Production of the Phytoalexin, Hydroxyphaseollin, in Soybean Leaves Inoculated with Tobacco Necrosis Virus

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ABSTRACT

The phytoalexin, hydroxyphaseollin (HP), was detected in soybean leaves 30 hr after inoculation with tobacco necrosis virus (TNV). Maximal concentration occurred 48 to 72 hr after inoculation, but HP decreased to approximately two-thirds of the maximum after 96 hr. Quantity of HP was proportional to the number of lesions per leaf provided sufficient uninfected tissue separated the lesions. When lesion number was high, HP concentration decreased. Hydroxyphaseollin was not detected in the noninoculated tissue, although it was noted that inoculation of one primary leaf with TNV interferred with the establishment of TNV lesions in the opposite leaf when inoculated 48 hr later.

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Klarman & Sanford (7) established that one major antifungal compound is produced by soybeans inoculated with various *Phytophthora* species, and that this compound is closely related to phaseollin, the phytoalexin produced by *Phaseolus vulgaris* (1). Sims et al. (9) characterized the soybean phytoalexin as 6a-hydroxyphaseollin (HP), and Keen et al. (6) quantitated HP by gas-liquid chromatography. Keen (5) determined that HP accumulates 20 to 50 times faster in resistant than in susceptible soybean hypocotyls when inoculated with *P. megasperma* var. *sojae*, and concluded that HP is the mechanism of expression of single gene resistance to *P. megasperma* var. *sojae*.

Hammerschlag & Klarman (2) demonstrated that soybean phytoalexin also can be induced by inoculation of soybean leaves and hypocotyls with tobacco necrosis virus (TNV), which produces local necrotic lesions on soybeans. In the present investigation, the relationship between TNV infection and HP production have been further studied.

MATERIALS AND METHODS.—Tobacco necrosis virus was maintained by the routine inoculation of tobacco, *Nicotiana tabacum* L. 'Kentucky 35'. Inoculum was obtained by grinding fresh or frozen infected leaves with a mortar and pestle and expressing the liquid through cheesecloth. The crude sap diluted 1:10 with a 0.01-M neutral

phosphate (Na) buffer was used as inoculum except where otherwise noted.

Purified TNV was prepared from infected tobacco leaves by the differential centrifugation technique of Kassanis (4). The supernatant of the final low-speed centrifugation was layered on top of a 10 to 40% sucrose density gradient and centrifuged for 90 min in an SW 25.1 rotor at 42,000 *g* in a Beckman L2-65 ultracentrifuge.

Seeds of soybean, *Glycine max* (L.) Merr. 'Harosoy 63', were planted in flats of steam-treated potting soil. Plants were maintained in the greenhouse for 8 days, then moved to a chamber maintained at 20 to 30 C, equipped with Gro-Lux fluorescent lamps that supplied 300 ft-c of continuous light. Leaves of 9- or 10-day-old plants were inoculated with either diluted crude sap or purified virus by the Carborundum gauze-pad technique. Control extracts were prepared from similar noninoculated leaves that had been wounded by rubbing with Carborundum.

Soybean leaves were harvested 24 hr after the first visible lesions appeared except where otherwise noted. The leaves were freeze-dried, and ground in a Wiley Mill. Weighed amounts (usually 0.5 g) were added to 150 ml boiling distilled water in a Waring Blendor. The slurry was filtered through cheesecloth and the filtrate centrifuged at 28,700 *g* for 90 min. The supernatant was filtered through Whatman No. 1 paper and extracted twice with half volumes of ethyl ether. Pooled ether extracts were evaporated to dryness under vacuum, and the residue was dissolved in 2.0 ml of ether.

Extracts were bioassayed for HP by the thin-layer chromatography (TLC) method of Klarman & Sanford (7). Silica Gel TLC plates, on which had been spotted 25- to 100-μliter portions of extract, were developed in ethyl acetate:benzene:methanol:water (5:4:1:1). Dried TLC plates were sprayed to saturation with a concentrated suspension of conidia from *Cladosporium cucumerinum* Ellis & Arth., and the plates were incubated at room temperature for 2 days in plastic-covered dishes containing small amounts of water to maintain high humidity. White areas of silica gel at R_F 0.8 where spore germination had been inhibited, surrounded by black areas of germinated conidia, indicated the presence of HP. Quantities of HP from induced soybean tissues were analyzed as the trimethylsilyl derivative by the gas-liquid chromatography (GLC) method of Keen et al. (6). At least three determinations of HP concentration were made with each extract and data are presented as mean concentrations.

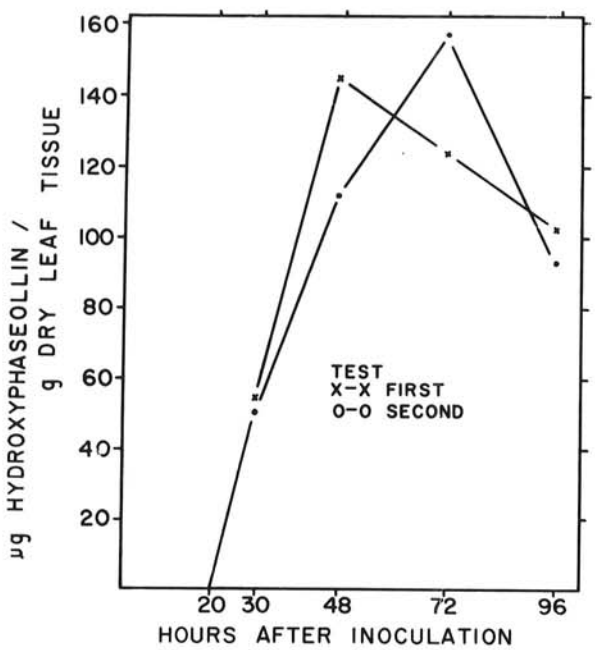

Fig. 1. Concentration of hydroxyphaseollin in soybean leaves at various times after inoculation with tobacco necrosis virus.

RESULTS.—*Tobacco necrosis virus induction of HP.*—Extracts were prepared from three 0.5-g portions of soybean primary leaves which had been inoculated with purified TNV. Fifty μliters of each extract bioassayed with TLC produced one inhibitory spot at R_F 0.8 which corresponded with the spot reported for soybean phytoalexin (7). No inhibitory spots were produced by the control extracts.

Time course study of HP induction by TNV.—Primary leaves of soybeans inoculated with TNV were harvested 20, 30, 48, 72, and 96 hr after inoculation in two tests, and HP concentrations were measured by GLC (Fig. 1). Appearance of the first visable lesions in the two tests varied by several hours, probably indicating different rates of virus multiplication and thus different rates of HP production.

Relationship between numbers of lesions and concentrations of HP.—Primary leaves of soybeans inoculated with TNV were divided into four groups of at least 25 leaves, each according to the number of lesions. Extracts were prepared from each group of leaves and quantities of HP were determined by GLC. Numbers of lesions and μg HP/g dry leaf tissue in each group were: 1 to 25 lesions, 22 μg HP; 26 to 50 lesions, 38 μg HP; 51 to 80 lesions, 107 μg HP; and 81 to 132 lesions, 52 μg HP.

Cross protection.—One primary leaf of each of 80 soybean plants was inoculated with TNV and, after 48 hr when abundant lesions had developed, the second primary leaf was inoculated. At the time of the second inoculation, one primary leaf of each of 80 similar but previously noninoculated plants was inoculated with TNV as a control. Seventy-two hr after the last inoculation, lesions on the last inoculated leaves were counted. Leaves from plants receiving the earlier inoculation had an average of 31 lesions; leaves from previously noninoculated plants had an average of 74 lesions.

The experiment was repeated, except that at the time of inoculation of the first primary leaves, one primary leaf of each control plant was removed. Leaves from plants receiving the earlier inoculation had an average of 74 lesions, and leaves from control plants 188. In both tests, the lesions on control leaves

were almost twice as large as those on leaves from previously inoculated plants.

Nontranslocation of HP.—One-half of one of the primary leaves on several soybean plants was inoculated with TNV. Abundant lesions were present 48 hr after inoculation and, 72 hr after inoculation, leaves were harvested and grouped as follows: (i) inoculated half of primary leaf; (ii) noninoculated half of inoculated primary leaf; (iii) entire opposite noninoculated primary leaf; and (iv) all tissues above primary leaves. Extracts were prepared from each group of tissues, and quantities of HP were measured by GLC. The inoculated half of the primary leaf contained 300 μg HP/g. No HP was detected in any of the other extracts.

Effect of HP on TNV infectivity.—Soybean leaves were harvested and freeze-dried 72 hr after inoculation with TNV, and the tissue was determined to contain 120 μg HP/g. Extract prepared from 2 g of this tissue was evaporated to dryness, and the residue dissolved in 50 ml sterile distilled water. A similar control extract prepared from 2 g of noninoculated leaf tissue contained no HP detectible by GLC. Equal portions of purified TNV were incubated in each of the extracts for 24 hr at room temperature and applied to opposite halves of eight tobacco leaves by the Carborundum gauze-pad technique. After 48 hr, half-leaves receiving TNV in extract containing HP had an average of 70 lesions. Half-leaves receiving TNV in control extract had an average of 66 lesions.

DISCUSSION.—Hydroxyphaseollin is produced in association with local necrotic lesions caused by TNV infection, but apparently has no direct effect in vitro on the infectivity of the virus. It could, perhaps, be indirectly involved in regulating lesion size, as phytoalexin formation is only one part of a complex of host changes, some of which might cause tissues immediately surrounding lesions to be unsuitable for further virus multiplication.

Quantity of HP is proportional to the number of lesions per leaf as long as they are separated by sufficient uninfected tissue. When large portions of the leaves become covered with lesions, HP concentration decreases, indicating that maximal production of HP is dependent upon a favorable ratio between infected and uninfected tissues, or HP breaks down readily in necrotic tissue.

Time-course data for HP accumulation in soybean leaves inoculated with TNV are generally consistant with those of Keen (5), who measured HP accumulation in hypocotyls of Harosoy 63 soybeans inoculated with *P. megasperma* var. *sojae*. In both cases, maximal production occurred 48 to 72 hr after inoculation.

Tobacco has been shown to be protected from fungal attack when infected with TNV (3). Hydroxyphaseollin, however, was not translocated from the site of production, suggesting that, at least in soybeans, phytoalexin induced by TNV infection is not directly involved in reduction of subsequent fungal infections. Some other chemical associated with local lesion formation is perhaps translocated, and does provide some resistance to additional TNV infection. A similar phenomenon has been reported in tobacco inoculated with TNV (8).

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