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Regulation of Phytoalexin Synthesis in Jackbean Callus Cultures

STIMULATION OF PHENYLALANINE AMMONIA-LYASE AND o-METHYLTRANSFERASE[1]

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ABSTRACT

Jackbean, *Canavalia ensiformis* (L.), callus tissues synthesized the phytoalexin, medicarpin (3-hydroxy-9-methoxypterocarpan), when treated with spore suspensions of *Pithomyces chartarum* (Berk. and Curt.) M. B. Ellis, a nonpathogen of jackbean. Medicarpin was isolated from treated callus tissue and identified by its ultraviolet and mass spectra. The minimum spore concentration found to elicit medicarpin synthesis after 26 hours was 1×10^5 spores/ml; levels of medicarpin in callus tissue increased linearly up to 1×10^7 spores/ml, indicating that the recognition sites for presumed elicitors were not saturated. Medicarpin was first detected in callus treated with 1×10^7 spores/ml, 6 to 12 hours after application, and the concentration reached a maximum at 48 hours, slowly declining thereafter to 72 hours. In callus treated with 3.15 mM $HgCl_2$, medicarpin concentrations were also maximum by 48 hours. Phenylalanine ammonia-lyase (EC 4.3.1.5) activity increased 2-fold in spore-treated callus after 36 hours. Isoliquiritigenin, daidzein, and genistein *o*-methyltransferase (EC 2.1.1.6) activities were increased 3- to 4-fold in treated callus. Caffeic acid and naringenin were more efficient substrates for *o*-methyltransferase activity than the other flavonoids or apigenin, but there was no increase in these *o*-methyltransferase activities in spore-treated callus. The phytoalexin response in this callus tissue culture system compares well with natural plant systems and should be an excellent system for investigating regulation of phytoalexin synthesis.

Phytoalexins are compounds produced by plants in response to microorganisms (13, 19). They vary from acetylenic compounds to pterocarpan isoflavonoids (13, 19). Evidence for the role of phytoalexins in disease resistance is accumulating. In some natural host/pathogen systems, phytoalexin production is thought to be initiated in response to certain microbial metabolites, designated specific elicitors (15). In laboratory systems, phytoalexin synthesis has been induced by a wide variety of nonspecific elicitors, including proteins, peptides, amino acids, RNA synthesis inhibitors, and heavy metal salts (17, 19, 21). Phytoalexin accumulation begins 6 to 24 hr after cells are treated with elicitor. Synthesis occurs only in treated plant cells, indicating that phytoalexin synthesis is regulated.

In a preliminary note, one of us (D. L. G.) reported that an isoflavonoid phytoalexin, medicarpin (3-hydroxy-9-methoxypterocarpan), was induced in jackbean callus issue by fungal spores or $HgCl_2$ (9). This tissue culture-induction system was developed to take advantage of the relatively uniform, achlorophyllous, nondifferentiated tissue in studies of phenylpropanoid phytoalexin regulation. Medicarpin induction was previously observed for a number of plants and may be an important factor contributing to disease resistance in alfalfa and red clover (6, 11-14).

The pathway of medicarpin biosynthesis is thought to involve the conversion of phenylalanine to cinnamic acid which condenses with acetate to form a chalcone. The chalcone is then isomerized to an isoflavone by an aryl migration and is then converted through a series of poorly understood steps to the pterocarpan structure (3-5), (Fig. 1). Some enzymes involved in the biosynthesis of isoflavonoids include PAL[2] (EC 4.3.1.5), hydroxycinnamate CoA ligase (EC 6.2.1.-.), cinnamic acid 4-hydroxylase (EC 1.14.13.11), and *o*-methyltransferase, OMT (EC 2.1.1.6) (10). The activity of these enzymes has been shown to increase when phytoalexins and flavonoids are induced (10, 14, 21), and the increases generally parallel phytoalexin concentration.

In this paper we report the spore concentration response and the time course of medicarpin accumulation in jackbean callus. We also report the stimulation of PAL and OMT activities during activation of the phytoalexin response.

MATERIALS AND METHODS[3]

Callus Tissue Culture. Cultures were started and subcultured on modified Miller's medium (18), but $Cu(NO_3)_2 \cdot 3H_2O$ and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ were omitted. Bactoager 10 g/l, α-naphthalene acetic acid 0.4 mg/l, and kinetin 0.25 mg/l were also added. Incubations were at 27 C under incandescent lights (21.4 $\mu E\ sec^{-1}m^{-2}$) and a 16-hr light cycle. Aseptic conditions were maintained at all times. Tissues were grown on 50 ml of agar medium in 125-ml Erlenmeyer flasks covered with aluminum foil.

Jackbean (*Canavalia ensiformis* [L.]) seed surfaces were disinfected by soaking for 5 min in 95% ethanol-H_2O-5.25%

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[2] Abbreviations: PAL: phenylalanine ammonia-lyase (EC 4.3.1.5); OMT: *o*-methyltransferase (EC 2.1.1.6); MEK: methyl ethyl ketone.

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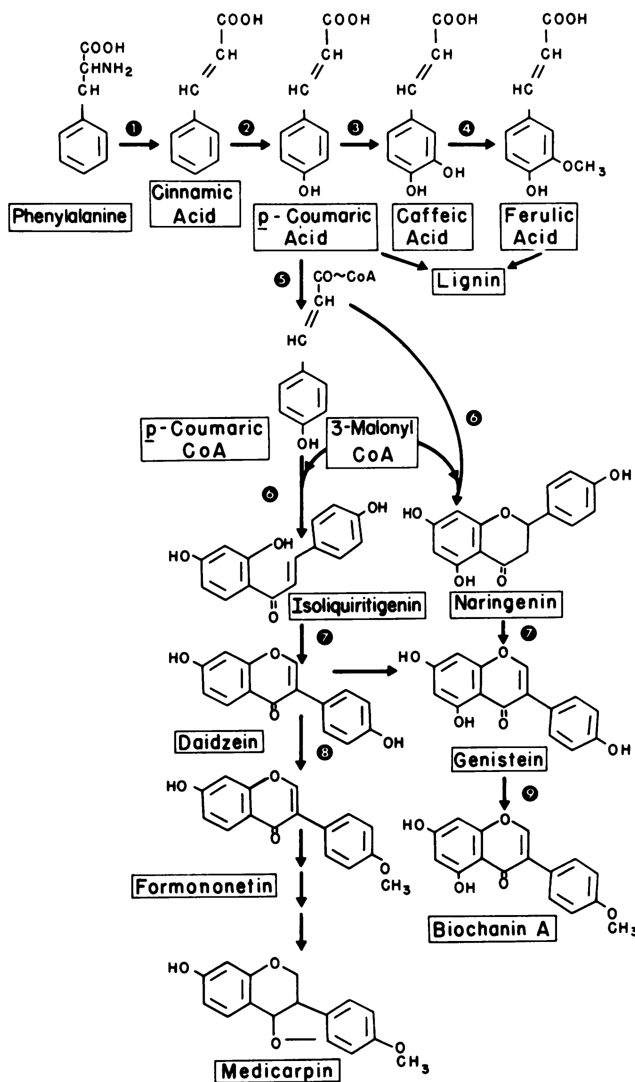

FIG. 1. Proposed pathway for the biosynthesis of medicarpin and the enzymes involved: (1) phenylalanine ammonia-lyase; (2) cinnamic acid 4-hydroxylase; (3) *p*-coumaric hydroxylase; (4) caffeic acid *o*-methyltransferase, (5) *p*-coumarate CoA ligase; (6) chalcone flavone synthetase; (7) aryl migration; (8) daidzein *o*-methyltransferase; (9) genistein *o*-methyltransferase.

NaClO (75:20:5, v/v). After seeds imbibed sterile H_2O for 24 hr, the seed coats were removed and surface sterilization was repeated. The seeds were germinated under dark conditions on agar medium until hypocotyls were about 2 cm long (3–5 days). Pieces of hypocotyl (6–8 mm long) were transferred to agar medium. Callus formed after 3 to 4 weeks. Subcultures were started every 3 weeks by transferring three pieces (5-mm cubes) per flask to new media flasks.

Preparation of Spores. Spores of *Pithomyces chartarum* (Berk. and Curt.) M. B. Ellis, a nonpathogen of jackbean plants, were obtained from cultures maintained on V-8 juice agar for 5 to 10 days at 24 C and in a 12-hr light cycle. Spores were scraped from the unwetted agar surface with a spatula and suspended in sterile H_2O. The suspension was filtered through four layers of cheesecloth, and the concentration of spores adjusted to 1×10^7 spores/ml after counting with a hemocytometer. Fresh preparations of spore suspensions were used in all experiments.

Identification of Medicarpin. A suspension of *P. chartarum* spores was applied to the surfaces of 3-week-old callus tissues (0.33 ml/callus piece). The foil covers were replaced and the flasks were incubated for 48 hr. The treated tissue (60 g) was homogenized with a Waring Blendor in 200 ml of 95% ethanol. Insoluble material was removed by centrifugation, the supernatant fraction was evaporated to dryness *in vacuo*, and the residue was extracted with ethyl acetate-methanol (5:1, v/v). This solution was evaporated to dryness *in vacuo*, dissolved in 70% ethanol, applied to a Sephadex LH-20 column (1 × 20 cm) (equilibrated in 70% ethanol) and medicarpin eluted with 70% ethanol. Medicarpin was detected in fractions by silica gel TLC; the solvent was hexanes-methyl ethyl ketone-formic acid (70:30:0.5, v/v) and visualized with diazotized *p*-nitroaniline. Fractions containing medicarpin were pooled, applied to a Brinkmann SILG-100/UV-254 (1-mm layer) preparative TLC plate, and developed in HMF solvent; medicarpin was located with UV light and by spraying a reference sample of medicarpin. The area corresponding to medicarpin was scraped from the plate and eluted with ethyl acetate-methanol (5:1, v/v); medicarpin was identified by MS, UV spectrophotometry, and TLC co-chromatography.

Spectrophotometric Determination of Medicarpin. Pieces of callus were individually weighed and homogenized in 3 volumes of MEK with a motor-driven Teflon pestle. The MEK extract was decanted, and 1 ml withdrawn; the aliquot was then evaporated to dryness *in vacuo*. The residue was dissolved in 0.2 ml of MEK, 0.1 ml of this solution was applied to a 1.9-cm band on a silica gel plate, and the plate was developed in HMF solvent. The area having the same R_F (0.45) as medicarpin (located by spraying reference medicarpin) was scraped from the plate, and the isolated silica gel extracted with 1.3 ml of ethyl acetate-methanol (5:1, v/v). After centrifugation, 1 ml of the supernatant solution was transferred to a test tube, evaporated to dryness, flushed with N_2 for 15 sec and 1 ml of spectral grade methanol was added. This solution was scanned from 250 to 320 nm and the concentration of medicarpin determined using the molar extinction coefficient reported by Smith *et al.* (25): $C = (A_{287} - A_{310}) \times (7{,}944\ l\text{mol}^{-1})$.

Concentration Response. The callus tissue in each of 12 flasks (three pieces of 3-week-old jackbean callus/flask) was irrigated with *P. chartarum* spore suspension (1×10^3 spores/ml to 1×10^7 spores/ml). After 26 hr of incubation, each callus piece was assayed for medicarpin.

Time Response. Nine flasks, each containing three pieces of 3-week-old jackbean callus tissue, were irrigated with *P. chartarum* spore suspension (1×10^7 spores/ml). At the end of each incubation period, three callus pieces were assayed for medicarpin.

Enzyme Assays. PAL activity was assayed by the procedure of Camm and Towers (2). The assay mixture contained 0.114 μCi of L-[1-^{14}C]phenylalanine (New England Nuclear, 40–60 mCi/mmol), and 2.5 μM of cold phenylalanine. OMT activity was measured by the procedure of Wegenmayer *et al.* (27) and Glass and Bohm (8). The assay mixture contained 0.068 μCi of S-adenosyl-[^{14}C-methyl]methionine (Schwarz/Mann, 56 mCi/mmol) and 200 nM of unlabeled substrate. The radioactive products of OMT assays were identified by co-chromatography with authentic standards. Caffeic acid was purchased, genistein, daidzein, and naringenin were supplied by R. T. Sherwood, and apigenin and isoliquiritigenin were kindly supplied by B. Bohm and G. H. N. Towers. Solvent systems used for silica gel and cellulose TLC were: (*a*) benzene-glacial acetic acid-H_2O (10:7:3, v/v, upper phase); (*b*) benzene-95% ethanol (98:2, v/v); and (*c*) chloroform-isopropyl alcohol (10:1, v/v). Compounds were located with diazotized *p*-nitroaniline and diazotized sulfanilic acid.

General Methods. Enzyme preparation was according to Vance and Sherwood (26) and protein was determined by the procedure of Lowry *et al.* (20). Radioactivity in products from

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enzyme assays was determined in a scintillation cocktail containing 4 g of Omnifluor (New England Nuclear) in 750 ml of toluene and 250 ml of 95% ethanol. Counting efficiency was 80% of ^{14}C.

RESULTS

Identification of Medicarpin. From 60 g of inoculated jackbean tissue, we isolated 0.89 mg of a compound that had the same TCL R_F as authentic medicarpin in solvent systems HMF, (*a*), (*b*), and (*c*). The UV spectrum was λ_{max}^{MeOH} nm 212, 225 sh, 282, 287. The MS diagnostic peaks were: m/e 270 (M+, $C_{16}H_{14}O_4$), 269, 255, 161, 148. These data agree with those published for medicarpin (14, 25) and were identical to data taken from authentic medicarpin.

Optimum *P. chartarum* Spore Concentration. Jackbean callus tissue was inoculated with concentrations of *P. chartarum* spores from 0 to 1 × 10^7 spores/ml. Medicarpin concentrations determined after 26-hr incubations are plotted in Figure 2 (panel A). At spore concentrations of 10^3 and 10^4 spores/ml, medicarpin was detectable, but at very low levels. From spore concentrations of 10^4 to 10^7 spores/ml, medicarpin levels increased sharply, and at a linear rate. We therefore used 10^7 spores/ml in all experiments.

Time Response. Medicarpin concentrations in jackbean callus tissue were determined at different times after treatment with *P. chartarum* spores. Medicarpin was detected in callus 12 hr after treatment, but not at 0 or 6 hr (Fig. 2, panel B). The phytoalexin concentration increased through 48 hr, and then slowly declined. We also tested the response of jackbean callus to $HgCl_2$ and found essentially the same time response as we observed for *P. chartarum* spores. Optimum production of medicarpin for a 48-hr time period was found at 3.15 mM $HgCl_2$.

PAL. PAL activity, as determined by conversion of [^{14}C]phenylalanine to *t*-[^{14}C]cinnamic acid, was consistently higher in jackbean callus tissue treated with *P. chartarum* spores (Table I) than in untreated callus tissue. The mean increase for the four experiments was nearly 2-fold (Table I). PAL was not assayed in spores. The jackbean callus tissues used in the four experiments contained medicarpin (Table I) at the time they were assayed for enzyme activity. Thus, the pathway for medicarpin synthesis was functioning when PAL was assayed. Medicarpin concentrations were consistent for all experiments, except for the higher concentrations in experiment 2 (Table I). These medicarpin levels were lower than shown in Figure 1A or Figure 1B, but were not from the same experiments.

OMT. The level of OMT activity in jackbean callus tissue (Table II) was a function of both the substrate and the physiological state of the tissue. The most effective methyl acceptors tested in noninoculated tissue were caffeic acid, a lignin precursor, and naringenin, a flavonoid precursor. OMT preparations from tissue incubated 36 hr with *P. chartarum* spores showed a slightly higher methylation of caffeic acid and naringenin as compared to preparations from control tissue (Table II). The other substrates tested with OMT preparations were much less effective than caffeic acid and naringenin in preparations from noninoculated tissue. In the presence of isoliquiritigenin, daidzein, and genistein, OMT preparations from inoculated tissues had a 3- to 4-fold increase in activity (Table II). OMT activity with apigenin as the substrate did not increase appreciably in inoculated tissue. OMT activity was not measured in the spores.

Products of the chalcone and two isoflavonoids could have been methylated at more than one hydroxyl position. TLC co-

FIG. 2. Panel A: effect of *Pithomyces chartarum* spore concentration on medicarpin concentration in jackbean callus tissue. Incubation time was 26 hr. Panel B: change in medicarpin concentration with time in jackbean callus tissue treated with *P. chartarum* spores. Spore concentration was 10^7/ml. Each point was the mean of three determinations; vertical lines denote the range of the values.

Table I. Phenylalanine ammonia-lyase activity and medicarpin concentration in jackbean callus tissue inoculated with *P. chartarum* spores*.

Exp. No.	PAL Control	PAL Treated	Increase	Medicarpin Control	Medicarpin Treated
	dpm cinnamic acid/mg protein/hr		%	µg/g fresh wt.	
1	19,044 ± 2651	39,450 ± 822	207	0	26.3 ± 2.2
2	19,578 ± 1570	31,794 ± 2050	162	0	42.5 ± 15.9
3	18,783 ± 428	30,212 ± 910	161	0	21.4 ± 10.1
4	18,995 ± 1171	40,112 ± 2119	211	0	23.8 ± 9.3

*Callus tissue was assayed for PAL and medicarpin before, and 36 hr after spores were applied. Results are mean of three determinations (± standard deviation). Substrate levels are given in the materials and methods.

Table II. O-methyltransferase activities in jackbean callus tissue inoculated with *P. chartarum* spores*.

Substrate	Experiment no. 1	2	3	4
	dpm product/mg protein/hr			
Caffeic acid				
Control	29,893 ± 2120	48,058 ± 1412	48,340 ± 7122	49,957 ± 5220
Treated	32,058 ± 1712	54,372 ± 8503	66,203 ± 6901	51,663 ± 4747
Isoliquiritigenin				
Control	---	2,112 ± 1110	1,898 ± 1197	2,752 ± 2190
Treated	---	6,419 ± 921	5,305 ± 2778	8,601 ± 2171
Daidzein				
Control	6,887 ± 2124	3,776 ± 820	0	7,886 ± 917
Treated	17,933 ± 2199	20,108 ± 2256	4,570 ± 854	33,987 ± 2444
Genistein				
Control	4,009 ± 1913	6,474 ± 890	175 ± 127	7,694 ± 4312
Treated	14,051 ± 1723	26,026 ± 2637	5,216 ± 872	29,127 ± 3392
Naringenin				
Control	---	40,496 ± 3751	27,518 ± 3174	18,863 ± 1521
Treated	---	47,333 ± 2802	30,193 ± 1722	20,548 ± 1058
Apigenin				
Control	---	3,914 ± 923	4,714 ± 903	---
Treated	---	5,127 ± 741	5,510 ± 1112	---

*Callus tissue was assayed for OMT activity for each substrate before and 36 hr after spore suspensions were applied. Results are the mean of three determinations (± standard deviation). Medicarpin concentrations in the callus are given in Table I. Substrate levels are given in the materials and methods.

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chromatography with authentic standards in three different solvent systems established that the chalcone was methylated at position 4, and the isoflavones at position 4′.

Medicarpin levels in inoculated callus tissue measured at the same time as OMT are given in Table I.

DISCUSSION

In this report, we have established that the phytoalexin, medicarpin, is not detected in jackbean hypocotyl callus tissue unless the tissue was inoculated with *P. chartarum* spores or treated with $HgCl_2$ solutions. During the 26-hr incubation period, the callus pieces appeared to lose water and to turn from a light tan to a tan color. This might suggest increased production of phenolic compounds, as would be expected during a hypersensitive response. There was no visible tissue necrosis. Results in Figure 2A suggest that: (*a*) the presumed elicitor substance in the spores must be present at a critical concentration before jackbean cells respond and initiate medicarpin synthesis; and (*b*) that the recognition site in the jackbean cells was not saturated. Medicarpin was not detected in inoculated tissue until 10 hr after addition of spores (Fig. 2B); this result was consistent with delays characteristic of phytoalexin accumulation in most systems studied (7, 19, 24). The time course for medicarpin accumulation (Fig. 2B) was similar to phytoalexin accumulation reported for other plant systems (7, 13, 19). The medicarpin level in spore-treated jackbean callus after 48 hr was calculated to be 420 μg/g of dry tissue. This value was consistent with medicarpin levels (300–700 μg/g) reported in spore-treated, detached alfalfa, and jackbean hypocotyls (12, 14).

Our results indicated that PAL had 2-fold higher activity in spore-treated callus incubated for 36 hr than in callus not inoculated (Table I). These observations are consistent with other reports indicating that PAL increases during isoflavonoid phytoalexin synthesis (21, 24). Although PAL apparently catalyzes the first step in medicarpin biosynthesis, the stimulation of this enzyme may not be a key step in regulation of medicarpin biosynthesis. In soybean and cowpea PAL was not shown to play a role in regulation of phytoalexin biosynthesis (21, 22).

The step in the medicarpin biosynthetic pathway at which methylation occurs is not established. A requirement for a methylation step during medicarpin synthesis was suggested by Dewick's studies on incorporation of isotopic precursors into medicarpin (3–5). He concluded that the methylation was an integral part of the aryl migration step (3). Our data (Table II) showed that isoliquiritigenin and daidzein were both methylated in the presence of S-adenosyl-[^{14}C-methyl]methionine and the OMT preparation. This activity was increased 3- and 4-fold, respectively, after 36 hr of exposure of the tissue to *P. chartarum* spores. This suggests that methylation could occur before or after the chalcone ring closure step.

Increased methylation of isoflavonoid precursors of medicarpin in inoculated tissue indicates also that *P. chartarum* spores selectively stimulate OMT activity: that is, isoflavonoid OMT activities were increased, but cinnamic acid and flavone OMT activities were not increased (Fig. 1 and Table II). This may be a function of altered substrate specificity or altered isozymes of OMT. Poulton *et al.* (23) have reported the occurrence of OMT isozymes in soybean suspension cultures, one specific for flavonoids, the other specific for cinnamic acids. A specific OMT has also been reported for isoflavonoids (27) in chickpea. We further found that genistein (4′,5,7-trihydroxyisoflavone) OMT activity was increased 4-fold in 36-hr treated tissue. Since it is unlikely that genistein could be a precursor to medicarpin, the increased amounts of this intermediate metabolite may be utilized for synthesis of other isoflavonoid or pterocarpanoid compounds. These results indicate OMT may be required for medicarpin synthesis. This is the first report of increased OMT activity in plant cells during phytoalexin synthesis.

In these experiments we have assumed that PAL and OMT activities before and after spore treatments were of jackbean origin, rather than *P. chartarum* or a combination of the two. At this time we cannot distinguish between these possibilities. We do have data which establish that PAL activity was unchanged in callus before and immediately after addition of spores, suggesting that PAL activity in spores was negligible compared to callus activity. Also, the high ratio of callus to spores (200:1, wt/wt) makes it unlikely that PAL or OMT specific activities would be high enough to contribute significantly to the measured activities. Final proof that these increased enzyme activities are not of fungal origin will require studies with purified elicitor fractions.

Although phytoalexin synthesis has been reported in callus culture systems, the levels produced were lower than in the corresponding plant systems (16), or the phytoalexin was produced continuously in untreated tissue (1). The phytoalexin, glyceollin, was synthesized in soybean cell suspension cultures at levels comparable to those reported in hypocotyls (7). Results presented here suggest that phytoalexin and enzyme responses in the spore-treated jackbean callus system compare well with "natural" plant systems. Jackbean callus provides a ready supply of tissue, free of contaminant organisms, for biological studies on the regulation of phytoalexin synthesis. Preliminary data suggest that two other phenylpropanoid enzymes, cinnamic acid 4-hydroxylase and hydroxycinnamic acid CoA ligase, also had higher activities in jackbean callus inoculated with *P. chartarum* spores than in control tissues (Vance and Gustine, unpublished data). Data presented here suggest that two of the presumed enzymes in the medicarpin biosynthetic pathway are increased upon spore inoculation, resulting in elevated catalytic activities. Future studies will attempt to determine if all of the enzymes in the pathway are regulated, if they are regulated in a coordinated fashion, and if such regulation accounts for increased medicarpin synthesis.

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