Hydroxyphaseollin Production by Various Soybean Tissues: a Warning Against Use of "Unnatural" Host-Parasite Systems

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ABSTRACT

Confirming previous reports, rates of production of the antifungal pterocarpan 6a-hydroxyphaseollin (HP) were greater in inoculated hypocotyls of Harosoy 63 soybeans (monogenically resistant to *Phytophthora megasperma* var. *sojae*) than in the near-isogenic susceptible cultivar Harosoy. Hydroxyphaseollin was also produced by soybean roots, cotyledons, pods, and tissue culture callus when inoculated with *P. megasperma* var. *sojae*, but rates of production by these tissues were similar in the two cultivars. The data further implicated HP with the *Phytophthora* resistance of Harosoy 63 soybeans, since accelerated production in this cultivar relative to Harosoy was only observed in hypocotyls, the organ in which resistance is expressed naturally. Our data therefore warn against the use of "unnatural" plant tissues such as pods and tissue culture callus in investigations concerned with the elucidation of naturally occurring disease resistance mechanisms in plants.

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Investigators studying disease resistance mechanisms in higher plants frequently employ "unnatural" host-parasite systems; namely, microorganisms in contact with host organs or tissues other than those normally attacked. Tuber tissues (15), fruit endocarp (2, 4), roots (14), and tissue culture callus (1, 6, 17) provide more controlled and manipulatable model systems, and eliminate errors due to the presence of organisms other than those under study (16), but may produce artifactual results irrelevant to the defense mechanisms occurring in "natural" host organ-parasite interactions.

Accelerated production of 6a-hydroxyphaseollin (HP) (13) appears to be the mechanism of expression of monogenic resistance in hypocotyls of soybeans (*Glycine max* [L.] Merr.) to *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildb. (5, 7, 8, 10). We report evidence here indicating that the disease resistance gene is only expressed in the natural hypocotyl-*P. megasperma* var. *sojae* system and not in inoculated roots, tissue culture callus, cotyledons, or pods.

MATERIALS AND METHODS.—The near-isogenic soybean cultivars, Harosoy (susceptible to *P. megasperma* var. *sojae*) and Harosoy 63 (resistant to *P. megasperma* var. *sojae*), were grown in 4-inch pots of soil in growth chambers as previously described (8). In some experiments, plants were grown in white silica sand (No. 16) for harvest of roots. Soybean pods were produced by growing plants in 8-inch pots. All plants were used when 7-12 days old except those grown for harvest of pods.

Tissue cultures of the two soybean lines were initiated from hypocotyl tips of seedlings obtained from surface-sterilized (10 min in 0.25% sodium hypochlorite) seeds germinated on water agar plates. The callus cultures were initiated and maintained on Linsmaier & Skoog's synthetic medium (11), amended to contain, per liter, 40 g sucrose, 100 mg myo-inositol, 140 mg L-glutamine, 10 mg L-cysteine-HCl, 1 mg kinetin, 1 mg 2,4-dichlorophenoxyacetic acid, and 10 g Bacto-agar (Difco), in addition to the original inorganic components and vitamins. Cultures were grown at 25 C in darkness and transferred at 3- to 4-week intervals.

A previously described (8) race 1 isolate of *Phytophthora megasperma* var. *sojae* (P174) was used for inoculations. The fungus was grown on a pea broth medium as previously described (9), and mycelial pieces or zoospore suspensions (ca. 10^5/ml) were used as inoculum. Zoospores were produced by washing mycelium grown for 48 hr on pea broth (8) 3-4 times with sterile distilled water, then inundating the mycelium with water or a modified sterile petri solution [0.4 g $Ca(NO_3)_2$, 0.15 g $MgSO_4$, 0.15 g KH_2PO_4, and 0.06 g KCl/liter of water]. The plates were maintained at room temperature under fluorescent lights for 12-24 hr, and the zoospore suspensions were recovered by decantation and diluted to desired concentrations with sterile water or petri solution.

Cotyledons were removed from 7- to 10-day-old plants, surface sterilized in 0.25% sodium hypochlorite for 5 min, and rinsed with sterile water. Depressions were made on the undersurface of the cotyledons with a razor blade, and mycelial pieces of *P. megasperma* var. *sojae* were placed into these "wells" with a drop of water. Inoculated and control-wounded cotyledons were then placed in petri dish moist chambers at 25 C in the dark.

Hypocotyls of intact 7-day-old soybean plants were inoculated with *P. megasperma* var. *sojae* by the method of Klarman & Gerdemann (9). The inoculated plants were maintained at 100% relative humidity after inoculation as previously described (8).

Tissue culture callus was placed in sterile petri

dishes in a transfer room, and 10 ml of sterile petri solution or zoospore suspension in petri solution were aseptically placed into each dish. The tissues were removed from the dishes after 24-72 hr, patted dry on absorbent paper, and weighed fresh or lyophilized. Sterility checks on these plates were made by direct microscopic observation of incubation fluids or by placing aliquots onto nutrient agar or into eugon broth tubes and observing for the formation of bacterial growth. Data were rejected for plates showing signs of contamination.

Immature seeds were removed from soybean pods, and water, mycelium, or zoospore suspensions of *P. megasperma* var. *sojae* (ca. 0.2 ml) were placed into the seed locules for 24-48 hr. The pods were incubated in petri dish moist chambers at 25 C in the dark. Fluids in the locules (diffusates) were then recovered, filtered through Whatman No. 1 paper, and extracted with ethyl acetate as described elsewhere (8) prior to quantitation of hydroxyphaseollin. The remaining pods were retained for fresh or dry weight determination.

The roots of soybean plants grown in sand were washed carefully with water, and the roots only were immersed in zoospore suspensions of *P. megasperma* var. *sojae* in water for 24 hr. The plants were then removed from the liquid and left in a darkened moist chamber for an additional 24-48 hr before the roots were removed and assayed for HP.

Hydroxyphaseollin was extracted from fresh soybean tissues with aqueous ethanol, or from lyophilized tissues with ethyl acetate/methanol (9:1, v/v) as previously described (7, 8). The ethanol extracts were bioassayed by the petri dish method using *P. megasperma* var. *sojae* (8), and by the thin-layer chromatographic (TLC) antifungal bioassay (8, 10). Chromatographic plates were developed with hexane/ethyl acetate/methanol (60:40:1, v/v) prior to bioassay. Because whole cotyledons contained sufficient concentrations of a compound which interfered with the gas-liquid chromatographic (GLC) analysis, they were harvested by excising ca. 0.5 mm of tissue underlying the originally wounded area with a razor blade. Such "excised wound areas" were also prepared from inoculated hypocotyls and analyzed for HP as in previous work (7).

RESULTS.–*Phytophthora megasperma* var. *sojae* rapidly rotted the hypocotyls of Harosoy, but Harosoy 63 hypocotyls exhibited typical hypersensitive resistance (Table 1). However, no other soybean tissue reflected such clear differentiation of the two cultivars. Pods and cotyledons of the two cultivars produced similar amounts of HP (Table 1). Browning and rotting were observed on roots and tissue culture callus of Harosoy, but occurred to a lesser degree on Harosoy 63.

Lyophilized mycelium of *P. megasperma* var. *sojae* did not contain antifungal compounds corresponding to hydroxyphaseollin, and the pterocarpan was not detected by GLC (minimum level of detection = 0.01 mg/g dry wt mycelium) or in the TLC bioassay.

Hydroxyphaseollin was not detected in any soybean tissue that had not been wounded or challenged with *P. megasperma* var. *sojae*. Low levels of HP were found in wounded tissues, and much higher levels were detected in fungus-challenged tissues (Table 1). In agreement with previous work (8), fungus-challenged whole hypocotyls of resistant Harosoy 63 accumulated from 5 to 10 times more hydroxyphaseollin than did the susceptible Harosoy, while excised, inoculated wound areas of Harosoy 63 hypocotyls contained 50-100 times more HP than did the corresponding tissue of Harosoy. Fungus-challenged roots or tissue culture callus of the two cultivars, however, accumulated similar amounts of HP, with the susceptible Harosoy often containing up to 2 times more than did Harosoy 63 (Table 1).

Low concentrations of HP were detected in diffusates from soybean pods challenged with *P. megasperma* var. *sojae* (Table 1), but no significant differences were noted between amounts produced by Harosoy and Harosoy 63.

Extracts from excised wound areas of inoculated soybean cotyledons contained large amounts of HP. As in experiments with hypocotyls (7, Table 1), HP was detected in wounded cotyledons, but much higher levels were detected in the inoculated cotyledons. Fungus-challenged Harosoy 63 cotyledons consistently contained ca. 1.5-2 times more HP than did Harosoy.

Extracts from challenged Harosoy roots or tissue culture callus frequently produced greater inhibition of growth of *P. megasperma* var. *sojae* in bioassay than did corresponding extracts from Harosoy 63. As found by Klarman & Sanford (10), only a single inhibitory spot was observed in the TLC bioassay from extracts of all fungus-challenged soybean tissues which corresponded to the R_F of authentic HP.

DISCUSSION.–All soybean tissues tested produced hydroxyphaseollin when challenged by *P. megasperma* var. *sojae* (Table 1), and this production was not due to its presence in the fungus. The fact that HP was produced by aseptic tissue culture callus when challenged by *P. megasperma* var. *sojae* minimizes the possibility (16) that its accumulation in the intact plant is due to the presence of contaminant microorganisms.

Although hydroxyphaseollin was produced more rapidly in the hypocotyls of *Phytophthora*-resistant Harosoy 63 soybeans than in susceptible Harosoy (7, 8) (Table 1), similar specificity in rates of HP production was not observed when soybean roots, pods, or tissue culture callus of the two near-isogenic soybean cultivars were challenged with the fungus. Excised lesion areas of Harosoy 63 cotyledons contained a maximum of twice the concentration of HP as in Harosoy, but under similar experimental conditions, inoculated Harosoy 63 hypocotyl lesions contained 50-100 times more HP than did Harosoy. These data confirm those of others (10) who found that cotyledons are good tissues for production of HP in relatively large amounts.

The accumulation of equal or slightly higher HP levels in roots and tissue culture callus of Harosoy as compared with Harosoy 63 was probably due to a

TABLE 1. Production of hydroxyphaseollin and disease reaction of various tissues of Harosoy and Harosoy 63 soybeans when challenged with *Phytophthora megasperma* var. *sojae*[a]

Tissue	Disease reaction[b]		Hydroxyphaseollin (mg/g dry wt tissue)[c]	
	Harosoy	Harosoy 63	Harosoy	Harosoy 63
Whole hypocotyls	Severe rotting	No rot, hypersensitive response	0.32–0.63	2.00–3.10
Excised hypocotyl wounds[d]			1.00–2.00	88.00–136.00
Roots	Considerable browning and rotting	Less browning and rotting	0.41–1.25	0.26–0.82
Tissue culture callus	Considerable browning and rotting	Less browning and rotting	0.50–0.98	0.43–0.70
Pod diffusates[e]	Browning of pods	Browning of pods	0.15–0.65	0.20–0.55
Excised cotyledon wound area[d]	Intense red pigmentation, little or no rotting	Intense red pigmentation, little or no rotting	24.00–32.00	41.00–48.00

[a]All data were obtained between 24-48 hr following inoculation; ranges denote a compilation of data from at least two to five experiments.

[b]Visible disease reaction.

[c]Analyses made on lyophilized tissues by gas-liquid chromatography (8); Hydroxyphaseollin concentrations in wounded but noninoculated tissues were (mg/g dry wt): whole hypocotyls, 0.04; excised hypocotyl wounds, 0.40; roots and tissue culture callus, <0.01; excised cotyledon wound areas, 4.0.

[d]Wound areas were excised and analyzed.

[e]Diffusates were extracted with ethyl acetate and analyzed; results were based on the lyophilized weight of the pods.

greater percentage infection of the Harosoy root systems by *P. megasperma* var. *sojae*. As found with hypocotyls (7) (Table 1), resistant responding cultivars accumulated from 50 to 100 times higher concentrations of HP at the infection site, but contained only 3 to 10 times more HP when whole hypocotyls were analyzed, presumably because greater fungus colonization occurred in the susceptible plants. Similar differences in fungus colonization rates may be a factor in infected roots, but data are lacking to support such a view. Meyer et al. (12) recently reported the production of an unidentified yellow, fluorescent substance by soybean roots inoculated with *P. megasperma* var. *sojae* that was considered to be a phytoalexin, but unfortunately did not present quantitative data comparing Harosoy to Harosoy 63.

In spite of possible effects due to unequal rates of fungus colonization of soybean tissues, the similarity in amount of HP produced and disease reactions (Table 1) of Harosoy and Harosoy 63 cotyledons, roots, pods, and tissue culture callus indicates that expression of the *Rps* resistance gene is minimal in these tissues. Instead, the gene appears to condition the capacity for more rapid HP accumulation, primarily in the hypocotyl. This is of interest, as the hypocotyl is the host organ in which the resistance gene was originally discovered by resistance screening (3).

These results thereby raise serious questions concerning the relevance of the widespread, unqualified use of unnatural host organ-parasite challenge systems in studies designed to elucidate naturally occurring plant disease-resistance mechanisms. Since such investigations are generally plagued by the lack of control, near-isogenic, susceptible responding host lines, it would appear impossible to determine whether observed resistance mechanisms were physiologically important or artifactual.

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