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CASBENE: AN ANTI-FUNGAL DITERPENE PRODUCED IN CELL-FREE EXTRACTS OF *RICINUS COMMUNIS* SEEDLINGS*

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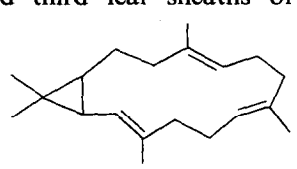
Key Word Index—*Ricinus communis*; Euphorbiaceae; castor bean; casbene; diterpene hydrocarbon; phytoalexin; biosynthesis; anti-fungal agent.

Abstract—The capacity of cell-free extracts of 2·5-day-old castor bean seedlings for synthesis of casbene from mevalonic acid were compared for seedings which had been germinated under sterile conditions and seedlings which were intentionally exposed to fungal cultures. Extracts from seedlings exposed to cultures of *Rhizopus stolonifer*, *Aspergillus niger* or *Fusarium moniliforme* produced much higher levels of casbene than extracts from sterile controls; the initial rates of casbene synthesis were 20–40 times higher in the extracts of seedlings that had been exposed to fungus. Although some variation in the capacity for synthesis of other diterpene hydrocarbons from mevalonic acid was seen in the two types of extracts, no consistent or striking stimulation in the synthesis of any of these was noted under these conditions of exposure of the seedlings to fungi. The potato–dectrose agar used as a fungal growth medium did not itself evoke the increase in casbene synthesis. Intact mycelia and cell-free extracts of mycelia of *Rhizopus stolonifer* gave no indication of diterpene biosynthesis from mevalonic acid. Purified casbene at concentrations of 10 $\mu g\ ml^{-1}$ or greater retarded the development of *A. niger* on potato–dextrose medium. Casbene was also found to inhibit the endogenous and gibberellic acid-stimulated growth of leaf sheaths of the *dwarf*-5 mutant of *Zea mays* and of the growth of the K-12 strain of *Escherichia coli* on glucose–minerals medium. It is suggested that casbene may serve the castor bean plant as a phytoalexin.

INTRODUCTION

Cell-free extracts of young seedlings of *Ricinus communis* L. (castor bean) produce a mixture of at least five diterpene hydrocarbons from mevalonic acid or geranylgeranyl pyrophosphate [1,2]. Four of these are the known substances *ent*-kaurene, *ent*-beyerene [(+)-stachene], *ent*-trachylobane and *ent*-sandaracopimaradiene. The fifth, called casbene, was assigned a tentative structure **(1)** as a macrocyclic compound containing a 14-member ring and a fused cyclopropane ring on the basis of spectral evidence [1].

While *ent*-kaurene may serve as an intermediate in the biosynthesis of the gibberellins as it does in other organisms, there was no indication of the physiological role which the other diterpenes might play. However, it was noted that exogenous casbene inhibited the elongation of the second and third leaf sheaths of the *dwarf*-5



(1) Casbene

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mutant of *Zea mays* L., in contrast to *ent*-kaurene and gibberellins which stimulate the growth of these seedlings [1]. Also it was observed that the ability of extracts of young castor bean seedlings germinated at 30° to convert mevalonate to casbene was greatly enhanced in comparison with extracts prepared from seedlings germinated at 25° [2]. The production of the other diterpene hydrocarbons was not so significantly influenced by germination temperature.

The results reported in this paper indicate that casbene production in cell-free extracts is greatly stimulated by exposure of the castor bean seedlings to fungi and further shows the casbene is antagonistic to fungal growth. Thus, a role is proposed for casbene as an anti-fungal agent or a phytoalexin.

RESULTS

Experiments to follow up earlier observations did not reveal a consistent effect of germination temperature on the capacity of cell-free extracts to produce casbene. Instead it was noted that the capacity of extracts for casbene production seemed to correlate better with the degree of spontaneous fungal contamination of the seedlings during germination. Thus the effect of temperature was a secondary consequence of its influence on the growth of contaminating fungi; the 30° temperature led to a much greater incidence of fungal contamination than did 25°. Isolates of the fungi which spontaneously contaminated the castor bean seedlings were obtained on potato–dextrose agar. The major species encountered were identified as *Rhizopus stolonifer* (also known as *R. nigricans*) and *Aspergillus niger*, two common saprophytic fungi.

A time course for the production of casbene in extracts of 60-hr-old seedlings exposed in the same Petri dish for the final 24 hr of the 60-hr period to *Rhizopus stolonifer* cultures in comparison with extracts from control seedlings which were not exposed to the fungus, showed an approximately 35-fold greater rate of casbene synthesis from mevalonic acid in extracts from seedlings which had been exposed to the fungus. Both types of seedlings produced approximately the same amount of total protein in extracts, so this result is the same whether expressed on the basis of rates of casbene formation per seedling or per mg of extracted protein. These results are typical; the increase varied from experiment to experiment, but generally ranged from 10-fold to over 100-fold. The main fluctuation was in the controls, which on occasion gave somewhat higher rates of casbene synthesis than seen here, but never approaching those of extracts which had been exposed to the fungus. An inverse relationship was usually (but not invariably) seen with the more polar metabolites formed from mevalonic acid-[2-^{14}C], i.e. the extracts from exposed cultures produced significantly less radioactivity in this fraction than the control extracts. Chromatographic studies of the polar fraction suggested that the majority of the radioactivity was associated with all-*trans*-farnesol and all-*trans*-geranylgeraniol along with lesser amounts of unidentified metabolites.

Cultures of *Aspergillus niger* or *Fusarium moniliforme* stimulated casbene production in a fashion similar to *Rhizopus stolonifer*. Table 1 shows

Table 1. The effect of exposure of castor bean seedlings to *Aspergillus niger* on the capacity of seedling extracts to incorporate 2-^{14}C-mevalonic acid into diterpenes

Product	Seedlings exposed to fungus* (nmol ^{14}C-incorporated†)	Sterile seedlings (nmol ^{14}C-incorporated†)
Polar compounds	0·58	2·67
Casbene	4·6	1·16
Beyerene	0·17	0·06
Sandaracopimaradiene	0·30	0·34
Kaurene	0·17	0·42
Trachylobane	0·09	0·02

* Seedlings after 44 hr of germination were exposed to *A. niger* for a total of 21 hr before extracts were prepared.

† Incubation mixtures were as follows: 2·0 ml of extract were incubated at 30° for 2·5 hr in a total vol. of 2·22 ml containing 2·70 mM ATP, 2·70 mM potassium phosphate, 1·35 mM $MgCl_2$, 1·35 mM $MnCl_2$ and 9·5 μM RS-mevalonic acid-[2-^{14}C] (2·35 × 10^5 dpm). The analysis for radioactivity associated with each product was as described in Experimental.

the results of one such experiment with *A. niger* as the contaminating organism. In this case the production of casbene in the control was higher than normal; this resulted in only a four-fold increase in casbene synthesis in the extracts of seedlings exposed to the fungus relative to the controls. Presumably this was a consequence of the long incubation period of 150 min employed. Time course experiments indicate that the maximum production of casbene under these conditions is seen after 30 min in extracts which have been exposed to fungus, whereas a linear rate of casbene synthesis is present for 120–180 min in extracts of control seedlings. Therefore, the 4-fold increase in casbene synthesis seen after 150 min in this experiment probably corresponds to a 20-fold difference in initial rates of casbene synthesis in the two types of extracts.

The data in Table 1 indicate some variation in the production of diterpenes other than casbene in the two types of extracts. However, no consistent or striking stimulation of the production of these other diterpenes corresponding to that seen with casbene was observed under these conditions of exposure of seedlings to any of the fungi employed.

If seedlings were exposed to slices of sterile potato dextrose agar which had not been inoculated with fungal spores, the extracts did not vary significantly in their capacity for mevalonic acid-[2-^{14}C] metabolism from those which were maintained under sterile conditions throughout. Thus, the alteration in seedling metabolism was due to the presence of the fungus rather than some component of the fungal growth medium.

Since there was contact between the fungus and seedling in these experiments, it was essential to evaluate the possible contribution of fungal enzymes to the metabolism of mevalonic acid in extracts. Cell-free extracts were prepared from *Rhizopus stolonifer* mycelia which had been grown for 8 days in 2 l. of glucose–NH_4NO_3 medium [3] and then frozen and ruptured by passage through a Sagers' press [4]. An aliquot of the 165000 *g* supernatant fraction was incubated with RS-mevalonic acid [2-^{14}C] under the same conditions employed with seedling extracts. Analysis of extracted materials from such incubation mixtures showed only a small amount of radioactivity associated with polar compounds and no radioactivity associated with casbene or other hydrocarbons. A similar incubation of intact mycelia with RS-mevalonic acid-[2-^{14}C] also failed to yield any radioactive diterpene hydrocarbons as products. Thus, contaminating mycelia are not the source of enzymes responsible for casbene synthesis.

Thus castor bean seedlings were being stimulated to produce by exposure to these fungi sharply increased quantities of casbene, which acted as a defense agent to protect the seedling from invasion by potentially pathogenic fungi. In order to test this, it was necessary to prepare mg quantities of pure casbene. This was accomplished essentially by the published procedure [1] by biosynthesis from RS-mevalonic acid in pooled extracts of castor bean seedlings. One difference in this case was the utilization of seedlings which had been exposed to fungus in order to increase the yields of casbene. The mass spectrum of the purified sample was found to be essentially identical to that obtained in the earlier study.

Casbene in Me_2CO solution (1 mg/ml) was then applied at 5, 10 and 20 μg levels to the center of 1 ml of sterile potato dextrose medium in a stainless steel planchet held in a sterile Petri dish. Acetone (10 μl) was added to another 1 ml portion of potato dextrose agar as a control. Each plate was inoculated with an amount of *A. niger* spore suspension which had been shown to be adequate to produce overgrowth of the control plate after 2 days incubation at 30°. The presence of 5 μg of casbene did not appreciably retard the growth of the fungus, whereas 10 and 20 μg progressively inhibited fungal growth in comparison with the control. Therefore the development of *A. niger* was consistently inhibited at concentrations of casbene of 10 μg/ml or greater.

The effect of casbene on the development of the *dwarf*-5 mutant of *Zea mays* L. was also reinvestigated. The application of 20 μg of casbene per plant was found to inhibit significantly (by 35–40%) both the endogenous elongation and the elongation induced by 1 μg of gibberellic acid in the second and third leaf sheaths during a subsequent 7-day growth period in comparison with appropriate controls. This inhibition in elongation occurred without any obvious toxic effect to the seedling. It was further determined that the presence of 1–10 μg casbene per ml of glucose-

minerals medium inhibited the growth of *Escherichia coli* K12 by greater than 95% (measured turbidometrically) after 12 hr. Thus, casbene at low concentrations inhibits the growth of a variety of organisms.

DISCUSSION

We conclude that casbene is probably a substance which participates in the resistance of castor bean plants to invasion by potentially pathogenic fungi. It has two of the important characteristics of phytoalexins, namely (i) the capacity for its production is greatly increased after exposure of the plant to fungi and (ii) its anti-fungal properties. The complete statement of the phytoalexin theory as summarized by Cruickshank [5] includes some postulates about the site of production of the phytoalexin, the non-specificity of the anti-fungal action, and the basis for differences in host susceptibility or resistance which have not been examined in detail with respect to casbene. This appears to be the first suggestion that a diterpene may function as a phytoalexin. However, the sesquiterpenes ipomeamarone and rishitin have been identified as phytoalexins in sweet potato roots infected by *Ceratocystis fimbriata* (Ell. and Halst) Elliott [6] and potato tubers infected by an incompatible strain of *Phytophtora infestans* [7], respectively. Rishitin has recently been reported to be a phytoalexin produced by tomato plants as well [8]. The biosynthesis of ipomeamarone has been extensively investigated by Uritani and his co-workers [9].

Casbene is structurally, and probably biogenetically, related to a number of macrocyclic diterpenes which have been identified from diverse sources including several which have interesting physiological activities [see 10]. Cembrene [11,12] and some of its oxidized derivatives have been identified in plant sources but have no known biological roles; however, the isomeric neocembrene-A has been identified as a termite trail pheromone [13] and as a constituent of a crude ayurvedic drug derived from the resin of *Commiphora mukul* [14]. A number of more oxidized macrocyclic diterpene derivatives have been isolated from other Euphorbiaceae including some with irritant and possibly cocarcinogenic activity [15–18] and jatraphone [19] which reportedly has inhibitory activity for the growth of tumor cells *in vitro* and tumors *in vivo*. And crassin acetate and eunicin, two macrocyclic diterpene lactones identified as major organic constituents of gorgonians, have been reported to have antimicrobial activity and to inhibit the development of fertilized sea urchin eggs [20,21].

The biosynthesis of casbene results from the cyclization of all *trans*-geranylgeranyl pyrophosphate [2]. This acyclic intermediate is also the precursor of *ent*-kaurene and probably other diterpenes and carotenes needed in the normal growth and development of the castor bean plant. Thus, a single step diversion from a normal flow of carbon through the isoprenoid pathway results in the formation of this specialized defense agent in an efficient manner. The availability of a cell-free system from castor bean seedlings which catalyze the synthesis of casbene along with other isoprenoid products should permit a study of this phenomenon and some of the factors which regulate the flow of carbon to different end products.

EXPERIMENTAL

Growth of fungi. Isolates of *Rhizopus stolonifer* and *Aspergillus niger* originally obtained from spontaneous infections of castor bean seedlings were identified with the assistance of N. Keen, Department of Plant Pathology, University of California, Riverside. A gibberellin-producing strain (Lilly-1) of *Fusarium moniliforme* Sheld. was originally obtained from B. Phinney of the Department of Biology, University of California, Los Angeles. These cultures were maintained on 2% potato extract-10% dextrose agar slants. Fungal cultures to which seedlings were intentionally exposed were grown in the dark for 30 hr at 30 ± 1° on 25 ml potato-dextrose agar in sterile Petri dishes from an inoculum of spores transferred from a slant. At the end of the 30-hr period the agar plates were cut into six radial slices for introduction into the dishes of seedlings.

Growth of castor bean seedlings and preparation of cell-free extracts. Seeds of *Ricinus communis* L. cv. Hale were kindly supplied by The Baker Castor Oil Company (now an affiliate of N L Industries) through its Oilseeds Production Division. Seeds mechanically freed of their coats were decontaminated by immersion in 0·01% NaClO for 1 min followed by several rinses in sterile dist H_2O. 10–12 seeds were transferred aseptically to the periphery of each of several sterile Petri dishes on a bed of 3 pieces of filter paper and a double layer of cheesecloth soaked with 15 ml dist H_2O. The dishes were placed in a dark incubator at 30 ± 1° for germination and growth of the seedlings. A radial slice of agar overgrown with fungal mycelia was transferred to the center of the Petri dish containing 36-hr-old seedlings in cases where seedlings were to be exposed to fungus. Incubation of these seedlings was continued for an additional 24 hr at 30 ± 1° to give a total incubation time of 60 hr. Control seedlings were maintained under aseptic conditions throughout the 60-hr period without

exposure to the fungal mycelia. Cell-free extracts were prepared essentially according to a previously published procedure [1] from batches of 8–12 viable seedlings which had received the same treatment. All steps were carried out at 2–4°. The combined seedlings were mixed with one-third of their weight of Polyclar AT. Tris–bicarbonate buffer (0·05 M; pH 6·8) containing 0·014 M 2-mercaptoethanol was added in the ratio 2·2 ml/g of seedlings. This mixture was ground at top speed in a Virtis "45" homogenizer for 30–45 sec and the resulting homogenate was expressed through several layers of cheesecloth. The filtrate was subjected to centrifugation at 15000 *g* for 15 min and the supernatant fraction, from which the floating lipid layer was first removed, was then subjected to recentrifugation at 165000 *g* for 1 hr. The supernatant fraction from the second centrifugation served as the source of enzyme.

Incubations of cell-free extracts with RS-*mevalonic acid-*[2-^{14}C] *and analysis of radioactive products.* A typical experiment was as follows: 1·4 ml of enzyme extract was incubated in a total vol of 1·62 ml with 3·70 mM ATP, 3·70 mM sodium phosphate, 1·85 mM $MnCl_2$, 1·85 mM $MgCl_2$ and 13 μM RS-mevalonic acid-[2-^{14}C] ($2{\cdot}35 \times 10^5$ dpm) at 30°. After the length of time indicated in individual expts, the mixtures were heated 1–2 min at 100°. Products were extracted from the ppt and supernatant fractions into a mixture of C_6H_6 and Me_2CO and analyzed for radioactivity associated with hydrocarbons and more polar products by a TLC method previously described [1]. This method employs a combination plain and $AgNO_3$-impregnated Si gel plate developed with *n*-hexane–C_6H_6 (7:3). The more polar compounds remain at the origin of the plain portion of the plate, casbene is immobilized at the $AgNO_3$ origin, and the other diterpene hydrocarbons are found at various zones in the $AgNO_3$-containing region.

Bioassays with the dwarf-5 *mutant of* Zea mays *L.* Solns of casbene and gibberellic acid (gibberellin A_3) were prepared by dissolving the samples in a few drops of Me_2CO and diluting with 0·05% Tween 20 in H_2O to final concentrations of 200 μg per ml and 10 μg per ml, respectively. Assays were conducted essentially as described by Phinney and West [22]. Dwarf seedlings after 7 days of growth were treated with either 0·1 ml of the casbene soln (20 μg) or 0·1 ml of the gibberellic acid solution (1 μg) or a combination of these two. Control plants were treated with 0·1 ml of 0·05% Tween 20 in H_2O containing an amount of Me_2CO equivalent to the above. The lengths of the second and third leaf sheaths were determined after an additional 7 days of growth.

Bioassays with E. coli. *E. coli* (strain K-12; wild type) was grown in 10 ml of medium containing 40 mg glucose, 20 mg $(NH_4)_2SO_4$, 100 mg KH_2PO_4, 260 mg $K_2HPO_4.3H_2O$ and 2 mg $MgCl_2.6H_2O$ adjusted to pH 7·1. Amounts of casbene sufficient to give a final concentration of either 1 or 10 μg per ml in the growth medium were added in 10 μl of Me_2CO. An equivalent amount of Me_2CO without casbene was present in control tubes. A culture in log phase growth was used as the inoculum and growth was allowed to proceed at 37° with shaking. The turbidity was estimated as a measure of growth by recording the absorbancy at 540 nm at hourly intervals over a 12 hr period after inoculation.

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