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Ethylene: Symptom, Not Signal for the Induction of Chitinase and β-1,3-Glucanase in Pea Pods by Pathogens and Elicitors[1]

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ABSTRACT

Infection of immature pea pods with *Fusarium solani* f.sp. *phaseoli* (a non-pathogen of peas) or f.sp. *pisi* (a pea pathogen) resulted in induction of chitinase and β-1,3-glucanase. Within 30 hours, activities of the two enzymes increased 9-fold and 4-fold, respectively. Chitinase and β-1,3-glucanase were also induced by autoclaved spores of the two *F. solani* strains and by the known elicitors of phytoalexins in pea pods, cadmium ions, actinomycin D, and chitosan. Furthermore, exogenously applied ethylene caused an increase of chitinase and β-1,3-glucanase in uninfected pods. Fungal infection or treatment with elicitors strongly increased ethylene production by immature pea pods. Infected or elicitor-treated pea pods were incubated with aminoethoxyvinylglycine, a specific inhibitor of ethylene biosynthesis. This lowered stress ethylene production to or below the level of uninfected controls; however, chitinase and β-1,3-glucanase were still strongly induced. It is concluded that ethylene and fungal infection or elicitors are separate, independent signals for the induction of chitinase and β-1,3-glucanase.

Many plants respond to an attack by pathogens with an enhanced ethylene production (20, 23, 26). Exogenously applied ethylene has been found to activate or enhance biochemical defenses against potential pathogens in a number of cases (4). It has been hypothesized, therefore, that the endogenously produced stress ethylene may function as a signal for the plant to enhance or activate its defenses against pathogens (4, 20, 26).

Two recent studies tested this hypothesis by manipulation of stress ethylene biosynthesis in diseased plants (18, 23). In soybean cotyledons treated with a fungal elicitor, suppression of ethylene biosynthesis by AVG[2] did not reduce phytoalexin production (18), while in diseased melon seedlings, an AVG treatment only slightly reduced the biosynthesis of hydroxyproline-rich cell wall glycoprotein, a substance implicated in defense (23).

These studies indicate that endogenous stress ethylene has little importance in the induction of biochemical defenses against pathogens. However, interpretation of their results is difficult since exogenously applied ethylene had only a small effect (9, 23) or none at all (18) on the defense reaction in question.

Exogenously applied ethylene induces a large increase of chitinase and β-1,3-glucanase in a number of plants (1, 5). These two enzymes may act as defenses against pathogenic fungi since chitin and β-1,3-glucans are major components of many fungal cell walls (2). Their potential to degrade cell walls of pathogens has been established (5, 13, 25, 27). Chitinase acts also as a lysozyme (5); it has no known function in the plant's own metabolism since there is no chitin-like substrate present in higher plants (5).

Activities of chitinase (19, 21) and of β-1,3-glucanase (15, 16, 19, 21) have also been found to increase strongly in a number of different plant-pathogen interactions. Because of the strong effect of exogenous ethylene, it is an obvious question to ask whether or not endogenous stress ethylene formed in the course of a pathogen attack is a signal for the induction of chitinase and glucanase.

In the present work, we used immature pea pods infected with pathogens and nonpathogenic fungi or treated with elicitors to examine and answer this question.

MATERIALS AND METHODS

Biological Material. *Fusarium solani* f.sp. *pisi*, strain P-A (ATCC 38136) and *Fusarium solani* f.sp. *phaseoli* W-8 (ATCC 38135) were obtained from R. J. Cook and D. J. Burk, respectively. The *Pisum sativum* pods were from the Alaska-type variety 'Dot'.

Chemicals. Colloidal chitin was prepared from crab shell chitin (Fluka, Buchs, Switzerland), as described (5). Unlabeled and ^3H-labeled regenerated chitin were synthesized as described (14), using crab shell chitosan from Sigma as the starting material. Snow crab chitosan (Madera Products Inc., Albany, OR) was used for the treatment of pea pods. Glycol chitosan was from Sigma. Laminarin was purchased from the United States Biochemical Corp. Snail gut juice (Helicase) was obtained from IBF, Clichy, France. AVG was from Maag, Dielsdorf, Switzerland. All other reagents were analytical grade.

Inoculation of Plant Material. Macroconidia of *F. solani* f.sp. *pisi* (a pea pathogen) and *F. solani* f.sp. *phaseoli* (a non-pathogen of peas), grown on pea pod-supplemented potato dextrose agar, were suspended in sterile water at a concentration of 1.5×10^6 spores per ml. Immature pea pods, about 2 cm long, were carefully split in half. Each half pod (fresh weight 170 mg ± 15 mg) received 25 μl of one of the following solutions on the freshly exposed endocarp tissue: sterile water, macroconidial suspensions of *F. solani* f.sp. *pisi* or f.sp. *phaseoli*, chitosan (0.1% w/v), colloidal chitin (0.2% w/v), $CdCl_2$ (0.5 mM), actinomycin D (10 μg/ml), AVG (3 mM), ACC (1 mM), $CoCl_2$ (1 mM), IAA (0.2 mM), polylysine (1 mM).

The inoculated samples, consisting of 10 half pods per treatment, were incubated at 25°C in a moist, dark chamber for the appropriate time period. The pea pods were then frozen at −80°C.

Crude Enzyme Preparation. A weighed portion of pea pods

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[2] Abbreviations: AVG, aminoethoxyvinylglycine; ACC, 1-aminocyclopropane-1-carboxylic acid; GlcNAc, *N*-acetylglucosamine.

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was ground in liquid nitrogen and homogenized in 0.1 M sodium citrate buffer, pH 5.0, at a ratio of 1:2 (w/v) with a pestle and mortar. The homogenate was centrifuged (10 min, 10,000*g*), and the supernatant was used as crude enzyme preparation. A 2-ml portion of the supernatant was desalted on a Sephadex G-25 column (7 × 2 cm) equilibrated with 10 mM sodium acetate buffer, pH 5.0 (5). For each preparation, protein was measured (6) before and after desalting to account for the dilution during chromatography in calculations.

For enzyme extraction from fungi, spores from *F. solani* f.sp. *pisi* and *phaseoli* were cultured 48 h in Vogel's medium as described (10). The mycelia were harvested by filtration, washed with water, frozen in liquid nitrogen, and then extracted as described above.

Enzyme Assays. Chitinase activity was measured with either the colorimetric or the radiometric assay for endochitinase described earlier (5). Preliminary results using uninfected and infected pea pods showed that the two assays yielded identical results, as had been demonstrated earlier for chitinase from bean leaves (5). For the colorimetric assay, the reaction mixture contained, in 0.5 ml: 50 µl appropriately diluted crude enzyme preparation, 0.8 mg colloidal chitin, 10 µmol sodium phosphate buffer (pH 6.4). The mixture was incubated in a shaking water bath at 37°C for 1 h. The reaction was stopped by centrifugation. Of the resulting supernatant, 0.3 ml were incubated with 0.02 ml 3% (w/v) desalted snail gut juice (5) to hydrolyze the liberated, water-soluble chitin oligomers to GlcNAc. The resulting GlcNAc was determined according to Reissig *et al.* (22), using internal standards in the assay mixtures for calculations (5). The radiometric assay employed ^{3}H-labeled regenerated chitin as a substrate. The reaction mixture consisted of appropriately diluted crude enzyme, 0.8 mg [^{3}H]chitin, 5 µmol sodium phosphate buffer (pH 6.4) in a final volume of 0.25 ml. The reaction was stopped after 1 h incubation at 30°C by the addition of 0.25 ml 10% (w/v) TCA. After centrifugation, the radioactivity was determined in 0.3 ml of the supernatant.

Because product formation was not a linear function of enzyme concentration, activity was calculated for an enzyme concentration approaching zero using standard curves (5). The amount of enzyme producing 1 nmol min^{-1} GlcNAc equivalents at infinite dilution was defined as one milliunit (mu). Each value determined is the mean of four replicate assays; with the enzyme dilutions employed, the average SD of the replicates was 9% of the mean.

β-1,3-Glucanase activity was determined by measuring the release of reducing sugars (8) from laminarin, treated with sodium borohydride to eliminate its high background of reducing sugars (7). The assay mixture contained in a total volume of 0.5 ml: 100 µl appropriately diluted desalted enzyme extract, 1 mg reduced laminarin, 10 µmol sodium acetate buffer (pH 5.0). After incubation (20 min at 37°C), the reducing sugar content was determined with the neocuproine method (8). Product formation was a linear function of enzyme concentration from 10 to 200 nmol glucose equivalents. One milliunit (mu) was defined as the amount of enzyme which liberates 1 nmol min^{-1} glucose equivalents. All assays and calculations included internal standards and enzyme and substrate blanks. Each value determined is the mean of two replicate assays that varied 5% in the average, and is expressed per ml crude enzyme preparation, using the protein values before and after chromatography to account for the dilution during desalting.

Ethylene Determination. Immature pea pods were treated as described and incubated at 25°C on wet filter paper in the dark in sealed 150-ml flasks. Gas samples (1 ml) were withdrawn at intervals, and ethylene concentration was determined on a gas chromatograph equipped with an aluminum oxide column and a flame ionization detector. Ethylene production (per g fresh weight) was calculated as the mean of three independently incubated samples of six half pods which varied 10% in the average. Each experiment was repeated at least once.

RESULTS

Induction of Chitinase and β-1,3-Glucanase. Freshly excised, split immature pea pods contained low but easily measurable activities of chitinase and β-1,3-glucanase (Fig. 1, zero time). When the pods were treated with sterile water and incubated in a moist chamber in the dark, chitinase activity changed little for up to 30 h (Fig. 1A), while β-1,3-glucanase activity increased

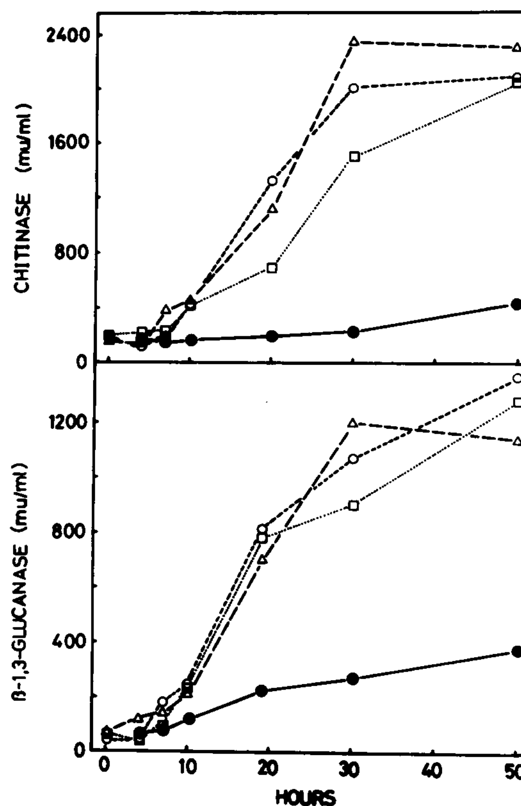

FIG. 1. Induction of chitinase activity (A) and of β-1,3-glucanase activity (B) in immature pea pods by a compatible pathogen (Δ), by an incompatible pathogen (O), and by chitosan (□). Controls (●) received sterile water. Chitinase activity was measured by the colorimetric assay.

Table I. *Induction of Chitinase and β-1,3-Glucanase in Pea Pods by Exogenously Applied Ethylene or Inoculation with F. solani f.sp. phaseoli*

Pea pods were incubated either in 10 nl ml^{-1} ethylene or in ethylene-free air after inoculation with water or with a spore suspension of *F. solani* f. sp. *phaseoli*. After 24 h of incubation, chitinase (radiometric assay) and β-1,3-glucanase were measured. The experiment was repeated twice with similar results.

Treatment	Chitinase		β-1,3-Glucanase	
	mu/ml	*% of control*	*mu/ml*	*% of control*
Water, ethylene-free air (control)	180	100	160	100
F. solani, ethylene-free air	890	495	820	510
Water, 10 nl ml^{-1} ethylene	520	290	650	405
F. solani, 10 nl ml^{-1} ethylene	940	520	1050	655

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considerably (Fig. 1B). Infection of the pods with *F. solani* f.sp. *phaseoli* or *F. solani* f.sp. *pisi* or a treatment with chitosan resulted in a strong increase of both chitinase and β-1,3-glucanase above the control level, starting 4 to 6 h after inoculation (Fig. 1). The rates at which the activities of both enzymes increased were similar for all treatments. The enzymes were both of plant origin since most fungal tissue had been removed prior to the assay, and since chitosan treatment induced them in the absence of the fungi. Furthermore, no chitinase and β-1,3-glucanase activities could be detected in mycelial extracts and culture filtrates from young cultures of the *F. solani* strains. The experiments were repeated twice and yielded similar kinetics. The levels of enzyme activities recovered varied 15 to 20% (SD) between experiments performed on different days with different lots of pea pods.

Chitinase and β-1,3-glucanase were also induced by exogenously applied ethylene (Table I). The dose applied (10 nl ml^{-1}) was saturating for induction (data not shown). The inducing effects of *Fusarium solani* f.sp. *phaseoli* and of ethylene were not additive when both stimuli were used in combination (Table I).

Ethylene Production. The rate of ethylene production strongly increased in excised, split pea pods after a lag of about 1 h (Fig. 2). This was a typical wound response (26); excised but unsplit pods produced little ethylene during incubation (Fig. 4). In the uninfected, split pods, ethylene production reached a maximum after 5 h and fell back to the initial, low values after 15 h. In pea pods infected with *F. solani*, ethylene was produced at much higher rates during a period of 3 to 15 h after infection (Fig. 2). Autoclaved spores of both fungal strains also induced ethylene production above the control values (Fig. 2), indicating that factors other than mechanical wounding played a role in pathogen-induced ethylene formation. Fungal elicitors might be one such factor since chitosan and colloidal chitin caused an increase in ethylene production as well (Fig. 3). Autoclaved spores and chitin not only increased stress ethylene formation but also induced chitinase and β-1,3-glucanase activities (Table II), like living spores and chitosan (Fig. 1). An abiotic elicitor, $CdCl_2$, and IAA strongly induced ethylene formation (Fig. 4).

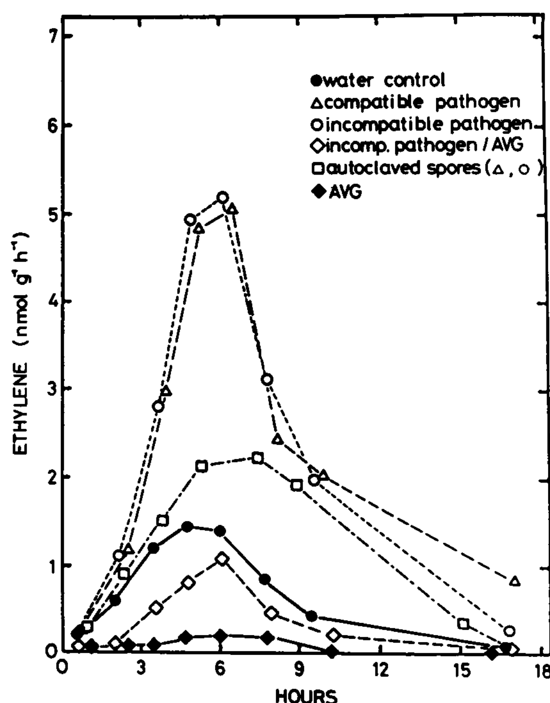

FIG. 2. Induction of ethylene production in pea pods by living and by autoclaved *F. solani* spore suspensions and its suppression by 3 mM AVG.

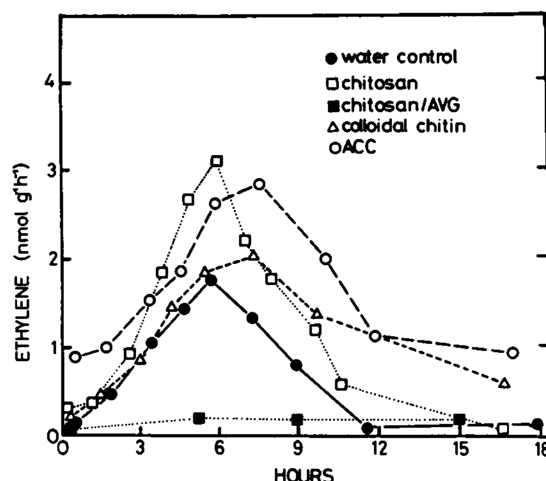

FIG. 3. Ethylene production in pea pods treated with biotic elicitors and with ACC. Concentrations employed were: Chitosan, 0.1% w/v; chitin, 0.2% w/v; AVG, 3 mM; ACC, 1 mM.

Table II. *Ethylene Formation and Induction of Chitinase and β-1,3-Glucanase in Pea Pods Inoculated with Autoclaved F. solani Macroconidia, or Treated with Chitin or ACC*

Ethylene formation and activities of chitinase (radiometric assay) and β-1,3-glucanase were determined after 24 h. Control values were 10.5 ± 2.0 nmol g^{-1} d^{-1} for ethylene formation, 250 ± 30 mu ml^{-1} for chitinase, and 230 ± 50 mu ml^{-1} for β-1,3-glucanase activities. The experiments were repeated at least once with similar results.

Treatments	Ethylene Formation	Chitinase	β-1,3-Glucanase
	% of control		
Water	100	100	100
F.s. phaseoli, autoclaved	211	287	252
F.s. pisi, autoclaved	211	270	253
Chitin (0.2% w/v)	147	225	180
ACC (1 mM)	360	168	154

Treatment of the pods with ACC resulted in increased ethylene production, particularly during the first hours (Fig. 3). This indicates that ethylene synthesis in untreated pods is limited by the supply of endogenous ACC, and that the increase in ethylene production upon wounding and infection is due, at least in part, to increased biosynthesis of ACC. Results with AVG, a specific inhibitor of ACC synthase (3), supported this: AVG inhibited ethylene formation in uninfected controls and in infected pea pods (Fig. 2) as well as in elicitor-treated (Fig. 3) or IAA-treated (Fig. 4) pea pods.

The shape of the curves relating ethylene production rate to incubation time was similar for all treatments (Figs. 2–4). The time sequence of ethylene production in infected pods relative to the induction of chitinase and β-1,3-glucanase (Fig. 1) indicates that endogenous stress ethylene could act as a signal triggering the subsequent enzyme induction.

Induction of Chitinase and β-1,3-Glucanase following Manipulation of Ethylene Production. A treatment of split pea pods with ACC strongly increased endogenous ethylene production (Fig. 3) and also caused an increase in chitinase and β-1,3-glucanase, although to a smaller degree than treatment with autoclaved spores or chitin (Table II). This indicated that an increase of the endogenous ethylene production above control levels was sufficient to induce chitinase and β-1,3-glucanase in pea pods. The question arose whether, in infected or elicitor-treated pods, such an increase in stress ethylene production was necessary for the subsequent induction of the two enzymes. AVG

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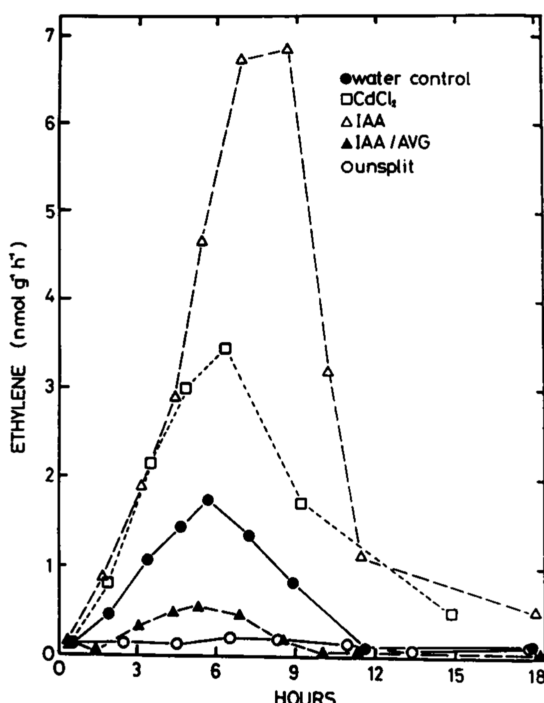

FIG. 4. Ethylene production in pea pods treated with the abiotic elicitor, $CdCl_2$, or with IAA. Concentrations employed were: $CdCl_2$, 0.5 mM; IAA, 0.2 mM; AVG, 3 mM. Data for excised, unsplit pea pods are also shown.

was used to test this. Infected pea pods treated with 3 mM AVG (25 µl/half pod) had stress ethylene production at or below the level of untreated control half pods (Fig. 2). At 1 mM AVG or less, stress ethylene production was not lowered to control levels in infected pods (data not shown). The AVG treatment in itself did not induce chitinase and β-1,3-glucanase (Table III). To test if the AVG treatment had side effects on the induction of chitinase and β-1,3-glucanase, pea pods were incubated with exogenous ethylene in the presence and absence of AVG. The two enzymes were slightly less induced in the presence of AVG than in its absence (Table III), indicating a marginal unspecific effect of AVG on enzyme induction.

In pods infected with *F. solani* f.sp. *pisi* or *phaseoli*, AVG inhibited the induction of chitinase and β-1,3-glucanase only slightly, although it lowered stress ethylene to or below the level of the water-treated controls (Table III). Similar results were obtained for the biotic elicitor, chitosan, and for the abiotic elicitors, $CdCl_2$ and actinomycin D: all induced chitinase and β-1,3-glucanase in the absence of AVG, when stress ethylene was formed, as well as in its presence, when ethylene production was lowered to levels considerably below the control (Table III).

The effect of auxin was different. High concentrations of IAA strongly stimulated ethylene production and resulted in an induction of chitinase and β-1,3-glucanase (Table III). However, in this case, induction depended on the increased ethylene formation since AVG prevented the induction of the two hydrolases (Table III).

$CoCl_2$ strongly inhibited ethylene formation; it did not affect the activities of chitinase or β-1,3-glucanase (data not shown). Different size classes of polylysine, polycations like chitosan, had little or no effect on ethylene production and no effect on enzyme induction (data not shown).

Table III. *Stress Ethylene Formation and Induction of Chitinase and β-1,3-Glucanase in Infected or Elicitor-Treated Pea Pods in the Presence or Absence of AVG*

Samples of pea pods were inoculated with living spores of *F. solani* f.sp. *phaseoli* or *pisi* or with elicitors or IAA (25 µl of the concentrations indicated per half pod). One half of the samples received, in addition, 25 µl 3 mM AVG per half pod (+AVG treatment), the other half 25 µl sterile water per half pod (−AVG treatment). Ethylene formation and activities of chitinase (radiometric assay) and β-1,3-glucanase were detemined after 24 h. Control values were 10.5 ± 2 nmol g^{-1} d^{-1} for ethylene production, 250 ± 30 mu ml^{-1} for chitinase, and 230 ± 50 mu ml^{-1} for β-1,3-glucanase activities. The experiments were repeated at least once with similar results.

Treatment	AVG	Ethylene Formation	Chitinase	β-1,3-Glucanase
			% of control	
Water (control)	−	100	100	100
	+	13	104	75
Ethylene (15 nl ml^{-1})	−	ND[a]	340	440
	+	ND	320	400
F.s. phaseoli (1.5 × 10^6 spores ml^{-1})	−	304	452	400
	+	50	384	395
F.s. pisi (1.5 × 10^6 spores ml^{-1})	−	352	500	430
	+	106	433	345
Chitosan (0.1% w/v)	−	175	380	232
	+	34	325	207
$CdCl_2$ (0.5 mM)	−	324	212	204
	+	51	212	186
Actinomycin D (10 µg ml^{-1})	−	167	206	247
	+	17	176	220
IAA (0.2 mM)	−	366	219	203
	+	27	87	93

[a] Not determined.

DISCUSSION

In immature pea pods infected with pathogens or treated with elicitors, the activities of chitinase and β-1,3-glucanase are rapidly and reproducibly increased after a lag phase of 4 to 8 h (Fig. 1). An earlier study of infected immature pea pods indicated considerable changes of the activities of the same two enzymes in the first 6 h after infection (17). We were unable to reproduce these results; we were also unable to detect the chitosanase activity that had been reported previously from the same tissue (17). The erratic results of the earlier study on chitinase (17) may have been due to the use of an exochitinase assay (1) which proved to be unsuitable for pea pods like for bean leaves (5).

In infected pea pods, chitinase and β-1,3-glucanase were induced with similar kinetics. Similar results have been obtained in ethylene-treated bean leaves (1, 5) and, albeit on a more extended time scale, in tomato plants infected with *Verticillium albo-atrum* (19, 21). This indicates a strongly coordinated regulation of the two enzymes and is in line with their postulated defensive function (1, 4, 19).

There were no significant differences in the induction of the two hydrolases between compatible and incompatible interactions. Thus, the enzymes do not seem to be directly involved in determining disease specificity in the pea-*Fusarium* interactions. This does not exclude the possibility that they are important for the resistance against the incompatible fungus. Resistance of pea tissue to *F. solani* f.sp. *phaseoli* appears to be associated with the synthesis of about 20 major pea proteins (11, 24). If the enhanced synthesis of these 'resistance-response proteins' is blocked or altered by inhibitors of protein synthesis or heat shock (12), the

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tissue becomes susceptible to infection. We are presently investigating the possibility that chitinase and β-1,3-glucanase are two of the twenty 'resistance-response proteins'.

With regard to ethylene production, pea pods show a wound response (26) when split for experimentation. Fungal infection or elicitor treatments lead to a large additional increase in ethylene production, as has been found in other instances (18, 23, 26).

Since ethylene (Table I) and ACC (Table II) induced chitinase and β-1,3-glucanase, it appears that enhanced ethylene, both exogenously applied and endogenously formed, is sufficient to induce the two enzymes. Is enhanced stress ethylene production also necessary, as a 'second messenger', for the induction of the two enzymes by infection or elicitors? We examined this by inhibiting ethylene production with AVG. A high concentration of AVG was used (25 μl 3 mM AVG/half pod, corresponding to about 0.4 mM AVG in the total amount of tissue water). At this concentration, AVG had only a marginal unspecific effect on the induction of chitinase and β-1,3-glucanase, but lowered the production of stress ethylene in all treatments tested to or below the level of controls treated with water only (Table III). In the infected and elicitor-treated pods, chitinase and β-1,3-glucanase were still strongly induced in the presence of AVG (Table III). In contrast, the induction of the two hydrolases by IAA was prevented when ethylene formation was inhibited by AVG (Table III). Thus, enhanced ethylene production appears to act as a second messenger for the induction of chitinase and β-1,3-glucanase by IAA but not for the induction by fungal infection and by biotic or abiotic elicitors. The enhanced stress ethylene production observed normally with the latter treatments is not a necessary signal for the induction of the two enzymes.

A similar conclusion was reached in the case of the induction of phytoalexins by fungal elicitors in soybean cotyledons (18). There, AVG treatments eliminated stress ethylene production but did not affect phytoalexin accumulation. Thus, as in our case, ethylene was only an 'indicator' but not an inducer of phytoalexin production. It should be noted, however, that exogenous ethylene does not induce phytoalexin accumulation in soybean cotyledons (18), and thus, endogenous ethylene is not really expected to be an inducer. In contrast, work on the accumulation of hydroxyproline-rich glycoprotein, a substance possibly involved in defense, came to a different conclusion (23). In melon seedlings infected with *Colletotrichum lagenarium*, AVG inhibited the biosynthesis of hydroxyproline-rich glycoproteins in the cell walls by about 20%, and this was taken as an indication that endogenous ethylene functioned as an inducer. Unfortunately, the effect seems so small, compared to the 9-fold stimulation of the synthesis of hydroxyproline-rich glycoprotein in the course of infection, that its significance remains somewhat doubtful. Again, a problem of this system is the small effect of exogenously applied ethylene: At a concentration of 500 nl ml^{-1}, it increased the amount of hydroxyproline-rich glycoprotein in the cell walls by a factor of only 2 in 7 d (9).

Chitinase and β-1,3-glucanase have a defense potential against fungi; thus, it may be advantageous for the plant to induce the two enzymes in direct response to a fungal infection. However, β-1,3-glucanase (15) and chitinase (A. Gehri, unpublished) are also induced when plants display defense reactions (the hypersensitive response) against virus infections. It will be of interest to examine whether or not the hydrolases are induced indirectly in response to endogenous stress ethylene in this case.

CONCLUSION

From these results, we conclude that ethylene and elicitors are separate, independent stimuli for the induction of chitinase and β-1,3-glucanase, and that enhanced stress ethylene production is a symptom, but not a necessary signal for the observed induction of the two hydrolases in infected pea pods.

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