Phytoalexin Production in Locally Cross-Protected Harosoy and Harosoy-63 Soybeans

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ABSTRACT

Hypocotyl tissues of 5-day-old Harosoy soybeans were cross-protected against local infection by *Phytophthora megasperma* var. *sojae* race 1 (*Pms*$_1$) by prior inoculation of the hypocotyl with a nonpathogen, *Phytophthora cactorum*. *P. cactorum* remained alive in the hypocotyl lesion during plant development, continuously triggering the production of phytoalexin (PA_k) inhibitory to the growth of the *Pms*$_1$ pathogen. The maintenance of the PA_k protective mechanism against infection by *Pms*$_1$ extended to a stage of plant development in which a form of adult plant resistance may become the predominating protective mechanism. Quantitatively, the PA_k reached peak concentration at 6 days after inoculation with *P. cactorum*; after 6 days, the concentration of PA_k remained relatively constant. No spectrophotometrically detectable breakdown product of the PA_k was found during the period studied. In the same manner, Harosoy-63 (H-63) soybean hypocotyls were cross-protected against local infection by an isolate of *P. megasperma* var. *sojae* (*Pms*) which can attack them. H-63 was cross-protected by prior inoculation of the hypocotyl with *P. megasperma* var. *sojae* race 1 (*Pms*$_1$), a nonpathogen of H-63. *Pms*$_1$ failed to persist in the hypocotyl of H-63 after 3 days. During this time, the initially induced PA_k broke down, allowing *Pms* infection to develop. With the cross-protective mechanism lost, the plants died.

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A model has been devised for a type of disease resistance for Harosoy-63 (H-63) soybeans to early stem infection by the Phytophthora stem and root rot pathogen *Phytophthora megasperma* var. *sojae* race 1 (*Pms*$_1$). This model implicates a phytoalexin, PA_k, in the protective mechanism (2, 3, 6, 8). Intensive studies have been made on the two soybean cultivars Harosoy and Harosoy-63. These two cultivars differ by a single dominant gene, *Rps*, conditioning Phytophthora stem and root rot resistance (1). This has made possible the partial elucidation of an *Rps*-activated Phytophthora rot resistance mechanism in H-63 hypocotyls. This mechanism leads to the formation of the precursor products for PA_k inducer formation in *P. megasperma* var. *sojae* (4).

The phytoalexin, PA_k, has been characterized as a yellow-colored compound with an R_F of 0.56 in the organic phase of butanol:acetic acid:water (4:1:5, v/v) solvent on paper or silica gel thin-layer chromatography. It fluoresces bright yellow under ultraviolet irradiation, has an absorption maximum at 489 nm at pH 7 (3), and was implicated in the acquired local resistance of Harosoy soybeans to infection by *Pms*$_1$ with prior inoculation with *P. cactorum* (8).

The purpose of this study was twofold. First, with respect to Harosoy cross-protection, the aim was to determine whether both the protection and the PA_k production were maintained until adult plant resistance becomes the predominating protective mechanism (9). Secondly, with respect to the H-63 cross-protection, the aim was to record duration of protection and correlate the results with the PA_k concentration flux reported by Frank & Paxton (3).

MATERIALS AND METHODS.—*Soybean seedling culture*.—Soybean, *Glycine max* (L.) Merr. 'Harosoy' and 'Harosoy-63' (H-63), were grown in builder's sand in the greenhouse during the early trials of this study. Healthier, more vigorous seedlings were propagated faster by germinating seeds on a wire screen over aerated Hoagland solution. Five-day-old seedlings (4 to 5 cm in height, emerging primary leaves) were used for inoculations. After initial inoculations, we maintained the seedlings by suspending the plant roots in aerated Hoagland solution

with support screens. Plants were held in a growth chamber on a 12 hr-12 hr diurnal cycle of light and dark at about 22 C. Plastic bags inserted over the hydroponic apparatus were used to maintain high humidity for 12 hr after all inoculations. In one trial, potted seedlings were used to verify prior results under more natural conditions. Six plants were grown in each 4-inch-diam pot. Soil used was a 2:1:1 silty-clay-loam:peat:sand mixture.

Inoculum.—Harosoy is resistant to *P. cactorum* (Leb. & Cohn) Schroet., while susceptible to *P. megasperma* Drechs. var. *sojae* A. A. Hildeb. races 1 and *Pms*. Harosoy-63 is resistant to race 1 and susceptible to *Pms*. Cultures of *P. megasperma* var. *sojae* race 1 were obtained from Morgan & Hartwig (7), and of *Pms* (an isolate from subterranean clover), from Johnson & Keeling (5). These isolates will be referred to as Pms_1 and *Pms*. *Phytophthora cactorum*, Pms_1, *Pms* inoculum was produced in V-8 broth for 7 to 10 days with *P. cactorum* and Pms_1, and for 3 days with the faster growing *Pms*. We prepared V-8 broth by centrifuging the pulp from commercial V-8 juice, diluting the clarified juice 20:1 with deionized water, and adjusting the pH to about 7 with 2 g $CaCO_3$/liter. We avoided possible loss of pathogenicity with Pms_1 and *Pms* and cultures by always inoculating broth cultures with plugs from active growth on plate cultures. Known pathogenic plate cultures were held in a cold room on lima bean agar and periodically transferred. Purity of the cultures was determined by differential cultivar inoculations and microscopic observation. The method used for hypocotyl inoculation was to insert a small piece of mycelium in a puncture wound 1 cm below the cotyledonary node using a curved needle or curved forceps.

Harosoy cross-protection.—A small puncture wound was made with forceps in the hypocotyls of 90 Harosoy seedlings. A small piece of mycelial mat of *P. cactorum*, a nonpathogen of soybeans grown on V-8 broth, was placed in this wound. These plants were then held in a moist chamber for 6 hr. At 24-hr intervals, 10 of these inoculated plants were inoculated with the pathogen Pms_1 in puncture wounds made at the same area on the hypocotyl, but at a 90° angle to the initial inoculation puncture wound. Again, plants were held in a moist chamber for 6 hr. After 5 days, plant reaction was recorded (Table 1). An additional 90 seedlings were inoculated with *P. cactorum* for time sequence PA_k analysis. At each 24-hr interval, a control of 10 noncross-protected plants was inoculated with Pms_1 to make sure patho-

TABLE 1. Prolonged cross-protection of Harosoy soybean plants by inoculation with *Phytophthora cactorum* (Pc) followed at various times with inoculation by *Phytophthora megasperma* var. *sojae* race 1 (Pms_1)

Initial inoculation	Second inoculation	% Plants killed when second inoculation was at indicated days after initial inoculation								
		1	2	3	4	5	6	7	8	15
None (wound only)	Pc	0[a]	0	0	0	0	0	0	0	0
None	Pms_1	90	90	87	80	80	80	80	70	7
None (wound only)	Pc	0	0	0	0	0	0	0	0	0
None	Pms_1	97	97	90	90	80	87	80	77	10
Pc (autoclaved)	Pms_1 (autoclaved)	0	0	0	0	0	0	0	0	0
Pc	Pms_1 (autoclaved)	0	0	0	0	0	0	0	0	0
Pc (autoclaved)	Pms_1	100	97	97	90	90	90	87	80	10
Pc	Pms_1	0	0	7	0	0	0	7	7	0

[a]Per cent of plants killed out of 90 inoculated in each treatment.

TABLE 2. Limited cross protection of Harosoy-63 soybean plants by inoculation with *Phytophthora megasperma* var *sojae* race 1 (Pms_1) followed at various times with inoculation by *Phytophthora megasperma* var *sojae* race 3 (*Pms*)

Initial inoculation	Second inoculation	% Plants killed when second inoculation was at indicated days after initial inoculation								
		1	2	3	4	5	6	7	8	15
None (wound only)	Pms_1	0[a]	0	0	0	0	0	0	0	0
None	*Pms*	90	97	100	100	97	97	80	77	30
None (no wound)	Pms_1	0	0	0	0	0	0	0	0	0
None	*Pms*	97	100	100	97	97	87	87	70	20
Pms_1 (autoclaved)	*Pms* (autoclaved)	0	0	0	0	0	0	0	0	0
Pms_1	*Pms* (autoclaved)	0	0	0	0	0	0	0	0	0
Pms_1 (autoclaved)	*Pms*	100	97	90	97	97	87	87	77	20
Pms_1	*Pms*	0	0	10	30	47	70	60	50	10

[a]Per cent of plants killed out of 90 inoculated in each treatment.

genicity was not lost during the investigation. All other necessary controls and the above treatments were run in 3 replicates of 30 plants each.

H-63 cross-protection.—Using the same methods, 90 H-63 seedlings were inoculated with Pms_1. At 24-hr intervals, 10 of the inoculated plants were inoculated with the pathogen *Pms*. Five days after inoculation with the pathogen, plant reactions were recorded (Table 2). Controls were run in all trials. Ninety additional seedlings were inoculated with Pms_1 for time sequence PA_k production analysis. All treatments and controls were run in triplicate.

Analysis.—Relative amounts of PA_k present in cross-protected plants were determined daily for an 8-day period and at 15 days after the initial inoculations. Crude PA_k extractions were done by autoclaving (15 min/121 C) 10 stem lesions in 20 ml of deionized water. Samples were concentrated to 10 ml and adjusted to about pH 7 with HCl; 3 ml of this solution was used for spectrophotometric analysis. We determined relative amounts of PA_k present at each 24-hr interval by comparing spectrophotometric results of the samples to a previously analyzed 4 = μg/ml ethanol standard of PA_k (Fig. 1).

Thin-layer chromatography (TLC) was used to verify the presence of PA_k in the lesions at each 24-hr interval. Extracts from three trials were combined; and each day, extracts were concentrated to 3 ml. One ml of this was spotted on silicic acid chromatogram plates. The plates were developed in the organic phase of butanol:acetic acid: water (4:1:5, v/v) solvent. Presence of PA_k was shown by a yellow fluorescing band near R_F 0.56 under ultraviolet irradiation. This fluorescing band was removed from the plate, added to 3 ml of water, and analyzed on the spectrophotometer for absorption at 489 nm.

The crude extracts from three trials were combined and concentrated to a 3-ml sample for each 24-hr interval. One ml of this concentrated sample was autoclaved in each of three small culture tubes with 10 ml of V-8 broth for bioassay. A 5-mm-diam plug from an actively growing culture of Pms_1, or *Pms* in the second series, was placed in each of the three tubes. The tubes were closed with parafilm. The height of growth for all experimental and control tubes was measured after 4 days. Assays were run in triplicate for each of the sample extracts from day 1 through day 8 (Fig. 2).

Another bioassay was set up like the above except that the partially purified PA_k from TLC plates was used instead of crude extracts. The TLC plate band containing the PA_k band was scraped off the glass plate into a small culture tube with 10 ml of V-8 broth. Height of growth was recorded after 4 days. The 0.56 R_F area from TLC chromatograms of healthy hypocotyl extracts was assayed as controls (Fig. 2).

Viability of *P. cactorum* in Harosoy and Pms_1 in H-63 inoculated hypocotyls was determined for 8 days. We surface-sterilized hypocotyl pieces with lesions by shaking 3 min in 0.525% sodium hypochlorite solution with a drop of Tween 20 (polyoxyethylene sorbitan monolaurate) added. Sections

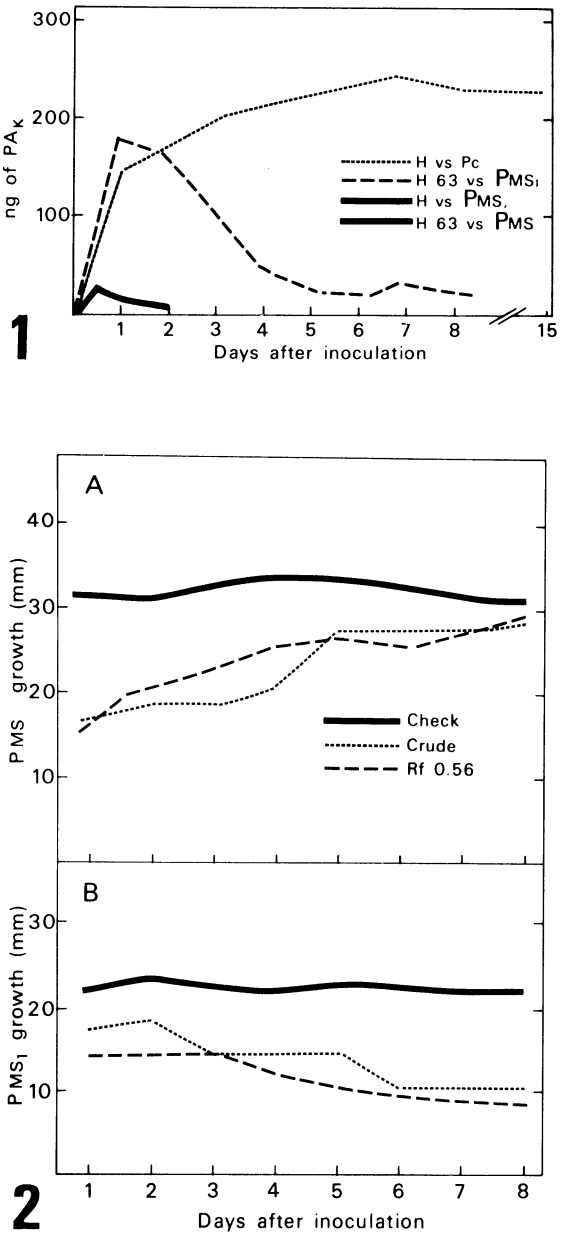

Fig. 1-2. 1) Nanograms of phytoalexin, PA_k, isolated from each Harosoy 63 (H-63) or Harosoy (H) plant inoculated with *Phytophthora cactorum* (Pc) or *Phytophthora megasperma* var.*sojae* race 1 (Pms_1) at various days after inoculation. **2) A)** Four-day growth of *Phytophthora megasperma* var. *sojae* race 3 (*Pms*) in V-8 broth supplemented with crude or chromatographed extracts (R_F 0.56) of Harosoy-63 soybean hypocotyls harvested at various days after inoculation with *Phytophthora megasperma* var. *sojae* race 1. **B)** Four-day growth of *Phytophthora megasperma* var. *sojae* race 1 (Pms_1) in V-8 broth supplemented with crude or chromatographed extracts (R_F 0.56) of Harosoy soybean hypocotyls harvested at various days after inoculation with *Phytophthora cactorum.*

were rinsed in sterile distilled water. Five longitudinally split sections were placed on an antibiotic lima bean agar medium. We prepared this medium by adding 50 μg/ml potassium salt of penicillin and 25 μg/ml dihydrostreptomycin sulfate to standard lima bean agar at 45 C. After 7 days, fungal growth from the hypocotyl sections was transferred to hemp seed broth to induce sporulation. Resulting fruiting bodies were identified.

RESULTS.—*Harosoy cross-protection.*—The artificially induced resistance in Harosoy against Pms_1 infection extended for at least 15 days after the PA_k production was initiated by *P. cactorum*. During this time, there was no evident PA_k breakdown. Maximum concentration of PA_k in the plant lesion occured 7 days after inoculation, after which it remained at a level of about 240 ng/lesion (Fig. 1).

TLC of concentrated extracts from each 24-hr period showed a yellow fluorescent band at R_F 0.56 under ultraviolet irradiation. Spectrophotometric analysis of this band showed a strong peak at 489 nm.

Results from the bioassays of the chromatogrammed PA_k and crude extract against growth of Pms_1 corresponded well with data from absorption intensity at 489 nm. The bioassays showed increasing inhibition of Pms_1 mycelial growth from day 1 to day 6 (Fig. 2-B). Likewise, absorption at 489 nm of the lesion extracts increased. PA_k extracted from day 6 to day 15 revealed no significant change in the level of inhibition. Spectrophotometric data correspondingly showed no significant change in the level of PA_k production of day 6 to 15 extracts.

The viability of *P. cactorum* in the lesions extended throughout the 15-day period.

Harosoy-63 cross-protection.—The induced resistance in H-63 against *Pms* infection extended only to 3 days following initial PA_k production initiated by Pms_1. Peak buildup of PA_k was recorded at 24 hr, after which breakdown began occurring (Fig. 1).

Analysis of hypocotyl extracts by TLC at 1-day intervals showed a distinct reduction in the yellow fluorescent band at R_F 0.56 after day 2. The bioassay of the TLC purified PA_k and the crude extract against growth of *Pms* correlated well with the PA_k amounts determined spectrophotometrically (Fig. 1). The bioassay showed decreasing inhibition from day 2 to day 6 (Fig. 2-A). Decreasing PA_k production was recorded during this same period by marked decreases in absorbance at 489 nm of lesion extracts.

Viability of Pms_1 in the lesion extended only to day 2 to 3.

DISCUSSION.—The cross-protection of two cultivars of soybeans against Phytophthora stem rot pathogens has been shown to differ significantly. The different reactions appear to depend on when PA_k is produced and the amount produced. The levels of PA_k recorded daily by bioassay and absorption at 489 nm coincided.

The differences in PA_k level in Harosoy and Harosoy-63 may be explained by the differential viability of the protecting fungi, *P. cactorum* and Pms_1, respectively, in the hypocotyl. Metabolites or enzymes from the actively growing fungus are necessary to stimulate production of PA_k by the soybean. The death of the fungus is followed by a sharp decrease in PA_k level. Apparently PA_k is unstable and without continuous production in the plant, the level of PA_k drops.

Without PA_k local protection, the pathogenic *Phytophthora* introduced on the second inoculation infects the plant. The viability of Pms_1 in H-63 lasted a maximum of 3 days, after which the PA_k level fell rapidly. Without Pms_1 present to induce additional PA_k H-63 becomes susceptible to *Pms*. The viability of the nonpathogen, *P. cactorum*, in Harosoy remained essentially constant over the 15-day period after inoculation. The fact that it does remain alive may explain the maintenance of cross-protection. The role of PA_k in the protective mechanism may become rather insignificant in the 3-week-old plant because of other forms of adult plant resistance (9). It appears the PA_k forms and accumulates beyond a level tolerable to Pms_1 growth. Accumulation of levels of PA_k fungicidal to *P. cactorum*, on the other hand, does not occur in Harosoy. Bioassays also indicate the difference in phytoalexin levels. Work is continuing on the interaction of different *Phytophthora* species, varieties, and races with soybean and the role PA_k plays in disease resistance.

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