Time Sequence for Phytoalexin Production in Harosoy and Harosoy 63 Soybeans

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ABSTRACT

Examination of soybean hypocotyls inoculated with *Phytophthora megasperma* var. *sojae* indicated that both Harosoy (susceptible) and Harosoy 63 (resistant) soybean plants produced a phytoalexin in response to infection. Within 4 hr after inoculation, phytoalexin production and fungal development is similar in both varieties. Differences in the host-parasite interactions of the two varieties become apparent between 4 and 8 hr after inoculation. Phytoalexin is no longer detectable in Harosoy after 8 hr, and the disease develops, resulting in the collapse of the hypocotyls within 48 hr after inoculation. Phytoalexin production continues in the resistant variety, and after 24 hr the plant cells surrounding the fungus become discolored and fungal invasion is halted. After 72 hr the pathogen is killed, phytoalexin production ceases, and the existing phytoalexin begins to disappear. Therefore, since plant reactions to the pathogen are identical in the first 4 hr after invasion, the reactions responsible for resistance or susceptibility appear to occur between 4-8 hr, when phytoalexin production either increases or declines. Phytopathology 60:315-318.

Disease resistance in some plant-pathogen interactions has been attributed to the production of phytoalexins. Phytoalexins are considered to be antibiotics produced as a result of the interaction of two metabolic systems, host and parasite, which inhibit the growth of the parasite (3). They have been found in a variety of host plants. The rate of phytoalexin formation has been postulated as a basis for differentiating susceptible and resistant hosts (3). The inability of the infecting fungus to stimulate the formation of phytoalexins or the fungus' capacity to tolerate the level of phytoalexin produced also have been postulated as alternative explanations for disease susceptibility of certain plants (1).

Phytoalexins produced in Harosoy 63 soybeans inoculated with *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildeb., a soybean pathogen causing root and stem rot, were reported by Klarman & Gerdemann (2). One of the phytoalexins produced in Harosoy 63 soybeans is an unidentified yellow-colored compound with an R_F of 0.56 in the organic phase of a butanol:acetic acid:water (4:1:5; v/v) solvent, which fluoresces a bright yellow under ultraviolet irradiation and has an absorption maximum at 485 nm (2). This study was undertaken to determine the rate of production of this phytoalexin (PA_K) in resistant and susceptible soybeans in relation to disease development.

MATERIALS AND METHODS.—Two soybean varieties, Harosoy (susceptible) and Harosoy 63 (resistant), were used in all experiments. These varieties are designated as H_S and $H63_R$, respectively, throughout this paper. All plants were grown in the greenhouse in washed sand, harvested after 6 days' growth, and inoculated. Inoculum was prepared by growing *P. megasperma* var. *sojae* in soybean broth for 7 days. A small wound was made on each plant hypocotyl, approximately 5-10 mm below the cotyledonary node, and a piece of mycelium was inserted into the wound. Plants were maintained in a beaker of water and covered with a plastic bag for 4 hr to maintain high relative humidity.

Inoculated H_S and $H63_R$ hypocotyls were studied histologically to observe disease development. Stem sections were cut from inoculated hypocotyls at 4, 8, 12, 18, 24, and 48 hr after inoculation and fixed in formalin:acetic acid:alcohol. Sections were infiltrated with paraffin, sectioned on a rotary microtome, mounted on glass slides, and stained with safranin and fast green.

To determine the time of phytoalexin production, stem sections were removed from inoculated hypocotyls of H_S and $H63_R$ plants at 1, 2, 4, 8, 12, 18, and 24 hr after inoculation and cut on a freezing microtome (30-μ sections). The sections were dry-mounted on glass slides and immediately observed, using a fluorescence microscope. The fluorescence wavelength of the yellow-green phytoalexin was measured with a fluorimeter. Observations were also made of noninoculated hypocotyl sections and of the fungus.

Inoculated H_S and $H63_R$ hypocotyl sections were cut from plants at 2, 4, 8, 12, and 24 hr after inoculation. The sections were placed in boiling water immediately to inactivate enzymes, ground in a Waring Blendor, and filtered through cheesecloth. The plant juice was adjusted to pH 2.0 with HCl, and extracted 3 times with equal volumes of *n*-butanol. Extracts were combined and re-extracted 3 times with equal volumes of .01 M potassium phosphate buffer (pH 7.0). The buffer extracts were concentrated to 10 ml, and 3 ml of this final sample were used for spectrophotometric studies.

Samples from each time interval were compared to a 4 μg/ml ethanol standard of PA_K and the relative amounts of PA_K produced were calculated. Thin-layer chromatography was also used to detect PA_K after these same time intervals. One ml of the final sample was spotted on thin-layer silicic-acid chromatogram strips next to a 40 nanogram (ngm) standard of PA_K. The strips were developed in the organic phase of butanol:acetic acid:water (4:1:5; v/v) and observed with ultraviolet irradiation.

Fungal viability was determined by placing infected hypocotyl sections of H_S and $H63_R$ previously surface-

sterilized with 0.1% sodium-hypochlorite on commercial lima bean agar plates 48, 72, 96, and 120 hr after inoculation. Twenty sections of each variety were plated for each time interval, and the fungi isolated were identified microscopically. Ten other hypocotyl sections from each variety were also ground and extracted for phytoalexin after these same time intervals according to the procedure previously outlined. Samples were concentrated to 3 ml and observed spectrophotometrically. One ml of this sample was also chromatographed on a thin layer strip to confirm the presence of PA_K.

RESULTS.—Development and progression of the pathogen in H_S and $H63_R$ were comparable within the first 4 hr after inoculation. Hyphae extended from the mycelial mat (inoculum) and intercellularly invaded the first row of healthy cells surrounding the wound area. Haustoria were not observed in this early developmental stage. Within 8 hr, the hyphae had penetrated the second cell layer surrounding the wound area in both varieties. The invaded tissue appeared water-soaked, with some darkening of individual cells.

Fungal invasion after 12 hr in the H_S plants had progressed into the 3 or 4 cells distant from the wound in the surrounding tissue. The rate of invasion began to increase at this time, and the fungus was progressing very rapidly, moving in 24 hr as far as 3 mm distant from the wound in some sections. Newly invaded cells were dark and water-soaked, while the cells surrounding the wound area had disintegrated and the cell walls had collapsed. Haustoria were numerous and fungal growth was very profuse, entering the cortex and vascular system. The H_S plants generally collapsed after 48 hr so that good sections showing cell structure could not be made.

Fungal invasion had progressed more rapidly in the $H63_R$ plants after 12 hr and the fungus had invaded five or six cell layers. A definite orange discoloration was apparent in the invaded $H63_R$ cells that had previously been water-soaked and were now behind the ad-

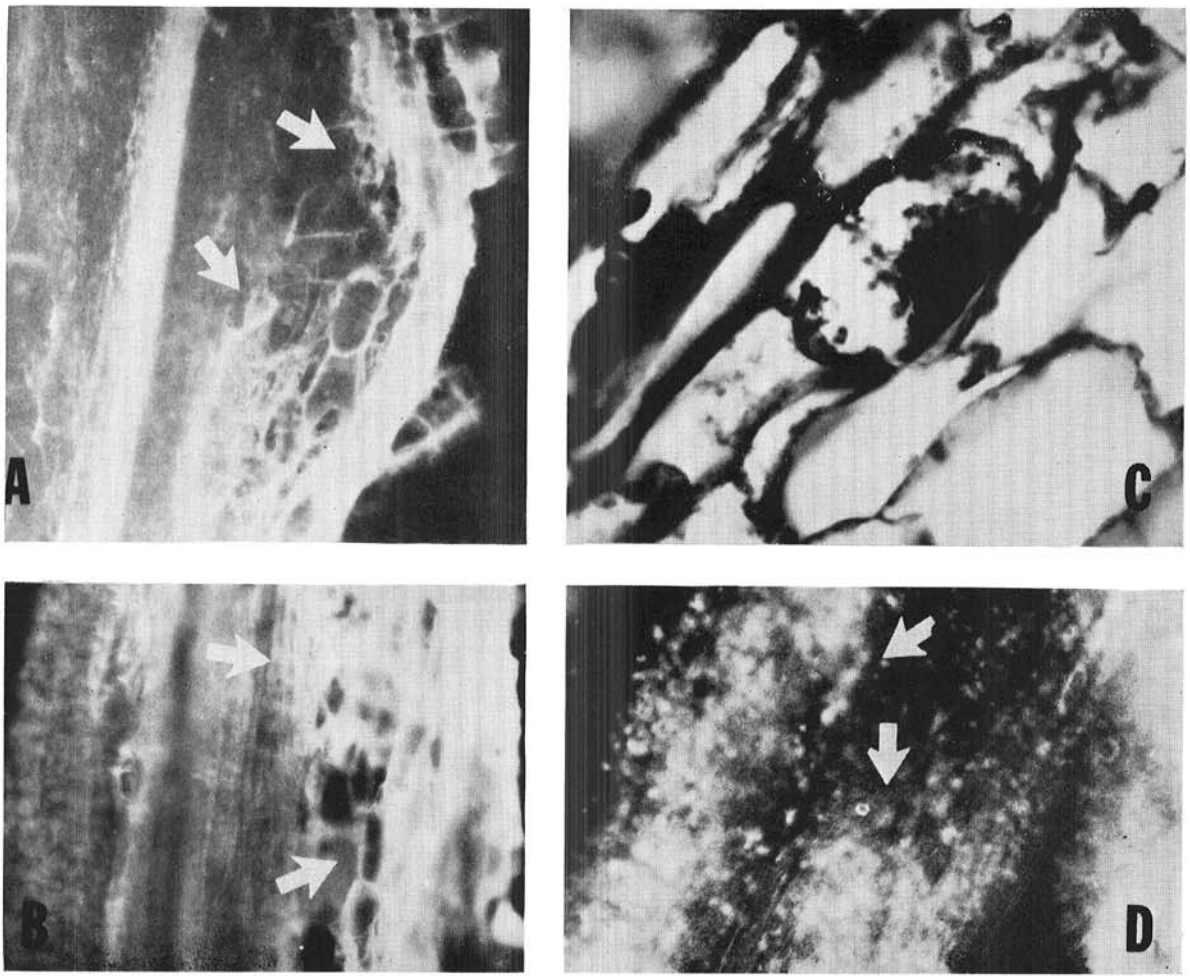

Fig. 1. A) Ultraviolet fluorescence of resistant Harosoy 63 hypocotyl at 4 hr after inoculation with *Phytophthora megasperma* var. *sojae* (× 400). **B)** Ultraviolet fluorescence of a resistant Harosoy 63 hypocotyl at 24 hr after inoculation (× 100). **C)** Granulation in resistant Harosoy 63 cells at 24 hr after inoculation (× 400). **D)** Resistant Harosoy 63 hypocotyl at 24 hr after inoculation showing diffusibility of water-soluble PA_K (× 400).

vancing fungal margin. In the newly invaded cells, granular materials were formed which were not confined to any specific region within them. The advance of the fungus in the $H63_R$ stems apparently slowed after 24 hr, and the orange discoloration then appeared in the darkened, granular cells. This orange discoloration extended outward from the mycelial mat so that the most recently infected cells were not yet discolored, but showed extreme amounts of granulation (Fig. 1-C). The granules appeared to collect along the inside of the cell wall. The fungus was confined to the cortex with very little vascular invasion. No further advance of the fungus after 48 hr occurred, but there was an increase in orange discoloration and a few traces of hyphae.

Freezing microtome sections of noninoculated H_S and $H63_R$ hypocotyls fluoresced red when observed with the fluorescence microscope, whereas the fungus did not fluoresce. Inoculated H_S hypocotyls fluoresced a pale yellow-green color at the inoculation site within 4 hr after inoculation, but fluorescence was not detectable after 8 hr. Inoculated $H63_R$ hypocotyls fluoresced a yellow-green color at the inoculation site after 4 hr (Fig. 1-A), and this color increased in intensity with time and progressed to other cells. The entire hypocotyl section adjacent to the inoculation site fluoresced a bright yellow after 24 hr (Fig. 1-B, D) and had a fluorescence wavelength of 529 nm, which corresponded to that of PA_K.

The only chromatographic detection of PA_K in H_S plants occurred at 4 hr after inoculation, and the yellow fluorescence from 600 lesions appeared to be less than that of the 4 μg PA_K standard. PA_K was detectable in the $H63_R$ plants by chromatography at 4, 8, 12, and 24 hr after inoculation. The intensity of the fluorescence increased as the time after inoculation increased so that a detectable amount of PA_K could be obtained from 10 lesions at 24 hr after inoculation. The only spectrophotometric detection of PA_K in H_S plants occurred at 4 hr after inoculation, and the amount of PA_K per plant was estimated as 5.1 ngm. PA_K was spectrophotometrically detectable in $H63_R$ plants at 4, 8, 12, and 24 hr after inoculation, and quantitative estimates per plant were determined (Fig. 2).

The pathogen could be isolated from H_S plants until the entire hypocotyl had completely deteriorated. The pathogen could be isolated from $H63_R$ plants at 48 hr after inoculation, but was isolated from only one of the 20 lesions tested at 72 hr. At 96 and 120 hr after inoculation, the fungus could not be isolated. PA_K was detectable in $H63_R$ lesions at 48 hr, but it decreased quantitatively by 72 hr and actually appeared as a shoulder of a peak located at 525 nm (Fig. 3). The shoulder was very small after 96 hr, and there was still a definite peak at 525 nm. After 120 hr, no discreet PA_K or 525 nm peaks were observed.

The material at 525 nm was pinkish-orange in color, fluoresced orange under ultraviolet irradiation, and had an R_F of 0.5 in the organic phase of butanol:acetic acid:water (4:1:5; v/v).

Discussion.—PA_K production is initiated within 4 hr after inoculation in the H_S and $H63_R$ soybeans, and coincides with initial invasion of host cells by the pathogen. Initial fungal invasion is similar in both varieties, and the difference in amount of PA_K produced in $H63_R$ and H_S is small. Differences in the host-parasite interactions occur between 4 and 8 hr after inoculation. The pathogen advances to a second layer of host cells in both varieties, but PA_K production increases in the $H63_R$ plants and declines in the H_S plants. H_S plants apparently contain no PA_K at 8 hr after inoculation. The actual mechanism involved in the disruption of PA_K production or the decomposition of PA_K is not known. The difference between susceptible and resistant soybean plants is not related to the rate of PA_K production or the inability of the fungus to stimulate the formation of PA_K. Resistance is re-

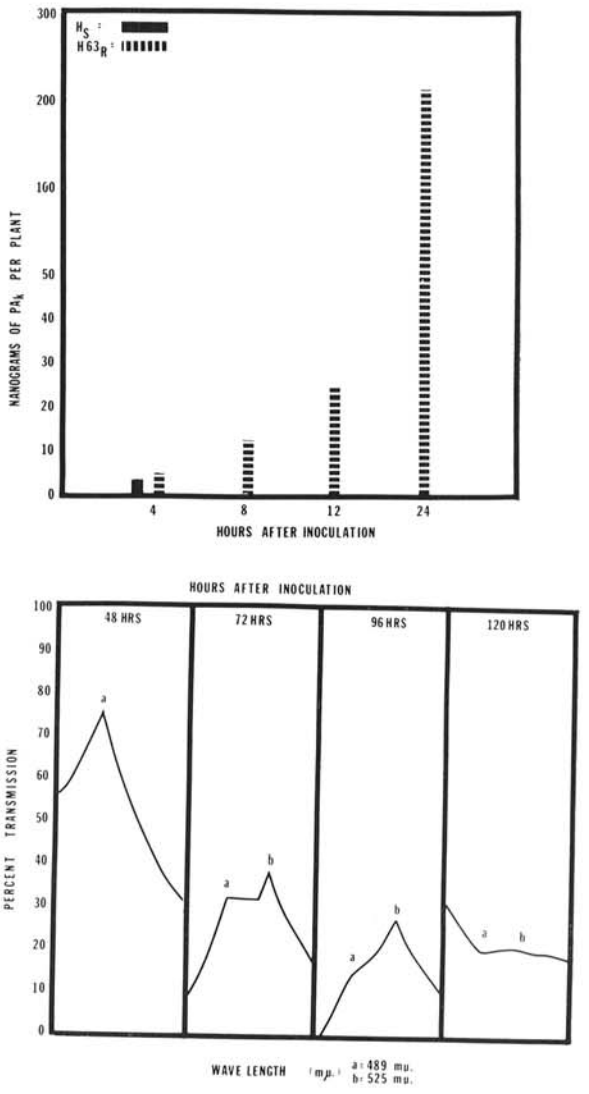

Fig. 2-3. **2)** Relative amounts of PA_K per resistant ($H63_R$) and susceptible (H_S) soybean plant at various time intervals after inoculation with *Phytophthora megasperma* var. *sojae*. **3)** Absorption spectra of $H63_R$ stem extracts at various time intervals after inoculation.

lated directly to the presence of phytoalexin, whereas the disruption of the phytoalexin-producing mechanism results in susceptibility.

After 12 hr, the pathogen appears to have progressed more rapidly in the $H63_R$ plants. This observation is significant and perplexing, since PA_K is not detectable in the H_S plants at this time, while the concentration of PA_K in the $H63_R$ has approximately doubled. The concentration present in $H63_R$ may have actually stimulated growth of the pathogen, indicating that PA_K is not detrimental to the pathogen at low concentrations. The appearance of granular materials in the invaded $H63_R$ plants could not be correlated with resistance. Studies with an electron microscope might help clarify the role of the granules in the cell.

The inoculation site on the H_S stems was dark brown in color after 24 hr, and in many cases this area had collapsed. $H63_R$ stem sections showed large, red borders surrounding the inoculum mat after 24 hr, and these borders surrounded all invaded host cells. This might indicate that the fungus was killed in the first cells invaded and was also prevented from advancing further. Destruction of the pathogen appears to be complete after 72 hr, and the existing PA_K breaks down. Preliminary results of a bioassay of this breakdown product indicate that the material is fungitoxic at relatively high concentrations (material from 5 plants/ml of H_2O/ml of soybean broth).

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