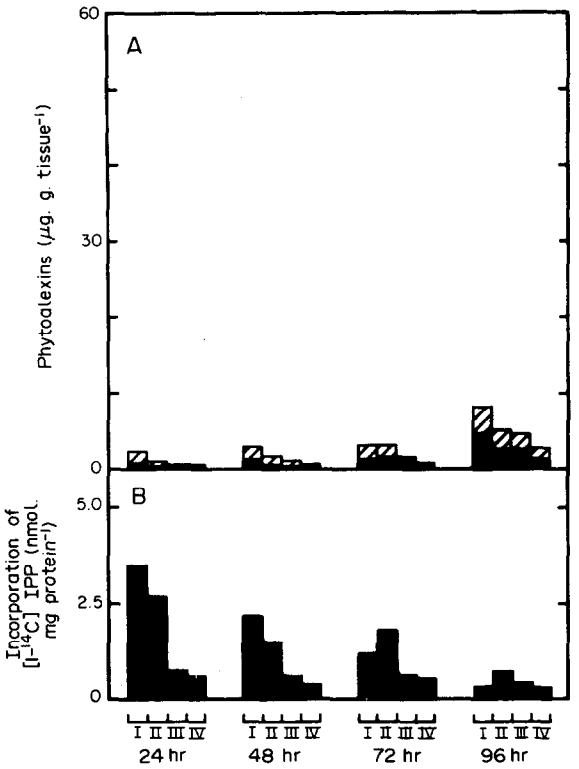

Fig. 3. Phytoalexin accumulation (A) and lubimin biosynthesis (B) in tissue from Kennebec potato tuber discs inoculated with zoospores of *P. infestans*, TY complex, and then incubated for 24 to 96 hr. Key to figure as given in Fig. 1.

oculated tissue. Incubations of cell-free preparations from layer 1 at this time point gave 0.35 nmole [1-^{14}C]IPP incorporated mg protein^{-1}. Similar incubations of cell-free preparations from layer 2 gave 75% of this activity, whereas activity from layers 3 and 4 presented under 20%. This pattern of activity was repeated in cell-free preparations from the layers at 48 hr after inoculation, but levels of activity were generally lower. Although the activity in the cell-free preparations from the various layers at 72 and 96 hr after inoculation progressively decreased, greater activity was found in those from layer 2 at both time points compared to those from layer 1.

Tissue inoculated with sodium arachidonate

As with race 4 zoospore treated tissue, phytoalexin accumulation in tissue inoculated with sodium arachidonate was extremely low after 24 hours, but rapidly increased over the subsequent 24 hr period (Fig. 4A). There was, however, a trend of increasing accumulation in phytoalexins over the entire 96 hr period, the total phytoalexin content being 1.5, 16.3, 27.1 and 38.1 μg·g fr. wt^{-1} at 24, 48, 72 and 96 hr after inoculation respectively. This represents an almost linear increase from 48 to 96 hr with a lag period from 24 to 48 hr. The proportion of phytoalexins in the various layers also changed over the 96 hr period. At 48 hr after inoculation levels in layer 1 were 31 μg·g fresh weight, with layers 2, 3 and 4 containing 60, 30 and 20% of this amount respectively. At 72 hr after inoculation layer 1 still contained the highest level but layers 2 and 3 both contained approximately 70% of this level and layer 4, 31%. By 96 hr, layer 2 contained the highest level of phytoalexins (49 μg·g fr. wt^{-1}), layers 1, 3 and 4 containing 80, 75 and 50% of this concentration. A further point is that at 48 hr the ratio of lubimin to rishitin was approximately unity whereas at the later time points lubimin was the predominant phytoalexin by at least 2.5×.

Biosynthetic activity in the cell-free enzyme preparations from sodium arachidonate treated tissue is presented in Fig. 4b. The highest levels of activity were found in preparations from layers of 48 hr treated tissue. There was significant activity in preparations from all layers from this tissue, that from layer 1 giving 0.3 nmole [1-^{14}C]IPP incorporated into lubimin·mg protein^{-1} compared to 0.31, 0.19 and 0.18 nmol·mg protein^{-1} in those from layers 2, 3 and 4 respectively. At 72 and 96 hr after treatment, however, biosynthetic activity was greater in cell-free preparations from layer 3 than those from the other layers. Thus incubations of preparations from layer 3, 72 hr after treatment gave 0.24 nmole [1-^{14}C]IPP incorporated mg·protein^{-1} whereas preparations from layers 1, 2 and 4 gave 30, 87 and 44% of this activity in similar incubations.

DISCUSSION

In our previous paper [23] we described the cell-free biosynthesis of lubimin from [1-^{14}C]IPP and commented on the lack of rishitin production in the system. The

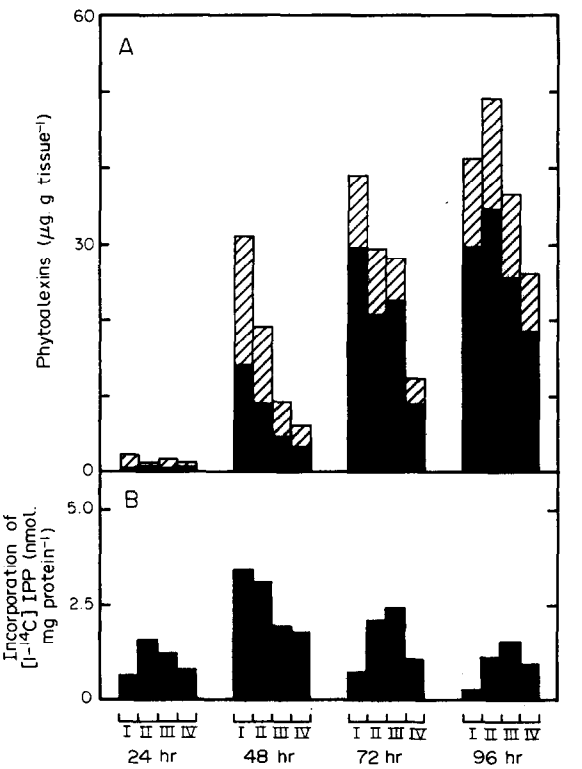

Fig. 4. Phytoalexin accumulation (A) and lubimin biosynthesis (B) in tissue from Kennebec potato tuber discs inoculated with sodium arachidonate, and then incubated for 24 to 96 hr. Key to figure as given in Fig. 1.

conversion of lubimin to rishitin has been suggested by other workers [24–27] to be the normal route of biosynthesis. Assuming this is correct, it is reasonable to compare the results we have obtained regarding the biosynthesis of lubimin in cell-free systems derived from different layers of tissue from stressed potato discs, with the combined accumulation of lubimin and rishitin in the layers.

Rishitin and lubimin are two of the major sesquiterpenoid phytoalexins produced by the potato, the predominant compound being dependent upon the physiological state of the tubers [28, 29]. Our own experience is that the potential for lubimin accumulation increases at the expense of rishitin as the tubers age in storage at 4°. This is reflected to a certain extent in the present study by the general difference in the ratio of rishitin to lubimin in tissue treated with elicitor preparation from *P. infestans* race 4, compared to that resulting from the other treatments. The former work was completed approximately two months before the latter was initiated.

A further general observation to make on our results is that the overall differences in the levels of phytoalexins accumulated and biosynthetic activity extracted cannot be realistically compared between treatments, since there is no way of determining the relative 'strength' of elicitor activity in each of the inocula. The suppression of other enzyme activities utilizing common precursor pools (i.e. acetate), such as those of steroid glycoalkaloid biosynthesis [30, 31] must also be considered.

Nakajima *et al.* [12] suggested that accumulation of phytoalexins occurred mainly in the necrotic, brown cells at the site of infection, whereas biosynthesis was localized in the adjacent, healthy cells. The biosynthetic activities were assessed by measuring the incorporation of radioactivity into rishitin when [2-^{14}C]acetate was fed to potato tuber slices cut at different levels from the site of infection by an incompatible race of *P. infestans*. The workers based their interpretation of their results on the assumption that the distribution of endogenous acetate pools was uniform throughout the tissue and did not increase in the top layer as a consequence of post-infectional events. In our own work on [2-^{14}C]MVA and [1-^{14}C]IPP feeding to similar slices [23] we found that the pattern of labelling of rishitin from [2-^{14}C]MVA resembled that found by Nakajima *et al.* using acetate, i.e. rishitin in the second layer more heavily labelled than that in the first layer. The labelling of rishitin from [1-^{14}C]IPP, however, was almost the same in the first and second layers of tissue. In comparing the feeding experiments, it must be borne in mind: (a) that the first and second layers used by Nakajima *et al.* were 0.7 mm and 0.41 mm thick respectively, whereas our slices were both 0.5 mm thick; (b) that the different substrates used may have penetrated the slices to different extents, particularly in the cases of the first layers the inoculated surfaces of which were necrotic and (c) that, as pointed out already, the endogenous pool sizes were unknown. On balance, however, our results seemed to indicate considerable synthesis of phytoalexins in the infected layers.

The results presented here regarding biosynthesis of lubimin from IPP by cell-free preparations obtained from the different layers of the discs inoculated with elicitor preparation or zoospores from *P. infestans* race 4 are also seemingly in conflict with the conclusions drawn by Nakajima *et al.* Tissue from the top layer of the discs inoculated with the elicitor preparation contained the majority of accumulated phytoalexins up to 72 hr after treatment. By 96 hr, the top layer still contained the highest concentration, but the lower layers also contained appreciable amounts. The cell-free preparations obtained from the upper layer at all the time points exhibited a capacity for the biosynthesis of lubimin from IPP which was equal to or higher than that in the second layer.

In the case of tissue inoculated with zoospores of race 4 the highest concentrations of phytoalexin were again observed in the upper layer, but substantial amounts were present in the second layer by 48 hr after inoculation and in the third layer by 96 hr. The capacity for biosynthesis of lubimin from IPP was similar in cell-free preparations from the first and second layers up to 72 hr after treatment, but by 96 hr the preparations from the second layer presented the higher activity.

Thus both these treatments gave results which are at variance with the work of Nakajima *et al.*; accumulation of phytoalexins was not restricted to the layer of tissue containing necrotic cells and the capacity for biosynthesis of lubimin, at least from IPP, was not as discretely segregated from the affected zone.

A further point regarding the above sets of results is that initially the increase in accumulation of phytoalexins reflects the increase in biosynthetic capacity. After 48 hr of treatment with the elicitor preparation, however, the biosynthetic capacity begins to decline whereas the actual accumulation increases slightly and then remains steady. In the case of treatment with race 4 zoospores, although the biosynthetic capacity at 72 hr is comparable to that at 48 hr, no further increase in accumulation was apparent in the third 24 hr period. All these results are discussed later in relation to those obtained from inoculation with race TY complex zoospores and with sodium arachidonate.

The results obtained from tissue inoculated with race TY complex zoospores are most interesting. In this case the levels of accumulation were extremely low. These low levels are in contrast to those found in this laboratory using another compatible race of *P. infestans* [32] but are consistent with the results of Bostock *et al.* [33] using race 1, 2, 3, 4. Biosynthetic activity in cell-free preparations from the top two layers of the discs was, paradoxically, higher at 24 hr after inoculation than at any time when elicitor preparation from race 4 was used. Thus although the enzymes required for lubimin biosynthesis are clearly induced by inoculation with race TY complex zoospores, the enzyme activity is either inhibited or is obscured by metabolism of any phytoalexins produced. It is perhaps relevant to note that the hyphae of race TY complex would have grown through the discs after germination of the zoospores (as opposed to those of race 4 which would have been contained) and would have been expected to contribute to the IPP isomerase and FPP synthetase activities of the cell-free preparations.

After treatment with sodium arachidonate the potato discs showed a total phytoalexin accumulation (i.e. the sum of the levels in all four layers) which increased almost linearly over the second, third and fourth 24 hr periods. This increase was apparent even though the biosynthetic activity of the corresponding cell-free preparations was decreasing, and is in contrast with the results obtained using the other treatments. A further feature is the apparent shift in the site of maximum accumulation to a lower layer of cells in the discs, paralleled by a change in

the layer yielding the maximum amount of cell-free biosynthetic activity. This may be the result of diffusion of elicitor activity through the cell layers. The lower levels of cell-free biosynthetic activity obtained 24 hr after inoculation compared to those at 48 hr is complimented by a corresponding difference in accumulation. This may indicate a delay in the response of the tissue to the inoculum due to an indirect mode of elicitation by sodium arachidonate e.g. by the release of constitutive (endogenous) elicitors [cf. 34–37, see 38].

The different patterns of accumulation and biosynthetic capacity obtained with the four treatments used can all be explained in terms of a combination of metabolism of products and inhibition of enzyme activity. The continued increase of accumulation in tissue treated with sodium arachidonate, compared to the steady state reached in the case of race 4 zoospore inoculation especially, would indicate that in the former synthesis outweighed metabolism of the phytoalexins, whereas in the latter metabolism balanced synthesis. The metabolism of rishitin by healthy tissue is documented [39]. A high rate of metabolism may also account for the low levels of accumulation of phytoalexins in the tissues treated with TY complex zoospores but whether enzymes inherent to this race of the fungus can catalyse such metabolism, or if the fungus induces such metabolism by the host, is unknown. We have no evidence, however, for the production of ether-extractable metabolites, although the possibility remains that water soluble compounds are produced.

Our observations on cell-free activities do not support this hypothesis in its entirety, since enzymes of metabolism may well be expected to be present in the cell free preparations, although our earlier work [23] does not suggest the presence of such activity. The lack of accumulation of phytoalexins in the experiments with race TY complex zoospores, despite the potential for lubimin biosynthesis, may therefore be more readily explained in terms of inhibition of biosynthesis by the fungus itself. The factor causing such inhibition would necessarily have to be removed during extraction of the cell-free preparations to account for the biosynthetic activity observed.

The discrepancies between our results and those of other workers with respect to the apparent compartmentalization of phytoalexin accumulation and synthesis have yet to be explained. It must be stressed that we are making a distinction between the actual biosynthesis *in vivo* and the potential for biosynthesis from IPP in the tissues. In view of our earlier results which indicate that lubimin itself inhibits cell-free incorporation of IPP into lubimin [23], the accumulation of phytoalexins in large amounts may cause a reduction in biosynthetic activity at the site of infection. Also, as mentioned earlier, pool size of endogenous precursors should also be taken into account. It is further possible, based on the contention that race TY complex has an inhibitory effect on biosynthesis, that incompatible races of the fungus also inhibit biosynthesis, but the growth of the fungus is restricted by other processes thus allowing lower layers to biosynthesize the phytoalexins which can be transported to the site of infection.

EXPERIMENTAL

Experimental methods were generally identical to those described previously [23] and therefore only necessary and additional exptl information is given here.

Media and chemicals. Bean agar was made as described for pea agar [23] except that prior to autoclaving 100 mg each of cholesterol and sitosterol were added in 40 ml CH_2Cl_2. The agar was stored in the dark. [1-^{14}C]Isopentenyl pyrophosphate (IPP) triammonium salt (53 mCi · mmole^{-1}) was from Amersham plc. Sodium arachidonate was from Sigma.

Fungal material. Phytophthora infestans (Mont) de Bary races 4 and TY complex (race 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11) were from the culture collection of the Department of Plant Biology, University of Hull and Dr. J. F. Malcolmson, Scottish Plant Breeding Station, Roslin, Midlothian, respectively. In order to obtain sporangia, race 4 and TY complex of the fungus were grown on sterol supplemented bean agar for 10 and 14 days respectively at 21°. The Petri plates were then flooded with sterile distilled H_2O and the mycelia rubbed with a glass rod to dislodge the sporangia. The spore suspensions were filtered through 2 layers of muslin to remove hyphal fragments and the concn of spores in the filtrate estimated with a haemacytometer. Zoospores were released by holding at 4° for 2 hr, then warming to room temp. The concn was subsequently adjusted with sterile distilled H_2O to give 10^5 zoospores ml^{-1}. A crude elicitor preparation from race 4 cell walls was obtained as described previously [23].

Treatment, incubation and extraction of potato tuber discs. Discs (1.0 cm dia. by 0.5 cm thick) were prepared from surface sterilized tubers of Kennebec (R1) potatoes and aged for 24 hr in the dark at 21°. The discs were then inoculated with one of the following, using 400 discs per treatment: (a) crude elicitor preparation, (b) race 4 zoospores, (c) TY complex zoospores or (d) sodium arachidonate (5 mM). All inoculates were applied at 20 μl per disc. The inoculated discs were replaced in the dark at 21° for up to a further 96 hr.

At 24 hr intervals 100 discs were taken and four successive 0.5 mm thick slices (designated layers 1 to 4) were removed, starting at the inoculated surface. 40 slices from each layer were extracted using $CHCl_3$–MeOH (2:1) and the rishitin and lubimin content determined by GLC. The rest of the slices from each layer were used to make cell-free preparations. These enzyme preparations were incubated with [1-^{14}C]IPP (0.2 μCi) and NADPH generating system (0.25 μmoles NADP) for 30 min, these conditions having been shown to be optimum for this system [23]. The protein content of the cell-free preparations from all layers was in the order of 0.6 mg protein ml^{-1}. Et_2O was used to terminate the incubations and to extract labelled lubimin, which was quantified by liquid scintillation counting after purification by TLC.

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