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Ethylene: Indicator but Not Inducer of Phytoalexin Synthesis in Soybean[1]

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ABSTRACT

Cell wall preparations (elicitors) from *Phytophthora megasperma* var. *sojae* increase C_2H_4 formation, phenylalanine ammonia lyase activity, and glyceollin accumulation in soybean cotyledons within about 1.5, 3, and 6 hours after treatment, respectively. The immediate precursor of C_2H_4, 1-aminocyclopropane-1-carboxylic acid, stimulates C_2H_4 formation like the elicitor within 1.5 hours after administration, whereas phenylalanine ammonia lyase activity and glyceollin concentration remain unchanged. Aminoethoxyvinylglycine, a specific inhibitor of C_2H_4 formation in higher plants, inhibits elicitor-induced C_2H_4 formation by about 95% but has no effects on phenylalanine ammonia lyase or glyceollin accumulation. It was concluded that C_2H_4 is a signal accompanying the specific recognition process which finally leads to the induction of phytoalexin formation, but it is not functioning as a link or messenger in the induction sequence of glyceollin accumulation.

Phytoalexins are fungitoxic compounds produced by the host in response to infection and are possibly involved in higher plant resistance to fungal pathogens. Fungal cell wall components, so-called elicitors (13), are active in the induction of enzymes involved in phytoalexin production (7, 23) and resistance (9, 11). Thus specific receptor sites in host cell walls and/or plasma membranes seem to produce signals or messengers which in turn increase activities of enzymes involved in the synthesis of individual phytoalexins. These phytoalexins, together with other factors and reactions, are responsible for resistance. One of the possible candidates for a role as messenger is the gaseous "plant hormone" C_2H_4 since (*a*) PAL[3], a key enzyme in flavonoid biosynthesis, is inducible by C_2H_4 (1, 21), (*b*) C_2H_4 is produced in response to fungal infections correlating either with resistance (21) or with the severity of the necrotic reaction (14, 18, 19, 21), (*c*) treatment with fungal elicitors of compatible hosts can yield resistance (11), and (*d*) C_2H_4 formation in response to treatment with fungal elicitors of hypocotyl segments correlates with the hypersensitivity of the individual bean variety the tissue was derived from (8, 19).

On the other hand, there are reports that: (*a*) PAL, peroxidase, and chalcone flavone isomerase occur in both resistant and susceptible cultivars of soybean after infection or wounding (only the resistant cultivars produce C_2H_4) (20); (*b*) C_2H_4 partly inhibited suberization and callus formation on injured surfaces of sweet potato roots (5); (*c*) C_2H_4 cannot mimic spore infection and chemical stress in respect to induction of pisatin synthesis (6); and (*d*) AVG, an inhibitor of C_2H_4 synthesis only partly inhibits PAL induction and phytoalexin formation in leaves of bean and tomato plants (4).

In this communication we report experiments undertaken to find out whether C_2H_4 is the messenger after elicitor treatment of soybean cotyledons.

MATERIALS AND METHODS

Chemicals. ACC was obtained from Calbiochem, Lahn, Federal Republic of Germany. AVG, batch No. RO 20-4468/001, was a gift from Hoffman-LaRoche Inc., Nutley, NJ.

Plant Material. Soybeans (*Glycine max.* L. cv. Harosoy 63) were grown on a day-night cycle of 11/13 h at 18/22 C and 8,000 to 10,000 lux.

Incubation of Plant Material. Incubations for C_2H_4 production by cut soybean cotyledons were done in Fernbach flasks with a volume of about 12 ml in 2 ml phosphate buffer (10 mM, pH 7.2), using the following concentrations of test substances: ACC, 10 μM; AVG, 250 μM; elicitor, 1 g/100 ml. Incubations for more than 5 h were done in Petri dishes. After the incubation in air for the indicated times (Fig. 2), the cotyledons were incubated for 1 h (C_2H_4 accumulation) in Fernbach flasks sealed with serum rubber stoppers.

Determination of C_2H_4 Formation. Samples of the gas phase were removed with a hypodermic syringe and analyzed for their C_2H_4 content in a Varian 1400 gas chromatograph equipped with a flame ionization detector, an activated alumina column (0.32 × 61 cm), and a Varian CDS 101 integrator.

Assay of PAL. Preparation of Pms elicitor and purification and determination of glyceollin was performed according to Zähringer *et al.* (23) as follows: Pms elicitor was derived from lyophilized mycelia of *Phythophthora megasperma* var. *soyae* (race 1) after lipid extraction. One g dried mycelium was suspended in 100 ml 10 mM phosphate buffer (pH 7.2) and autoclaved for 30 min at 120 C. After centrifugation, the supernatant was used as "cell wall released elicitor preparation."

Induction of C_2H_4, PAL, and Glyceollin. Induction was followed in soybean cotyledons. About one-third of the upper part of the cotyledons was cut off with a razor blade. The cut surfaces were treated with 0.7 ml of solution each, containing either elicitor (1 g/100 ml), ACC (10 μM), AVG (250 μM), or the respective com-

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[3] Abbreviations: PAL, phenylalanine ammonia lyase; Pms, *Phytophthora megasperma* var. *sojae*; elicitor, cell wall preparation from Pms; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine, the aminoethoxy analog of rhizobitoxin [systematic name: L-2-amino-4-(2′-aminoethoxy)-*trans*-3-butenoic acid (RO 20)].

binations. Controls were treated with 0.7 ml 10 mM phosphate buffer during the indicated times of incubation. After the incubations, the "wound droplets" were removed from each cut surface and gathered for phytoalexin determination. The incubations were done in Petri dishes in 100% humidity at 26 C in the dark. Six Petri dishes were used for each treatment, containing 15 cut cotyledons each; the experiments were repeated three times with essentially the same results.

Determination of PAL Activity. After incubations for the indicated times under the above conditions, the tissue next to the exposed wound surface was removed with a razor blade and homogenized in 100 mM phosphate buffer (pH 8.0) containing 4.2 mM mercaptoethanol (1.5 ml buffer/g tissue). The brei was filtered through cheesecloth and centrifuged for 30 min at 48,000*g*. One ml of the supernatant was stirred with 100 mg prewashed Dowex (1-X2; 50 to 100 mesh; Cl^- form; Roth-chemicals, Karlsruhe) for 20 min at 4 C. PAL was determined spectrophotometrically in the filtrate (23).

Glyceollin. Glyceollin was determined in the "wound droplets" from the cut surfaces of the cotyledons. Three combined extractions with each 50 ml ethyl acetate (90 wound droplets were combined for the individual extractions) were dried with Na_2SO_4 at 4 C. After filtration and evaporation to dryness, the extracts were redissolved in 10 ml ethanol. Pterocarpanes, including glyceollin, were determined spectrophotometrically at 285 nm in the ethanol extracts (23). Glyceollin was identified by TLC (see below) using an authentic standard (kindly provided by Dr. Zähringer, Freiburg).

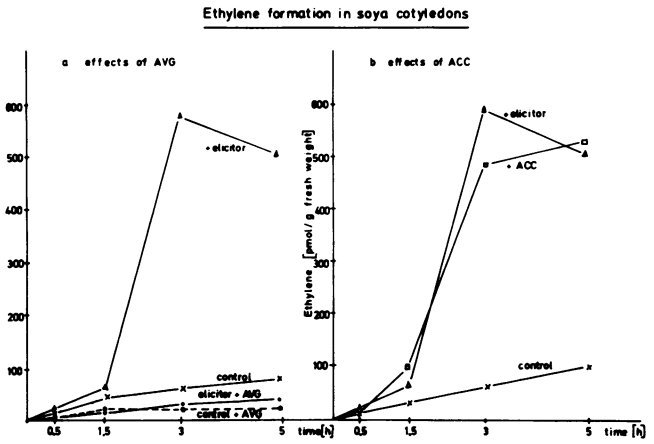

FIG. 1. Effects of elicitor, ACC, and AVG on C_2H_4 formation by soybean cotyledons. Cotyledons were incubated with the indicated compounds in closed vessels for the indicated times.

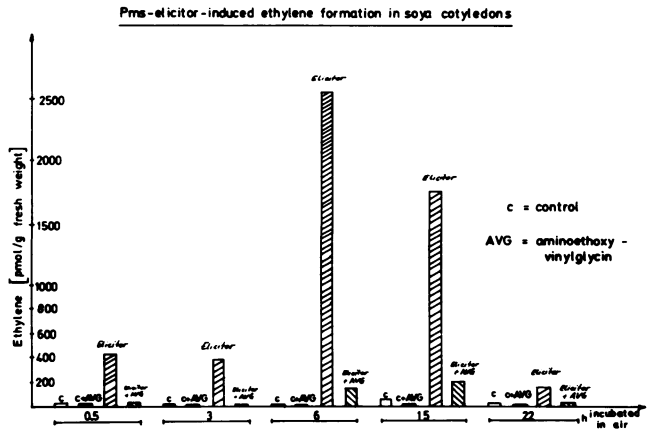

FIG. 2. Time course of C_2H_4 formation by elicitor- and AVG-treated soybean cotyledons.

Table I. *Effect of Elicitor, ACC, and AVG on PAL Activity and Glyceollin Accumulation by Soybean Cotyledons*

Incubation of soybean cotyledons, assay of PAL, purification of Pms elicitor, and glyceollin purification and determination were performed as described under "Materials and Methods" and in reference 23. Values were taken after 22 h incubation.

Treatment	PAL Activity[a]	Glyceollin
	μKat/kg	*μmol/ml*
Control	0.37	0.29
+ACC (10 μM)	0.28	0.13
+Elicitor (10 mg/ml)	9.4	4.05
Control	0.34	0.23
+AVG (250 μM)	0.35	0.23
+Elicitor (10 mg/ml)	6.8	3.1
+Elicitor + AVG	6.1	3.3

[a] 1 Kat represents an enzyme activity, converting 1 mol substrate in 1 s (IUPAC-IUB definition).

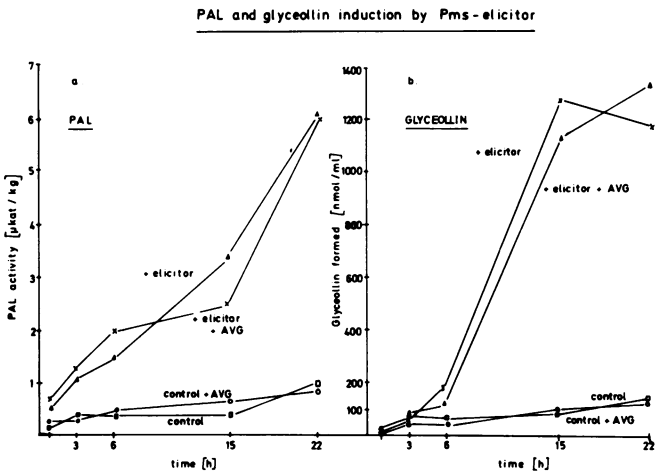

FIG. 3. Time course of PAL activity and glyceollin accumulation by soybean cotyledons incubated with elicitor and AVG. Soybean cotyledons were incubated in air (in Petri dishes) for the indicated times; PAL activity and glyceollin were determined as described (23).

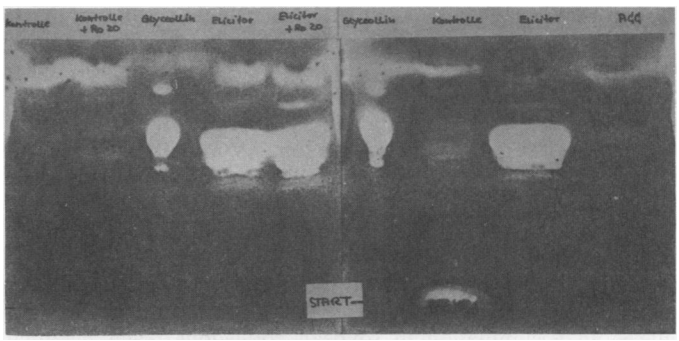

FIG. 4. *C. cucumerinum* growth on thin layer plates after chromatographic separation of glyceollin. For details, see "Materials and Methods" and reference 10.

Phytoalexin Bioassay. *Cladosporium cucumerinum* was cultured in the following medium: 0.7% KH_2PO_4, 0.3% K_2HPO_4, 0.4% KNO_3, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, and 2.5% glucose (w/v). Sixty ml of the above medium was distributed in 12 culture vials containing the *C. cucumerinum* cultures and spores + mycelium were suspended carefully in the medium. After filtration through

six layers of cheesecloth, the spore suspension was combined with 40 ml culture medium + 0.4 g agar which was heated briefly after mixing.

Thin-layer plates, for the separation of phytoalexins (Merck Silica Gel F_{254}, Merck, Darmstadt, Federal Republic of Germany) were developed in toluene-chloroform-acetone (40:25:35, v/v) (*cf.* ref. 10). The spore suspension (in 100 ml culture medium + agar) was sprayed onto a freshly developed thin-layer chromatogram. The sprayed plates were incubated in 100% humidity at 24 C for about 3 days. White spots on the dark plates indicate the presence of phytotoxic compounds.

RESULTS AND DISCUSSION

Using the well established Pms-soybean system, Zähringer *et al.* (23) recently demonstrated that increased PAL activity correlated reasonably well with glyceollin accumulation after incubation of soybean cotyledons with Pms elicitor. Wounding of cotyledons was excluded as a possible stimulus for the observed effects since [2-^{14}C]mevalonate was only incorporated into glyceollin after elicitor treatment. We tested the hypothesis that C_2H_4 may be involved as a link in the sequence of events between elicitor recognition and glyceollin formation.

C_2H_4 Formation by Soybean Cotyledons after Elicitor or ACC Treatment. C_2H_4 formation by soybean cotyledons is stimulated in the presence of elicitor (Fig. 1a). The stimulating effect of the elicitor is completely inhibited by appropriate concentrations of AVG, a specific inhibitor of C_2H_4 formation from methionine (3, 16). Incubation of soybean cotyledons with ACC, the immediate precursor of C_2H_4 in higher plants (2, 17), results in increased C_2H_4 formation, comparable to elicitor treatment (Fig. 1b).

In Figure 1, the incubation vessels were closed during the incubation time, yielding maximal C_2H_4 concentrations after 3 h. Maximal rates of C_2H_4 production of about 300 to 400 pmol g^{-1} fresh weight h^{-1} are observed between 1.5 and 3 h incubation in closed vessels. If the incubation is done in open vessels (Petri dishes), allowing free gas exchange, and C_2H_4 is trapped by closing the vessels for 1 h after the indicated time of free gas exchange, C_2H_4 formation is observed as biphasic: a clear stimulation during 0.5 to 1.5 h incubation which remains constant during h 3 to 4 incubation, followed by a strong increase during h 6 to 7 incubation, declining to about 10% of the maximum value at 6 after 22 h incubation in open vessels (Fig. 2).

C_2H_4 formation by cotyledons 3 h after inoculation in a closed system might be O_2-limited (Fig. 1). In addition to the biphasic nature of the "rates" of C_2H_4 formation (Fig. 2) form the beginning (0.5 h) of incubation, the percentage of AVG-inhibitable C_2H_4 formation is about 6 to 7%, with an eventually slight increase in noninhibitable C_2H_4 formation by AVG after longer times of incubation. There is good evidence that AVG inhibits the biosynthetic pathway of C_2H_4 between methionine and ACC (3, 16), and that enzymic oxidation of ACC yielding C_2H_4 is catalyzed by a constitutive enzyme (3, 12, 15, 22). Elicitor treatment seems to enhance synthesis of ACC in soybean cotyledons.

Effect of Elicitor, ACC, and AVG on PAL Activity and Glyceollin Accumulation in Soybean Cotyledons. PAL activity and glyceollin accumulation in soybean cotyledons reach maximal values about 22 h after elicitor treatment (23). In contrast to C_2H_4, neither PAL activity nor glyceolline accumulation are influenced by ACC or AVG after elicitor treatment, indicating that C_2H_4 formation is an event different from PAL and glyceollin induction at an early stage (Table I). The lack of effect of AVG on PAL (Fig. 3a) and glyceollin induction (Fig. 3b), in contrast to C_2H_4 formation (Fig. 2), was observed from 0.5 to 22 h after treatment.

Phytoalexin Bioassay. Growth of *C. cucumerinum* is inhibited by glyceollin and other phytoalexins (10). White spots with R_F values similar to authentic glyceollin (indicating total growth inhibition of *C. cucumerinum*) are visible in extracts from elicitor-treated soybean cotyledons (Fig. 4). H_2O-treated or ACC-treated cotyledon extracts did not produce such inhibition zones. AVG in combination with elicitor did not visibly influence the formation of an inhibition zone (AVG = RO 20).

The above data contradict the concept that C_2H_4 might be involved in phytoalexin induction, functioning as a messenger between the primary process of elicitor recognition and the final cellular processes yielding increased phytoalexin synthesis; C_2H_4 apparently is merely an accompanying indicator of the elicitor recognition process and is not involved as a direct link or a signal transmitter.

CONCLUSIONS

From these results, we conclude that C_2H_4 is not the signal in elicitor-induced phytoalexin formation in soybean cotyledons, although it is a sensitive indicator for certain host-parasite interactions.

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