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PHYTOALEXIN FORMATION IN CELL SUSPENSIONS OF *PHASEOLUS VULGARIS* IN RESPONSE TO AN EXTRACT OF BEAN HYPOCOTYLS

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; cell suspension culture; constitutive elicitor; phytoalexin; phaseollin; phaseollinisoflavan; conversion; metabolism.

Abstract—Suspension cultures of *Phaseolus vulgaris* accumulated phytoalexins after treatment with an extract of bean hypocotyls. Maximum production of phaseollin occurred during the early exponential phase of culture growth. Phaseollin was converted to phaseollinosoflavan by these cultures and this conversion occurred during accumulation of the phytoalexins. Factors affecting phytoalexin accumulation in these cultures are discussed.

INTRODUCTION

Phytoalexins are not present in healthy tissues of *Phaseolus vulgaris*, but accumulate when challenged by microorganisms [1–5] and after treatment with various abiotic stimuli [6] and fungal products [7, 8]. Their production in response to these agents is generally associated with cell damage [6] (see [9] for possible exception). Recently it has been shown that aqueous extracts obtained from bean hypocotyls which were damaged by freezing, macerating or autoclaving stimulated phytoalexin formation when applied to hypocotyl tissue [10]. The activity of these extracts was thought to be due to a component (termed constitutive elicitor) which is released, after damage, from cells in which it is normally restricted.

Suspension cultures of *P. vulgaris* synthesized phaseollin when grown on defined media in the absence of biotic or abiotic inducers [11]. Preliminary work confirmed these findings. but the amount of this and other phytoalexins produced was much reduced after continual subculture. In this paper we report that bean cell suspensions, which contained low endogenous levels of phytoalexins, accumulated large quantities of these compounds when treated with an aqueous extract of bean hypocotyl tissue. Factors influencing the accumulation of phytoalexins are described in an attempt to define optimum conditions for the assay of extracts for phytoalexin inducing activity.

RESULTS

Stimulation of phytoalexin formation in suspension cultures by hypocotyl extracts

The amounts of the phytoalexins in 7-day-old cultures 15 hr after the addition of the hypocotyl extract or distilled water were measured. Phaseollin was the major phytoalexin (505 μg/g) produced by cultures incubated with the hypocotyl extract; phaseollidin, (31 μg/g), phaseollinisoflavan (207 μg/g) and kievitone (151 μg/g) were also present but at lower concentrations. Only traces (< 5 μg/g) of these inhibitors were detected in cultures treated with distilled water.

Since phaseollin was the predominant phytoalexin, this compound was routinely measured in all further experiments. The other phytoalexins were monitored by spraying developed chromatograms with diazotised *p*-nitroaniline. When the pattern of their accumulation differed from that of phaseollin, the amounts of these compounds were also measured.

Stimulation of phaseollin formation by hypocotyl extracts during culture growth

When cultures were treated with the hypocotyl extract for 15 hr at different times during their growth, phaseollin increased with time after transfer of cells to fresh medium (Fig. 1). Maximum levels of phaseollin occurred at the onset of exponential growth. However, as the rate of growth increased the amount of phaseollin present was much less. The amount of phaseollin in treated cultures during the later stages of growth and also immediately after transfer to fresh medium was similar to those in untreated cultures. Phaseollin levels in the medium, although less than those in the cells followed a similar pattern.

Treatment of cultures with the hypocotyl extract at different stages of growth showed that during the lag phase (2-day-old cultures) the amount of phaseollin formed did not change with further incubation (Fig. 2). Six days after transfer of cells, treated cultures accumulated phaseollin at a greater rate and reached much higher concentrations. With the onset of exponential growth (8-day-old cultures), phaseollin followed the same pattern of accumulation as in 6-day-old cultures for 15 hr. However, this was followed by a rapid decrease in concentration during the next 9 hr after which levels became similar to those in untreated cultures. Again the concentrations of phaseollin in the cells was paralleled by those in the culture medium.

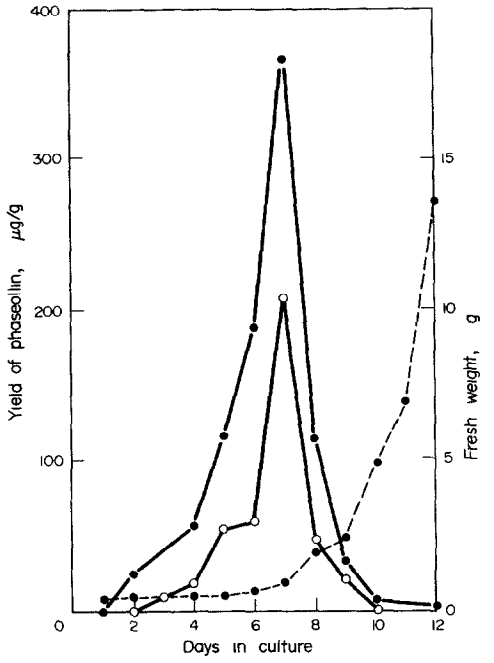

Fig. 1. Growth of cultures (●———●) and accumulation of phaseollin in cells (●———●) and culture media (○———○) after treatment with the hypocotyl extract for 15 hr.

The relationship between growth of cultures and phytoalexin accumulation

When 6-day-old cultures were treated with the hypocotyl extract their growth was prevented for 40–60 hr. During this time phaseollin accumulated to high levels (Fig. 3). Growth recommenced as the concentration of phaseollin decreased. At the same time there was a rapid increase in the level of phaseollinisoflavan but this also disappeared with the onset of higher growth rates. Subsequently, both compounds remained at levels similar to untreated cultures. The resumed rate of growth of these cultures was similar to that of untreated cultures.

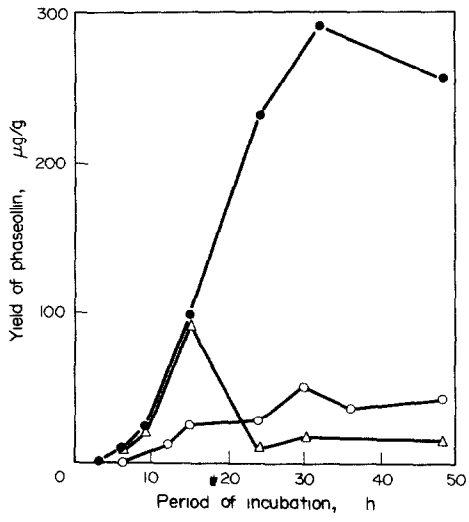

Fig. 2. Accumulation of phaseollin in cells after addition of the hypocotyl extract to 2 (○———○), 6 (●———●) and 8 (△———△)-day-old cultures.

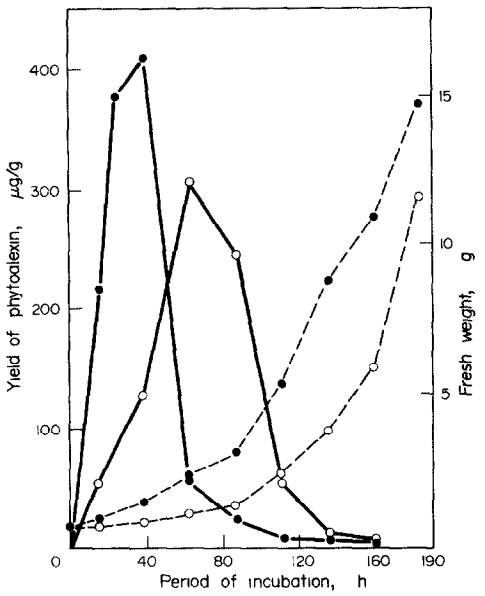

Fig. 3. Accumulation of phaseollin (●———●) and phaseollinisoflavan (○———○) in 6-day-old cultures after addition of the hypocotyl extract and growth of treated (○— — —○) and untreated (●— — —●) cultures.

Although cultures treated with the hypocotyl extract produced brown pigments along with the phytoalexins, the cells retained their ability to reduce 2,3,5-triphenyltetrazolium chloride to a red formazan [12].

Metabolism of phaseollin by suspension cultures

When phaseollin (5 μg/ml) was added to untreated cultures at different stages of growth (3, 6 and 9 days old) it disappeared from these cultures to a similar extent (90% loss after 5 hr). Time course studies of phaseollin metabolism by 6-day-old cultures revealed that the initial rapid loss of phaseollin was accompanied by an increase in the concentration of phaseollinisoflavan (Fig. 4). Thereafter, the amount of the isoflavan present

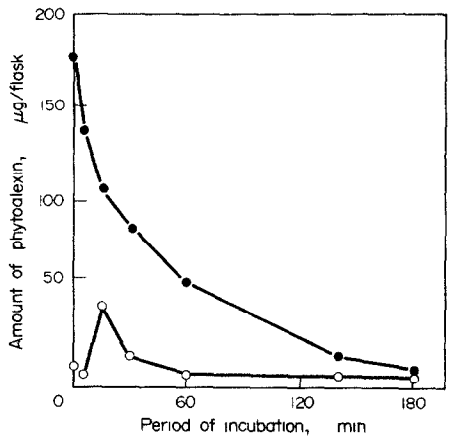

Fig. 4. Metabolism of phaseollin by 6-day-old cultures. phaseollin (●); phaseollinisoflavan (○).

also declined, reaching levels comparable to those before the addition of phaseollin.

Phaseollinisoflavan was shown to be metabolite of phaseollin using phaseollin-[^{14}C]. After addition of phaseollin (sp. act. 150700 dpm/mg) to 6-day-old cultures, extraction 30 minutes later afforded unchanged phaseollin (42% recovery) and phaseollinisoflavan (7.4% yield). The specific activities were calculated to be 151037 and 152982 dpm/mg respectively. Radioactive label was also detected in the residue remaining after EtOH extraction (11.3%) and in the aqueous phase after partitioning with Et_2O (6.7%).

DISCUSSION

The data presented here confirm parallel studies carried out using hypocotyl tissue, that a water soluble component of bean cells will elicit phytoalexin formation. Unlike previous findings [11] all the bean phytoalexins formed in plant tissues after infection with fungi [1–3], bacteria [5] and virus [4] were produced by cell suspensions treated with the hypocotyl extract, indicating that the potential to synthesise phytoalexins is maintained during the transfer of bean hypocotyl cells to axenic culture. The formation of brown pigments in treated cultures suggests that the hypocotyl extract stimulated biochemical pathways associated with general phenolic synthesis.

The pattern of stimulation of phaseollin formation by the hypocotyl extract during the growth cycle suggests that cultures only produce phytoalexins during the later stages of the lag phase and the early stages of the growth phase. Since maximum production of phaseollin only occurred for a short period during the growth of cultures, assays for phytoalexin inducing activity have to be carried out on cultures at a defined growth stage. Preliminary studies with the hypocotyl extract using 6-day-old cultures and an incubation period of 24 hr have proved to be a consistent and useful assay material. Results so far have shown that the active component of these extracts is of low molecular weight, being retained by Sephadex G-10. is neither basic nor acidic and with the exception of methanol is not soluble in organic solvents.

Although cultures can metabolize phaseollin at all stages of growth, it is unlikely that metabolism affects the accumulation of phaseollin during the lag phase, since phaseollin formed during this period did not disappear after prolonged incubation. However, phaseollin formed in growing cultures after treatment with the hypocotyl extract does eventually disappear indicating that metabolism of phaseollin may have an important part to play in determining the level of phaseollin attained at this time. It is not possible, at present, to to suggest any physiological or biochemical similarities between cell suspensions during the period when phytoalexins are produced, and hypocotyl cells.

The conversion of phaseollin to phaseollinisoflavan demonstrated here has also been shown using the fungus *Stemphylium botryosum* [13]. The changes in concentration of these phytoalexins in suspension cultures treated with the hypocotyl extract indicates that this conversion occurred during phytoalexin accumulation. The disappearance of the isoflavan from these cultures suggests that it is further metabolized to as yet undetected products. Whether similar conversions occur in either healthy or infected hypocotyl tissue remains to be determined.

Although culture growth was delayed by the addition of the hypocotyl extract, it seems probable that this was due not to direct effects of the hypocotyl extract but rather to the production of phaseollin which is known to be toxic to these cells [14].

These results show that further studies on phytoalexin formation in suspension culture will not only be of value to the study of their biosynthesis and enzymology but also to investigations of the induction and control of phytoalexin synthesis.

EXPERIMENTAL

Plant material. All experiments were carried out with suspension cultures of *Phaseolus vulgaris* cv Kievitsboon koekoek 18–21 months after initiation. The cultures were prepared and incubated as described elsewhere [14]. Transfer of cells was carried out by diluting cultures in the stationary phase of growth (14–16 g cells) with 1 l. fr. medium and dispensing aliquots (40 ml) into 150 ml conical flasks to give 0.4–0.6 g cells per flask. The growth of cultures was measured by weighing the cells after they were filtered through filter paper (Whatman No. 1) under vacuum, air being allowed to pass through the filter paper for 30 sec. Duplicate flasks were taken for each growth measurement and for analysis of phytoalexins.

Prepn of hypocotyl extract. Hypocotyls of *P. vulgaris* cv Prince (250 g) were excised from 5-day-old etiolated seedlings and autoclaved at 121° for 20 min in a 1 l. beaker. On cooling, the 'juice' released from the collapsed tissue was decanted under sterile conditions and stored at −20° until required. 2 ml. of this extract was added to each flask.

Isolation and measurement of phytoalexins. Filtered cells were extracted by boiling in EtOH for 10 min. After evapn *in vacuo* at 30°, the residue was taken up in H_2O. This extract and culture media filtrates were partitioned with Et_2O and after removal of solvent the residue was dissolved in Me_2CO for TLC. All extracts were initially separated by TLC in C_6H_6–MeOH (9:1). When only phaseollin was measured it was recovered from R_f 0.74. In experiments where both phaseollin and phaseollinisoflavan were measured, chromatograms developed as described above, were dried and redeveloped in C_6H_6–EtOAc–MeOH (25:4:2). Phaseollin and phaseollinisoflavan were recovered from R_fs 0.78 and 0.55 respectively. Kievitone and phaseollidin were measured after eluting the corresponding band and rechromatographing in $CHCl_3$–EtOH (100:3), developed five times and hexane–Me_2CO (2:1) respectively. All phytoalexins were eluted in EtOH and measured using the published extinction coefficients [4]. The results were not adjusted for extraction losses during preparation of extracts or subsequent TLC (20–40%).

Metabolism of phaseollin. Crystalline phaseollin, obtained from virus-infected hypocotyls [4], was dissolved in DMSO (2 mg/ml) and added to flasks to give 5 μg phaseollin and 2.5 μl DMSO per ml culture media. At intervals after addition of phaseollin, 50 ml EtOH was added to duplicate flasks to prevent further metabolism. Phaseollin and phaseollinisoflavan were extracted and measured as described above. Zero time readings were obtained by adding phaseollin to boiled cultures.

Isotope experiment. Five 6-day-old suspension cultures were each administered 200 μg phaseollin-[^{14}C] (sp. act. 150700) obtained from virus-infected hypocotyls [15]. EtOH was added after 30 min incubation and phaseollin and phaseollinisoflavan were recovered from the combined extracts of the cultures as previously described.

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REFERENCES

1. Bailey, J. A. (1974) *Physiol. Plant Pathol.* **4**, 477.
2. Smith, D. A., Van Etten, H. D. and Bateman, D. F. (1975) *Physiol. Plant Pathol.* **5**, 51.
3. Van Etten, H. D. and Smith, D. A. (1975) *Physiol. Plant Pathol.* **5**, 225.
4. Bailey, J. A. and Burden, R. S. (1973) *Physiol. Plant Pathol.* **3**, 171.
5. Gnanamanickam, S. S. and Patil, S. S. (1977) *Physiol. Plant Pathol.* **10**, 159.
6. Van Etten, H. D. and Pueppke, S. G. (1976) in *Biochemical Aspects of Plant–Parasite Relationships* (Friend, J. and Threlfall, D. R. eds) p. 239. Academic Press, London.
7. Anderson-Prouty, A. J. and Albersheim, P. (1975) *Plant Physiol.* **56**, 286.
8. Cruickshank, I. A. M. and Perrin, D. R. (1968) *Life Sci.* **7**, 449.
9. Paxton, J., Goodchild, D. J. and Cruickshank, I. A. M. (1974) *Physiol. Plant Pathol.* **4**, 167.
10. Hargreaves, J. A. and Bailey, J. A. (1978) in preparation.
11. Dixon, R. A. and Fuller, K. W. (1976) *Physiol. Plant Pathol.* **9**, 299.
12. Towill, L. E. and Mazur, P. (1975) *Can. J. Botany* **53**, 1097.
13. Higgins, V. J., Stoessl, A. and Heath, M. C. (1974) *Phytopathology* **64**, 105.
14. Skipp, R. A., Selby, C. and Bailey. J. A. (1977) *Physiol. Plant Pathol.* **10**, 221.
15. Burden, R. S., Bailey, J. A. and Vincent, G. G. (1974) *Phytochemistry* **13**, 1789.