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Influence of abscisic acid and the abscisic acid biosynthesis inhibitor, norflurazon, on interactions between *Phytophthora sojae* and soybean (*Glycine max*)

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Abstract

A comparison was made of the effects of abscisic acid (ABA) and the ABA biosynthesis inhibitor, norflurazon on the interaction between soybean leaves and *Phytophthora sojae*. Inoculation of leaves of cv. Harosoy resulted in a compatible interaction typified by the presence of spreading, water soaked lesions with ill-defined margins while inoculation of cv. Haro 1272 resulted in an incompatible interaction with lesions restricted to the inoculation site. Activity of phenylalanine ammonia lyase (PAL) slowly increased in the compatible interaction but in the incompatible interaction there was a rapid rise in activity within 8 h after inoculation. When Haro 1272 plants were treated with ABA the normally incompatible interaction with race 1 was changed to what resembled a compatible interaction and activity of PAL was reduced to control levels. There was no visible effect on the compatible combination. In contrast when plants of cv. Harosoy were treated with norflurazon the normally compatible interaction with race 1 was changed to that which resembled an incompatible interaction and PAL activity increased to high levels rapidly. There was no effect of norflurazon on the incompatible interaction of cv. Haro 1272 with race 1. Stomata on leaves of cv. Harosoy treated with norflurazon closed within 2 h of inoculation resembling the response of stomata in normal incompatible interactions but not compatible interactions where stomata remained open. On leaves of cv. Harosoy treated with norflurazon at sites 3 and 20 mm from the inoculation point stomata also closed. These results extend and confirm the idea that ABA is a molecule that may regulate the outcome of the interaction between soybeans and *P. sojae.*

Introduction

The plant hormone, abscisic acid (ABA), has been shown to be a potential regulator of the outcome of the interaction between specific cultivars of soybean and races of *Phytophthora sojae* (Cahill and Ward, 1989a,b; Cahill et al., 1993; Graham and Graham, 1996). Abscisic acid has also been implicated in several other plant–pathogen interactions (Dunn et al., 1990; Ryerson et al., 1993; Salt et al., 1986) and is known to regulate the expression of at least 150 genes (Anderson et al., 1994; Chandler and Robertson, 1994; Wu et al., 1994). It is commonly observed that an increase in ABA concentration is strongly correlated with its action (Willmer and Fricker, 1996).

In the soybean–*P. sojae* interaction it has been demonstrated that in incompatible interactions but not compatible interactions of dark-grown hypocotyls there are large decreases in ABA concentration at lesion sites and in tissues adjacent to the lesioned area. In addition, application of the abiotic elicitor, silver nitrate, which resulted in what phenotypically resembled an

incompatible interaction (restricted 'lesions', increased PAL activity and glyceollin synthesis) also resulted in lowered concentrations of ABA (Cahill and Ward, 1989a). Similarly, when treated with the acylalinine fungicide, metalaxyl, hypocotyls that normally reacted compatibly to inoculation with an avirulent isolate of *P. sojae* reacted incompatibly and showed dramatically reduced levels of ABA (Cahill et al., 1993). In experiments that used cDNA probes from bean to determine expression of PAL genes after treatment with ABA and inoculation it was shown that ABA suppresses PAL gene transcription directly and leads to development of compatibility in what should normally be an incompatible combination (Ward et al., 1989). In the soybean–*P. sojae* system ABA is clearly acting as a regulatory molecule.

There are two approaches that have been very useful in elucidating the role of a potential signal molecule – those that have used specific inhibitors of the synthesis of the molecule in question and those that use mutants that are either deficient in the molecule or have an impaired biosynthetic pathway. In this paper we use the pyridazinone herbicide, norflurazon, in the first approach to further define the role of ABA in the soybean–*P. sojae* interaction. Norflurazon inhibits phytoene synthesis and phytoene desaturase in carotenogenesis and therefore ABA biosynthesis (Bartels and Watson, 1978; Wilkinson, 1987). It is readily taken up by plant roots (Mersie and Singh, 1987), and is closely related to fluridone, a herbicide that has been used in a number of studies to elucidate the role of ABA (Belefant-Miller et al., 1994; Popova and Riddle, 1996; Steinbach et al., 1997; Kim et al., 1994; Pagano et al., 1997; Xu and Bewley, 1995). We also examine the effect of ABA and norflurazon on systemic stomatal closure, a characteristic of incompatibility in this system.

Materials and methods

Soybean cultivars, pathogen isolates and zoospore production

Seeds of soybean cultivars Harosoy (Rps 7) and the Harosoy isoline Haro 1272 (Rps 7, Rps 1a) and isolates of race 1 of *P. sojae* were kindly provided by Dr. Malcolm Ryley, Queensland Department of Primary Industries, Toowoomba, Queensland. The pathogen was maintained in culture on 20% V8 juice agar.

Zoospores were produced with the procedure of Cahill and Ward (1989a) and for inoculation were used at a density of 1×10^5 ml^{-1}.

Plant growth

Seeds of both cultivars were germinated in a commercial potting mix (Nurserymens' Brand, pH 5.5–6.0) in 15 cm plastic pots (5 seeds per pot). Plants were grown until 4 weeks of age in a greenhouse and then for a further 2 weeks in an environment chamber that enabled control of temperature and lighting conditions (25 °C, 14 h light). Plants were watered daily and provided with a complete fertiliser.

Treatment of plants with ABA and norflurazon

Six-week-old plants were carefully removed from the potting mix and roots washed gently with distilled water. The whole root system was then immersed in 100 ml of either distilled water (control) or a solution of 1×10^{-4} M ABA (± *cis–trans* isomers, Sigma Chemical Co., USA), or 3.4×10^{-7} M norflurazon (norflurazon technical, Novartis Crop Protection Australasia Limited, Pendle Hill, NSW). Plants were incubated in the solutions for 14 h within the controlled-environment chamber.

Abscisic acid was obtained as mixed isomers but the concentrations given here refer to the biologically active (+) isomer only. Abscisic acid was dissolved in methanol and diluted with sterile distilled water as required. The final methanol concentration in ABA solutions was adjusted to 0.2% v/v: 0.2% v/v methanol : water was used as the control. Norflurazon was dissolved in sterile distilled water.

Inoculation

Fully expanded trifoliate leaflets from plants treated with ABA, norflurazon or water alone as the control were carefully detached from the plant by severing the petiolule close to the petiole using a razor blade. Leaves were then placed abaxial side down onto a layer of moistened cotton wool (Combine Dressing, Smith & Nephew, Australia) within transparent plastic trays (29 cm × 21 cm × 6 cm). For enzyme extraction leaflets were inoculated by placing 3–4 10 µl drops of zoospore suspension, 2 cm apart onto the adaxial surface. For lesion diameter, and stomatal pore aperture measurements a single 10 µl drop of zoospore suspension was

placed on a leaflet blade, to one side of the central vein, approximately 1 cm from the junction of the petiole with the leaflet. Water or 0.2% v/v methanol (for ABA experiments) was used as the control treatment in each experiment. Trays were then placed into the controlled-environment chamber for the duration of the experiment.

Leaf epidermal peels and measurement of stomatal aperture area

Using fine forceps the epidermal layer was removed from the inoculation site and 3 and 20 mm from the inoculation site on the adaxial side of the leaf surface. Peels were immediately placed in a drop of distilled water on a microscope slide that was then placed in a humid atmosphere within a sealed plastic container. The time elapsed between removal of the epidermal peel and completion of the stomatal pore measurements was less than 15 min.

Light microscopy (Provis AX70, Olympus, Tokyo, Japan) in conjunction with data analysis software (Optimas Version 5.2, Optimas Corporation, Washington, USA) was used to calculate stomatal pore areas for individual stomata. For each treatment four epidermal peels from each of the three sites on the leaf were analysed. Twenty stomata were assessed at each site on each epidermal peel. Stomatal pore areas were calculated as $\mu m^2 \pm$ the standard error of the mean.

The viability of cells in epidermal peels was determined using the ability of functioning cells to take up neutral red stain (0.01% w/v in water).

Growth of P. sojae *in leaves*

Linear growth of *P. sojae* was determined in 10 leaves of each cultivar after inoculation with a single 10 µl drop of a suspension of zoospores (1×10^5 ml^{-1}) of *P. sojae* race 1. Visible lesion diameters were measured at 24 and 48 h after inoculation using a calliper with a vernier scale. To examine the effect of ABA and norflurazon on lesion development plants were treated with ABA or norflurazon as described above before inoculation and then lesion diameters measured at 24 h after inoculation.

Extraction of PAL from leaves

Extraction of PAL followed established procedures (Cahill and McComb, 1992). Forty lesions were excised from 10 leaves from each treatment or an equivalent area in uninoculated Harosoy leaves that served as controls and ground in cold sodium borate buffer (pH 8.8). The tissue slurry was then placed in a 1.5 ml Eppendorf tube and subjected to centrifugation (13,000 rpm) for 4 min. The supernatant was removed and stored at $-70\,^{\circ}$C until analysis. Activity of PAL in the extract was determined spectrophotometrically from the amount of cinnamic acid produced upon incubation with L-phenylalanine. An identical mixture with D-phenylalanine in place of L-phenylalanine served as the control. Activity was expressed as nmol cinnamic acid/g fresh weight/h by reference to a cinnamic acid standard curve. Duplicate samples were assayed and the data presented are based on the mean $\pm$ standard error of at least two separate experiments.

Results

Growth of the pathogen in leaves

Diameters of leaf lesions were measured at 24 and 48 h after inoculation (Table 1). Lesions were significantly smaller ($P < 0.01$) in the incompatible interaction of leaves of cv. Haro 1272 than in the compatible interaction of cv. Harosoy. In the incompatible interaction lesions were tightly defined and characterised by dark necrosis whereas in the compatible interaction lesions were lighter, less defined and spread throughout the leaf tissue.

Effects of ABA and norflurazon on pathogen growth in leaves

(i) ABA

At concentrations of ABA from 50 to 200 µM lesion diameters in the incompatible interaction of cv. Haro 1272 with race 1 were increased to the same size as those of the compatible interaction at 24 h post

Table 1. Lesion diameter on leaves of soybean cv. Harosoy and cv. Haro 1272 24 and 48 h after inoculation with *P. sojae* race 1

Time after inoculation (h)	Lesion diameter (mm)	
	cv. Harosoy	cv. Haro 1272
24	12.0 ± 0.4[a]	6.2 ± 0.5
48	17.5 ± 2.5	6.4 ± 0.8

[a]Data are the mean $\pm$ SE ($n = 10$).

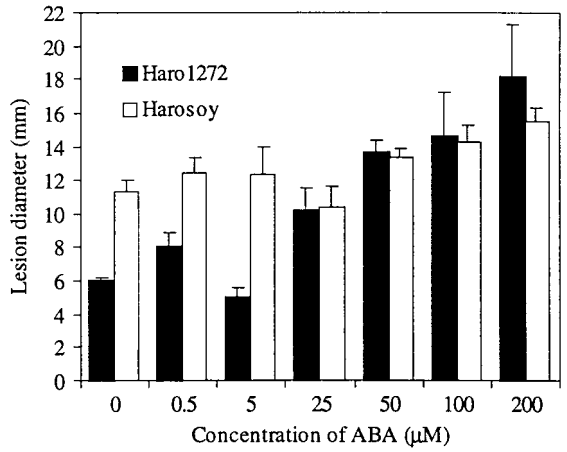

Figure 1. Diameter of lesions on leaves of soybean cultivars Harosoy (□) and Haro 1272 (■) 24 h after inoculation with a 10 μl drop of a suspension of zoospores (1×10^5 ml^{-1}) of *P. sojae* race 1 following treatment with ABA for 14 h. Standard errors of the mean are shown.

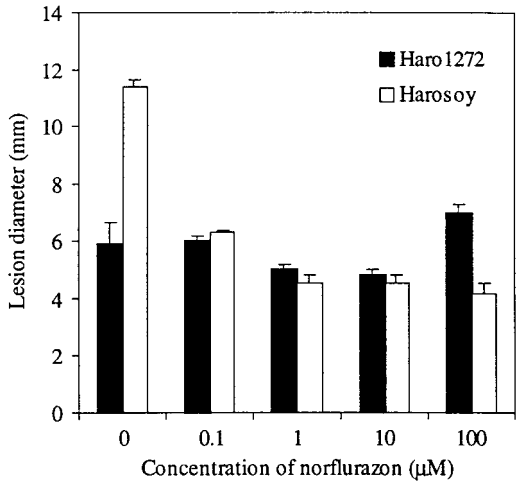

Figure 2. Diameter of lesions on leaves of soybean cultivars Harosoy (□) and Haro 1272 (■) 24 h after inoculation with a 10 μl drop of a suspension of zoospores (1×10^5 ml^{-1}) of *P. sojae* race 1 following treatment with norflurazon for 14 h. Standard errors of the mean are shown.

inoculation (Figure 1). Lesion diameters increased by up to 10 mm at the higher concentrations of ABA. There was no effect of ABA on lesion size in the compatible interaction.

(ii) Norflurazon

Treatment of the soybean plants with concentrations of norflurazon from 0.1 to 100 μM resulted in restriction of lesion diameter on leaves of cv. Harosoy measured 24 h after inoculation with *P. sojae* to a diameter that was similar to that in the cv. Haro 1272 (Figure 2). In cv. Harosoy, lesions were produced rapidly, were darkly necrotic and were typical of a hypersensitive response (HR). There was no effect of norflurazon treatment on leaf lesion diameters of cv. Haro 1272.

Activity of PAL after inoculation with P. sojae

There were very low levels of PAL activity in uninoculated cv. Harosoy control leaves throughout the course of the experiment (Figure 3). PAL activity in lesions from the incompatible interaction with cv. Haro1272 increased steadily to approximately 2×10^3 nmol cinnamic acid/g fresh weight/h at 24 h after inoculation. In contrast, PAL activity in the incompatible interaction rose rapidly within 4 h after inoculation and then increased to a maximum at 12 h after inoculation of 1.9×10^3 nmol cinnamic acid/g fresh weight/h. In the incompatible interaction PAL activity decreased

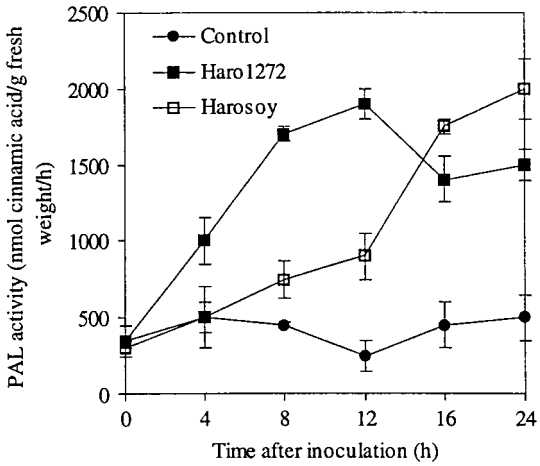

Figure 3. PAL activity in lesions taken from leaves of soybean cultivars Harosoy (□) and Haro 1272 (■) after inoculation with four 10 μl drops of a suspension of zoospores (1×10^5 ml^{-1}) of *P. sojae* race 1. Control levels were measured in non-inoculated Harosoy (●) leaves. Standard errors of the mean are shown.

between 12 and 16 h after inoculation and remained at this level for the duration of the experiment.

PAL activity in lesions after treatment with ABA and norflurazon

Incubation of seedlings of Harosoy with ABA had no effect on the levels of PAL activity in leaf extracts

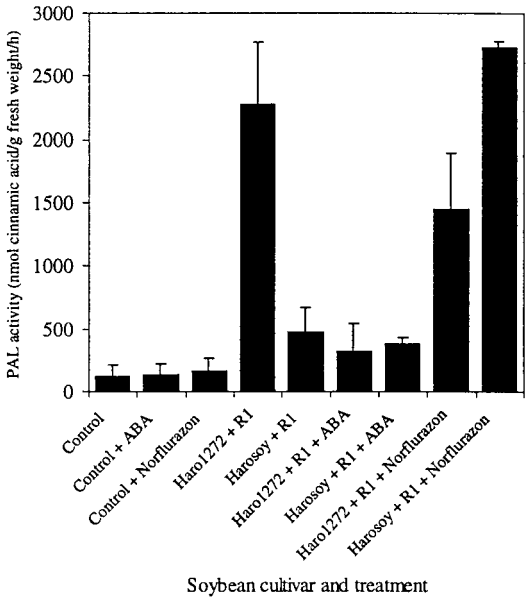

Figure 4. PAL activity in lesions of leaves of soybean cultivars Harosoy and Haro 1272 after treatment of plants with ABA (1×10^{-4} M) or norflurazon (3.4×10^{-7} M) for 14 h, followed by inoculation with four 10 µl drops of a suspension of zoospores (1×10^{5} ml^{-1}) of *P. sojae* race 1 for 8 h, or both treatment with ABA or norflurazon and inoculation. Harosoy served as the control. The standard errors of the mean are shown.

(Figure 4). Extracts from leaves of uninoculated Harosoy and Harosoy treated with 1×10^{-4} M ABA had activities of approximately 100 nmol cinnamic acid/g fresh weight/h. When inoculated with race 1, leaves of Harosoy produced low levels of PAL activity. In contrast inoculated seedlings of Haro 1272 showed activity of 2.3×10^{3} nmol cinnamic acid/g fresh weight/h. When treated with ABA, levels of PAL activity in inoculated leaves of Haro 1272 were reduced to levels equivalent to those found in the compatible interaction of Harosoy. There was no effect of ABA treatment on PAL activity in inoculated Harosoy.

Treatment of seedlings with norflurazon at a concentration of 3.4×10^{-7} M reduced the level of PAL activity in Haro 1272 leaves inoculated with race 1, but increased levels in the interaction with Harosoy to amounts above that found in the untreated incompatible interaction.

Stomatal responses to leaf inoculation before or after treatment with ABA and norflurazon

Stomatal pore areas of fully opened stomata varied depending on age of the leaf. Mean areas also varied from leaf to leaf but were generally within the range 20–30 µm^2 (data not shown). In uninoculated Harosoy control leaves the stomatal pore area remained relatively constant throughout the course of the experiment (Table 2, Figure 5A). After inoculation with *P. sojae*, pore areas remained constant in the compatible interaction but decreased markedly in the incompatible interaction. In the incompatible interaction, by 2 h after inoculation, stomata were almost fully closed at the inoculation site and 3 mm from the inoculation site and to one third of controls at 20 mm from the inoculation site (Table 2). In the compatible interaction, stomata were partially closed at the inoculation site but remained fully open at 3 and 20 mm from the inoculation point. By 8 h after inoculation stomata at 3 and 20 mm in the incompatible interaction had reopened.

Stomata at all sites examined on leaves from plants that had been treated with ABA were closed at the start of the experiment and remained closed over the 12-h observation period (data not shown). In leaves of Harosoy treated with norflurazon stomata were completely closed at the inoculation site within 2 h (Figure 5A). Stomata remained closed over the 12-h period of the experiment. Stomata also closed at the site 3 mm from the inoculation point but began to reopen after 6 h and were fully open by 12 h after inoculation (Figure 5B). A similar pattern was found at the site 20 mm from the inoculation point where stomata closed rapidly by 2 h and then reopened by 12 h after inoculation (Figure 5C).

Discussion

Inhibition of ABA biosynthesis by norflurazon had a dramatic effect on the outcome of the interaction of soybean with a compatible race of *P. sojae*. In leaves, lesion size was reduced, activity of PAL was increased and systemic closure of stomata occurred. All these responses phenotypically resembled a normal incompatible response. Expression of incompatibility in the cv. Haro 1272–*P. sojae* race 1 combination was, however, not affected by norflurazon. Previous results have shown that ABA decreases in concentration at the inoculation site in incompatible but not compatible interactions in soybean hypocotyls (Cahill and Ward, 1989a,b) and that PAL activity is regulated at the level of gene transcription by ABA (Ward et al., 1989). Together with the present results these data provide strong support for the hypothesis that ABA is a regulator of the defence response in the soybean–*P. sojae* system.

Table 2. Stomatal pore area of single stomata in soybean leaf epidermal peels of cv. Harosoy and cv. Haro 1272 after inoculation with *P. sojae*

Distance from inoculation point (mm)	Stomatal pore area (μm^2)					
	Harosoy control		Harosoy inoculated		Haro 1272 inoculated	
	2 h[a]	8 h	2 h	8 h	2 h	8 h
0	19.6 ± 0.2[b]	21.32 ± 4.2	13.05 ± 5.7	13.89 ± 3.2	1.73 ± 0.1	1.47 ± 0.1
3	20.1 ± 3.3	20.71 ± 3.5	19.07 ± 4.5	23.46 ± 1.9	2.37 ± 0.2	20.22 ± 1.9
20	20.01 ± 4.2	20.26 ± 3.1	22.01 ± 3.1	23.97 ± 2.8	7.05 ± 1.8	18.67 ± 2.5

[a]Time in hours after inoculation of leaves with *P. sojae* race 1.
[b]Values are the mean $\pm$ standard error ($n = 80$).

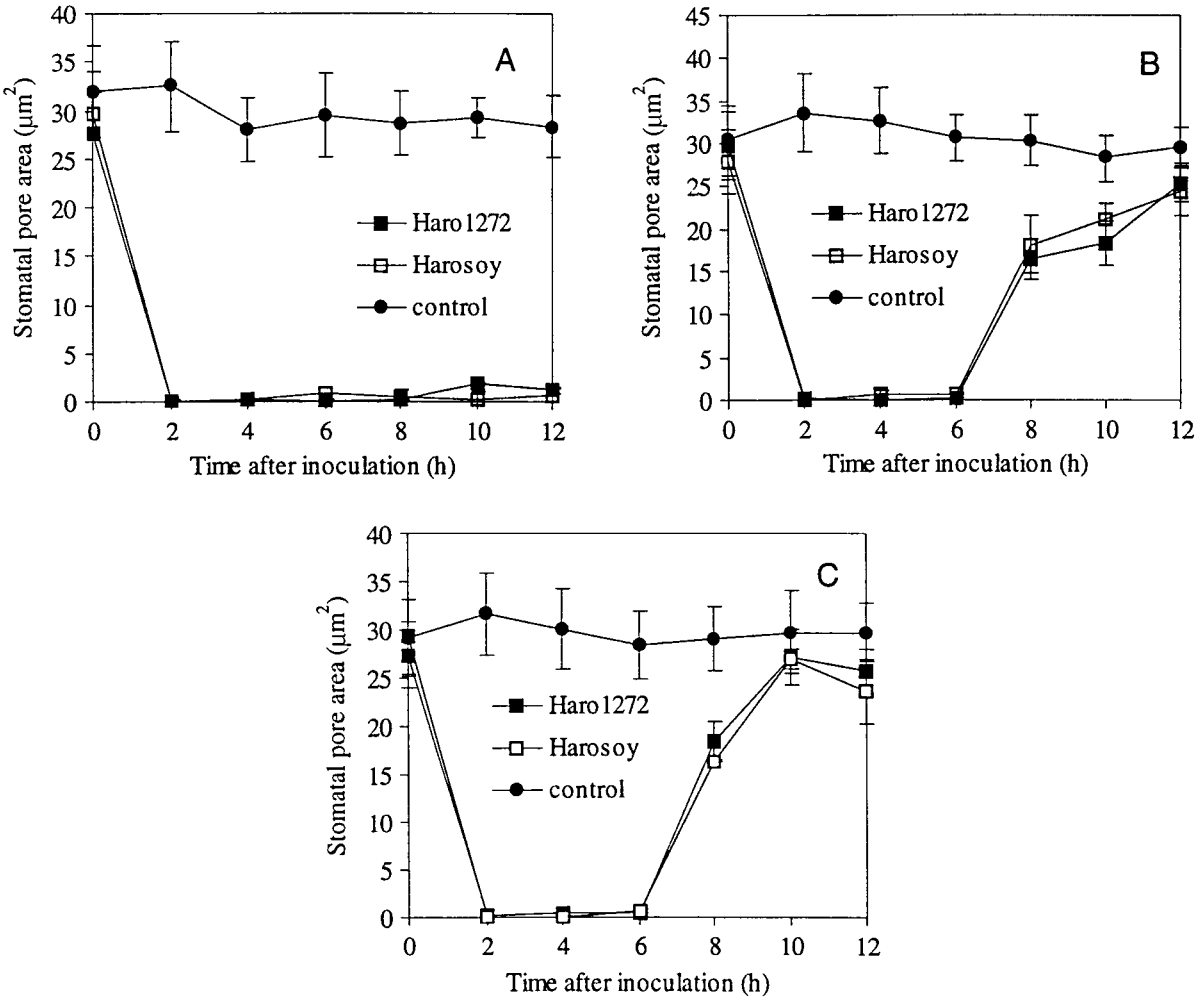

Figure 5. Areas of individual stomatal pores in leaves of soybean cultivars Harosoy (□) and Haro 1272 (■) that had been treated with norflurazon (3.4×10^{-7} M) and then inoculated with a 10 µl drop of a suspension of zoospores (1×10^5 ml^{-1}) of *P. sojae* race 1. Uninoculated Harosoy leaves treated with norflurazon (3.4×10^{-7} M) served as the controls (●). Stomatal pore area was measured on epidermal peels taken from A, the inoculation site; B, 3 mm from the inoculation site and C, 20 mm from the inoculation site. Standard errors of the mean for each data point are shown. Where standard errors are not shown they were smaller than the data point symbol.

The responses to inoculation of leaves that had been treated with norflurazon resemble also the responses of hypocotyls to treatment with the acylalanine fungicide metalaxyl. Metalaxyl, an inhibitor of oomycete RNA polymerase II (Davidse, 1984), changes compatibility in this system to what phenotypically resembles incompatibility (Cahill and Ward, 1989a; Lazorovits and Ward, 1982). In conjunction with this change is a rapid reduction in concentration of ABA in treated hypocotyls that normally react compatibly (Cahill et al., 1993).

As was found previously in dark-grown soybean hypocotyls, treatment with ABA of plants that normally reacted incompatibly with race 1 of *P. sojae* turned the incompatible interaction in leaves into a compatible interaction. Concentrations of PAL and glyceollin were also reduced as a consequence (data not shown). The effect of ABA on stomatal pore area following inoculation was not easily assessed because ABA is a potent activator of stomatal closure and in plants that were treated with ABA stomata were tightly closed in the controls and in compatible and incompatible interactions.

Closure of stomata upon infection with *P. sojae* has been shown previously to be a rapid and characteristic response of leaves during incompatible interactions (McDonald and Cahill, 1999). In contrast, in the compatible interaction stomata remained open after leaf inoculation. This response possibly indicates the presence of a transmissible factor that is produced during incompatibility. We have now shown that a normally compatible response, where stomata remain open, can be switched to what phenotypically resembles incompatibility with consequent rapid stomatal closure upon treatment of leaves with norflurazon before pathogen challenge. It would thus seem that norflurazon, by acting directly to reduce cellular concentrations of ABA, induces HR. Systemic stomatal closure occurs as a consequence of the interaction. We do not know at present whether ABA is the transmissible signal but it is possible that ABA in existing pools is transported away from the lesion, as occurs in hypocotyls (Cahill and Ward, 1989a) to the guard cells where accumulation causes stomatal closure. Measurement of the concentration of ABA in individual guard cells during incompatible interactions would elucidate the role of ABA.

The present results that show loss of ABA induces incompatibility contrast markedly with those of Dunn et al. (1990). In their work it was shown in the interaction between *Colletotrichum lindemuthianum* and *Phaseolus vulgaris* that there was an increase in symptom severity (i.e. susceptibility) when resistant hypocotyls were treated with fluridone, a molecule similar to that of norflurazon and with similar modes of action. In addition, they showed that an increase in ABA concentration in host tissue correlated with induced resistance and that application of ABA reduced the severity of symptoms on hypocotyls. The differences in the two systems may in part be due to timing of the interactions and subsequent events in pathogenesis. The interaction of *P. sojae* with soybean is very rapid and host recognition and penetration occur within 30 min. Compatible and incompatible states are thus established within 2 h after inoculation. In the *C. lindemuthianum–P. vulgaris* system an initial biotrophic stage lasts up to 4 days. Full development of resistant and susceptible responses may not be discernible until 10 days after inoculation (Dunn et al., 1990). ABA concentrations reached a maximum after 2 days and then declined to pre-inoculation levels after 8 days. Direct comparisons between the *C. lindemuthianum* and bean interaction and that of *P. sojae* and soybean are further complicated because concentrations of ABA in the present system were determined on localised levels in small pieces of lesioned tissue and not whole organs.

Abscisic acid has been shown to influence both the biochemistry of resistance and the outcome of the interaction in several other systems. For example in potato tuber slices Henfling et al. (1980) showed that addition of ABA suppressed the formation of terpenoid phytoalexins. Salt et al. (1986) showed that susceptibility of tobacco to *Peronospora tabacina* was increased upon stem injection of ABA. Reduced levels of ABA were also a characteristic of the interaction between two *Uromyces* species and *P. vulgaris* where significant decreases in ABA concentration were found 6 h after inoculation (Ryerson et al., 1993). However in the latter study challenge by the nonpathogenic or pathogenic variety led to similar reductions in ABA levels.

Conclusions

We have clearly shown that administration of ABA or the carotenoid biosynthesis inhibitor norflurazon to soybean plants shows opposing effects. The data support the hypothesis that ABA is a molecule that has an important role in expression of either compatibility or incompatibility in the soybean–*P. sojae* system. It is evident also that systemic stomatal closure during the

incompatible response is closely linked to the induction of the incompatible response.

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