Histopathology of Susceptible and Resistant Soybean Roots Inoculated with Zoospores of *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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Histopathological comparisons of the sequence and timing of pre- and postpenetration events of pathogenesis of *Phytophthora megasperma* f. sp. *glycinea* zoospores on resistant (Harosoy 63 [H-63]) and susceptible (Harosoy [H]) soybean roots revealed that resistance is effective during the colonization stage in the resistant cultivar. Roots were inoculated with zoospores and samples were excised at intervals to 72 hr after inoculation and prepared for light microscopy. Scanning electron microscopy was used to examine prepenetration events. Lateral roots of other plants were inoculated with zoospores and were examined macroscopically at 24-hr intervals for 8 days. Hypocotyl and tap root sections of these plants were examined for oogonia, oospores, or mycelium. The timing and sequence of pre- and early postpenetration phenomena were similar on H and H-63 roots. Zoospore accumulation, encystment, germination, and penetration from simple germ tubes between anticlinal walls of epidermal cells occurred within 1.5 hr after inoculation. Hyphae grew inter- and intracellularly within cortical tissue and the stele. Significant differences in ramification hyphae lengths in resistant vs. susceptible cultivars were apparent in roots at 48 hr. Ramification hyphal lengths in H roots were twice the lengths in H-63 roots at 72 hr. Fewer oogonia and oospores per root section were observed in H-63 roots than in the more susceptible cultivar. Hyphal ramification from lateral roots to tap roots and hypocotyls of H, but not H-63, plants occurred within 8 days after zoospore inoculation.

Additional key words: *Glycine max*, *Phytophthora megasperma* var. *sojae*.

Histological studies of events of fungal pathogenesis on near-isogenic cultivars differing in resistance may indicate the location and timing of expressions of resistance. This information can be helpful in guiding experiments and interpreting results of biochemical and physiological research on mechanisms of resistance. The objective of this work was to describe the sequence and timing of the pre- and postpenetration events of pathogenesis of zoospores of *Phytophthora megasperma* Dreschs. f. sp. *glycinea* (Kuan & Erwin) (*Pmg*) on roots of near-isogenic resistant and susceptible soybeans (*Glycine max* (L.) Merr.) and to determine when resistance is effective. A preliminary report of portions of this work has been published (4).

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Though zoospores of *Pmg* are effective inoculum for the development of Phytophthora root rot (10), we are not aware of any published studies of comparisons of the events of pathogenesis of zoospores on susceptible and resistant soybean roots. Other aspects of the events of pathogenesis of *Pmg* on soybeans have been described: the attraction and accumulation of zoospores on plant roots (9,11,12); penetration, growth and ultrastructural features of *Pmg* in hypocotyls after zoospore inoculation (22–24); *Pmg* development in roots after inoculation of resistant, susceptible, and field-tolerant cultivars with mycelium (21); the ultrastructure of the host-parasite interface in susceptible roots (20); and disease development in tap roots and hypocotyls of a susceptible cultivar (5). Histopathological studies of hypocotyls of near-isogenic resistant and susceptible soybeans inoculated with *Pmg* mycelium revealed limited fungal colonization in resistant hypocotyls (15).

MATERIALS AND METHODS

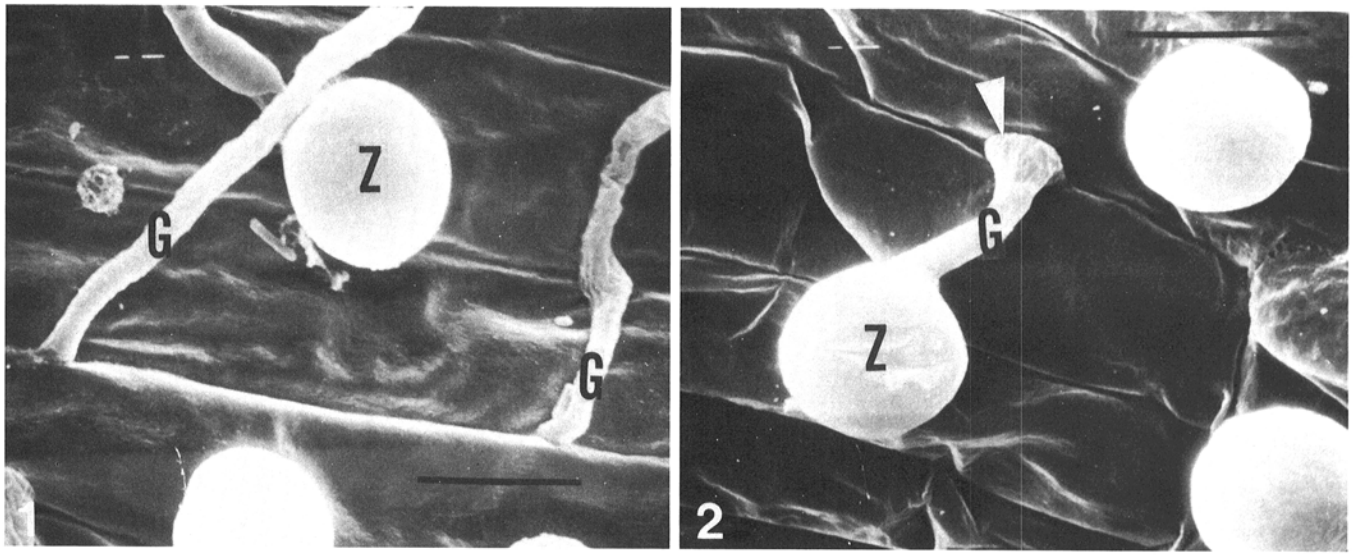
Soybean seed of cultivars Harosoy (H) and Harosoy 63 (H-63), susceptible and resistant, respectively, to *Pmg*-race 1, were surface disinfested (0.05% NaOCl for 10 min) and germinated on sterile filter paper for 3 days (27 C). H-63, a cultivar near-isogenic to H, differs in resistance to *Pmg*-race 1 (2). Seedlings without visible microbial contamination were transferred to plastic growth pouches (DiSPo-Seed Pack Growth Pouches, Scientific Products, Columbia, MD 21045), moistened with nutrient solution (19), and incubated at 25 C (16 hr light, 8 hr dark).

Isolates of *Pmg*-race 1 obtained from Maryland-grown diseased soybeans (3) were maintained on lima bean agar and transferred monthly to fresh medium. Zoospores were produced according to the methods of Eye et al (7).

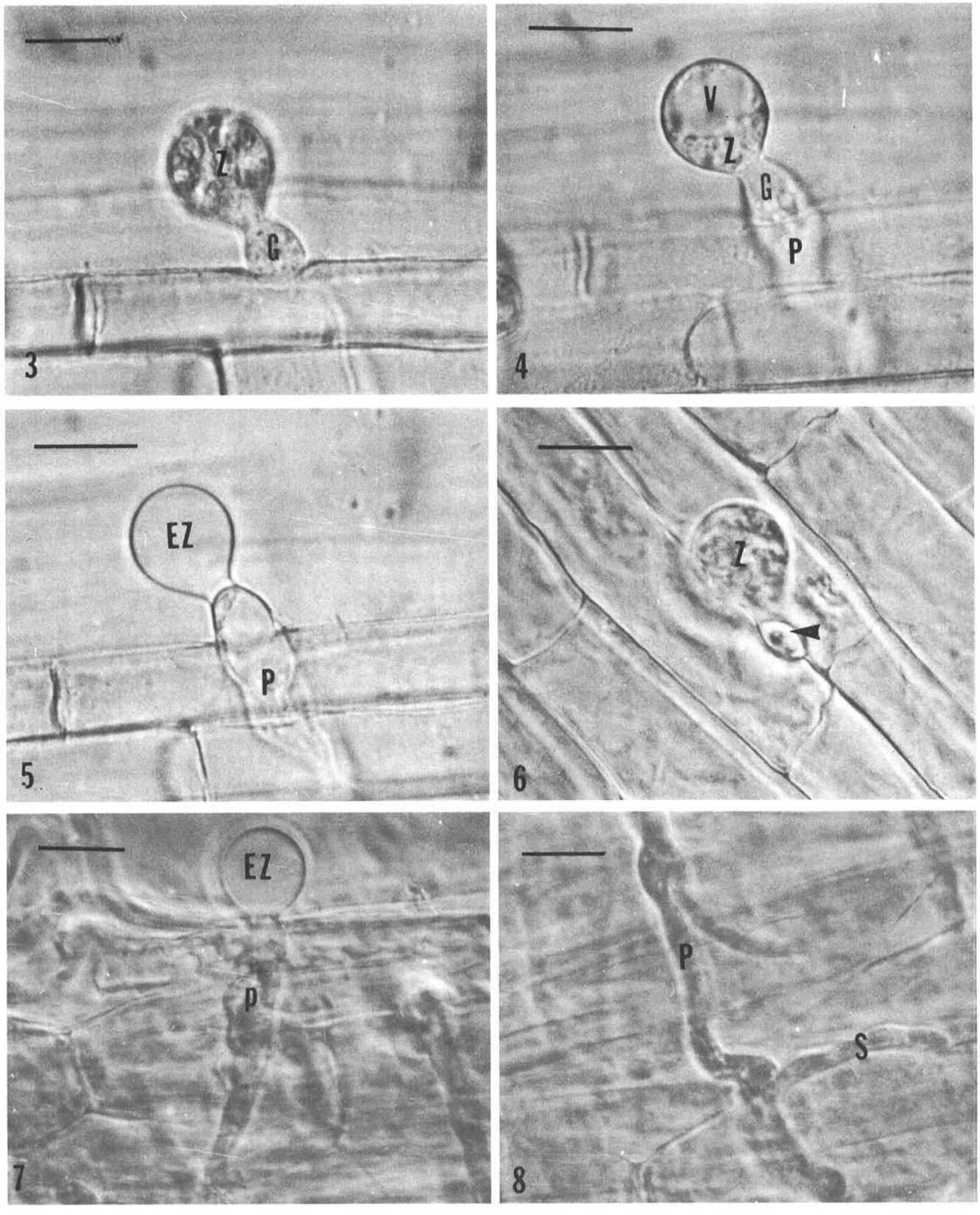
For light microscopy, 2-wk-old seedlings from pouches were placed in glass dishes and moistened with nutrient solution (19). Fifty-microliter drops containing approximately 2×10^3 zoospores were placed on portions of lateral roots on gelatin-coated (0.5% gelatin) glass microscope slides. Dishes were covered with plastic and incubated at 27 C in the dark. Root sections (5 mm long) were excised from the tip through the region of elongation of lateral roots at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 8, 12, 24, 48, and 72 hr after inoculation; examined without fixation or fixed for 24 hr in 3% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 7.4) after an initial 15-min vacuum infiltration; rinsed in buffer; stained in 0.05% aniline blue in lactophenol (lactic acid:phenol: glycerin:distilled H_2O, [1:1:1:1, v/v]); cleared in lactophenol; mounted on glass slides; slightly squashed; and examined under bright-field and phase-contract microscopy. Comparable control tissue was prepared similarly using sterile distilled water instead of zoospore inoculum. Cut roots were used to facilitate manipulation and observation. The following data were recorded from observations on entire 5-mm-long root sections: number of full zoospore cysts, germ tubes, swollen germ tube tips, germ tubes with primary hyphae, secondary hyphae, oogonia, and oospores; and lengths and widths of germ tubes, swollen germ tube tips, primary hyphae, secondary hyphae, and ramification hyphae. Primary hyphae were the first hyphae produced in roots after penetration. Secondary hyphae formed as branches from primary hyphae. Ramification hyphae were branched and unbranched hyphae more than 150 μm in length. These data were transformed to total number of zoospore cysts on root sections at a given sample time; percentage germination of cysts; mean lengths and widths of germ tubes, swollen germ tube tips, primary hyphae and secondary hyphae; mean lengths of ramification hyphae; mean number of oogonia and oospores per root section; percentage germ tubes with swollen tips; percentage penetration with swollen germ tube tips; and percentage penetration from germinated zoospore cysts. Fifty-four H and 52 H-63 plants were inoculated. Data presented are means from two replications of the experiment and include lateral root samples from at least three plants at each sample period. Standard errors of the means were calculated for data collected at later sample periods.

For scanning electron microscopy (SEM) studies, roots excised from 2-wk-old H and H-63 seedlings were inoculated by placing them in zoospore suspensions (4.7×10^4 zoospores per milliliter) in petri dishes. Control root tissue was placed in sterile distilled water. Samples were removed at 0.5-hr intervals up to 2.5 hr after immersion and fixed (3% glutaraldehyde in 0.025 M sodium phosphate buffer, pH 7.4), dehydrated in a graded ethanol series, dried in a Technics model CPA II critical point dryer, mounted on aluminum stubs, sputter coated with 0.01 μm (100 Å) of gold-palladium, and scanned on an International Scientific Instruments model 60 scanning electron microscope.

To determine whether *Pmg* mycelium ramified from lateral to tap roots and hypocotyls, 2-wk-old H and H-63 seedlings (12 per cultivar) were placed in glass dishes as before and were inoculated in five separate areas on lateral roots with 50-μl drops containing approximately 800 zoospores each. Controls included roots that were uninoculated or treated with drops of sterile distilled water or autoclaved zoospore suspensions. At 24-hr intervals, numbers of brown lateral roots were counted. Eight days after inoculation, hypocotyls and tap root samples were macerated, stained, and



Figs. 1 and 2. Scanning electron micrographs of the early events of pathogenesis of zoospores of *Phytophthora megasperma* f. sp. *glycinea* on Harosoy (H-63) soybean roots. **1,** Penetration of H-63 root by simple germ tubes between anticlinal walls of epidermal cells. **2,** Intercellular penetration of H-63 root from swollen germ tube (arrow). Z = zoospore cyst, G = germ tube. Bars = 10 μm.



Figs. 3–8. Light micrographs of events of pathogenesis of zoospores of *Phytophthora megasperma* f. sp. *glycinea* on Harosoy (H) roots. **3,** Zoospore cyst filled with cytoplasm and globular germ tube penetrating intercellularly. **4,** Vacuolation of zoospore cyst as cyst contents enter hypha. **5,** Empty zoospore cyst on H root surface. **6,** Highly refractive elliptical halo (arrow) surrounding point of penetration by germ tube. **7,** Empty zoospore cyst on H root surface and primary hyphae within root cortical tissue perpendicular to longitudinal root axis. **8,** Secondary hypha branching from primary hypha in H root. Z = zoospore cyst, G = germ tube, P = primary hypha, V = vacuole, EZ = empty zoospore cyst, S = secondary hypha. Bars = 10 μm.

cleared as above and examined for the presence of oospores, oogonia, and mycelium. The percentage of brown and/or infected tap roots and hypocotyls was determined for each cultivar. Standard errors of the means were calculated for these data. This experiment was replicated three times.

RESULTS

The sequence of the events of pathogenesis of *Pmg* zoospores on H and H-63 roots was accumulation and encystment of zoospores on root surfaces; cyst germination and germ tube elongation; penetration; primary hypha formation and elongation; secondary hypha formation; ramification hypha elongation; and oogonia and oospore formation. Pre- and early postpenetration phenomena were similar on H and H-63 roots. Zoospores accumulated and encysted in the region of root elongation and on cut ends of roots. Within 1.0 hr after inoculation more than 50% of the cysts had germinated (Fig. 9). While a single, simple germ tube was usually produced from a single zoospore cyst, branched germ tubes were occasionally observed. The greatest rate of increase in mean germ tube length occurred between 2.0 and 2.5 hr after inoculation on both cultivars (Fig. 9). Mean germ tube widths varied from 2.2 to 4.0 μm at any sample time.

Penetration was defined as the event characterized by the passage of germ tubes to the inside of the root surface. Greatest increases in the percentage of penetrations ([number of penetrations/number of germinated cysts] $\times$ 100) occurred between 0.5 and 1.5 hr after inoculation (Fig. 10). Changes in the percentage of penetrations were similar on both cultivars (Fig. 10). Ninety-four percent of the penetrations observed on soybean roots occurred from simple germ tubes between anticlinal walls of epidermal cells (Fig. 1). Occasional penetrations (5%) occurred from swollen germ tube tips (Fig. 2) which appeared to be closely appressed to the root surface. Less frequently (1%) penetration occurred from globular structures formed directly from zoospore cysts.

During the penetration process, vacuolation increased in zoospore cysts as the cyst contents entered hyphae in the root (Figs. 3-5). Empty, often-collapsed zoospore cysts were observed on root surfaces after penetration (Fig. 5). A septum usually separated empty zoospore cysts and germ tubes from hyphae in the roots. Highly refractive elliptical halos (Fig. 6) were consistently observed in host tissue around the point of penetration under bright-field optics.

Early postpenetration phenomena were similar on H and H-63 roots. Primary hyphae averaged 5 μm in width and were wider than germ tubes on the root exterior. Primary hyphae developed inter- and intracellularly within cortical tissue and the stele, most often perpendicular to the longitudinal root axis (Fig. 7). The

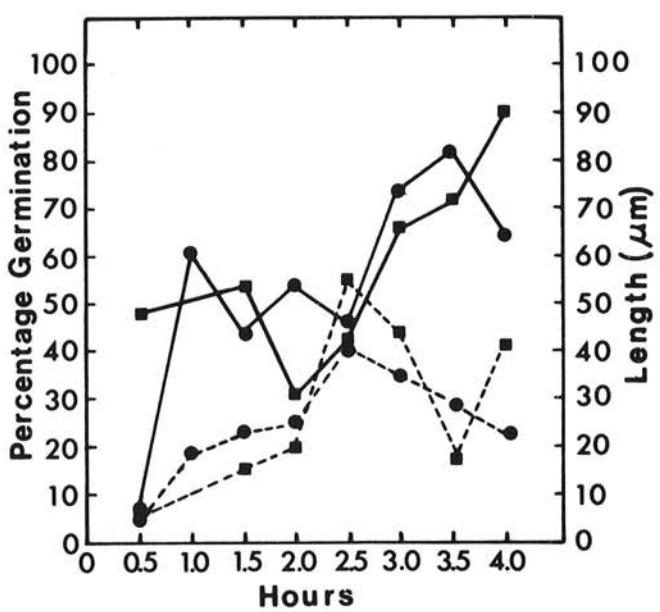


Fig. 9. Percentage germination of zoospore cysts and mean germ tube lengths of *Phytophthora megasperma* f. sp. *glycinea* on Harosoy and Harosoy 63 soybean roots from 0.5 to 4.0 hr after inoculation. Percentage germination (H = •——• , H-63 = ■——■); mean germ tube length (H = •·····•, H-63 = ■·····■).

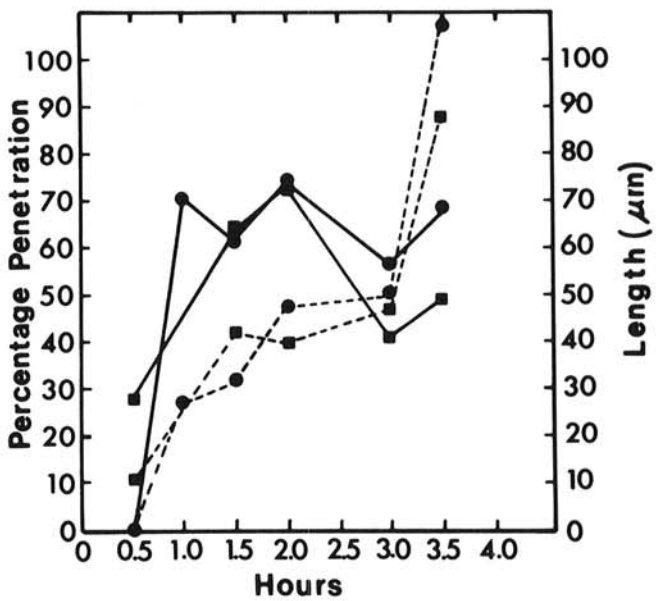


Fig. 10. Percentage penetration of germ tubes and mean primary hyphae lengths of *Phytophthora megasperma* f. sp. *glycinea* in Harosoy and Harosoy 63 soybean roots from 0.5 to 3.5 hr after inoculation. Percentage penetration (H = •——•, H-63 = ■——■); mean primary hyphae length (H = •·····•, H-63 = ■·····■).

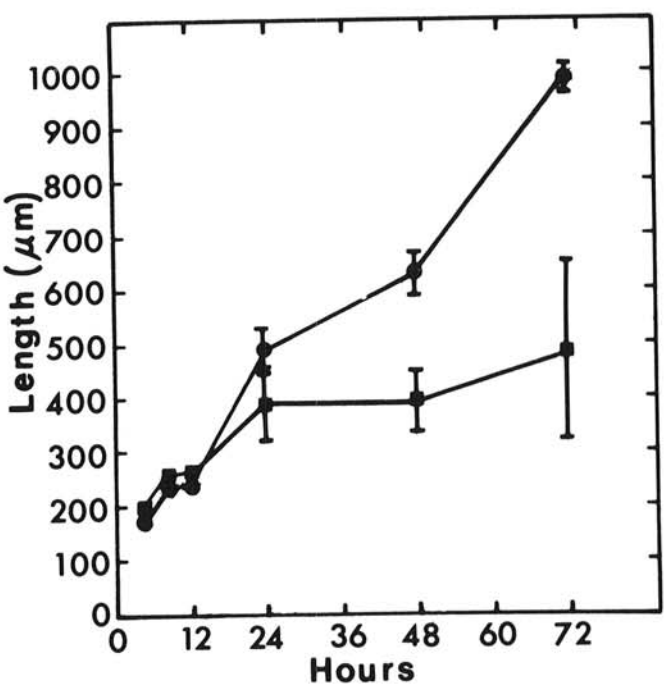


Fig. 11. Mean ramification hyphae lengths in Harosoy and Harosoy 63 soybean root tissue from 4.0 to 72 hr after inoculation with zoospores of *Phytophthora megasperma* f. sp. *glycinea*. Mean ramification hyphae length (H = •——•, H-63 = ■——■). Vertical bars represent standard errors of the means.

greatest rate of increase of mean primary hypha lengths occurred between 3.0 and 3.5 hr after inoculation of both cultivars (Fig. 10). Within 2 hr after inoculation, secondary hyphae (Fig. 8) formed as branches from primary hyphae.

During the later events of pathogenesis, ramification hyphae grew both perpendicularly and parallel to the longitudinal axis of the root in cortical tissue and the stele. By 72 hr, ramification hyphal lengths in H roots were twice the lengths in H-63 roots (Fig. 11). Harosoy root cells were distorted and visibly necrotic at this time. Differences in mean ramification hyphae lengths in H and H-63 roots were significant at 48 and 72 hr after inoculation. The rate of increase in ramification hyphae lengths between 24 and 72 hr averaged 10 μm/hr and 2 μm/hr in H and H-63 roots, respectively.

Numbers of oogonia and oospores differed in H and H-63 roots (Fig. 12). Oogonia were apparent initially in both H and H-63 root cortical tissue and the stele 24 hr after inoculation. Mean number of oogonia per root section increased up to 48 hr in H roots and then decreased by 72 hr as mean number of oospores per root section increased. Few oogonia or oospores and little change in mean number of oogonia and/or oospores per root section were observed between 24 and 72 hr after inoculation of H-63 roots (Fig. 12). Differences in mean number of oospores per root section in H and H-63 roots were significant at 48 and 72 hr after inoculation.

Hyphal ramification from lateral roots to tap roots and hypocotyls of H but not H-63 plants occurred by 8 days after inoculation. Ninety-seven percent of the H tap roots were brown and 81% contained mycelium, oogonia, or oospores at 8 days. Sixty-three percent of the hypocotyls on H plants were brown and infected. Tap roots and hypocotyls of H-63 plants were not brown or infected at 8 days. Initially, the percentage of brown lateral roots was greater on H-63 plants, but at 7 days after inoculation the percentage of brown lateral roots was greater on H plants (Fig. 13). Differences in the percentage of browning of H and H-63 lateral roots were significant at 144 and 168 hr after inoculation. No discoloration or infection of control plant tissue was observed.

DISCUSSION

This study reveals that resistance in roots of H-63 is apparently effective during the colonization stage of pathogenesis by *Pmg*. The sequence and timing of prepenetration and early postpenetration events were similar on both cultivars. However, as early as 24 hr after inoculation with *Pmg* zoospores, ramification hyphal length was less in roots of the resistant cultivar. By 72 hr, with hyphal length in the resistant cultivar one half the lengths in the susceptible, it was apparent that fungal ramification was slowed in the resistant cultivar. Fungal development was limited to the cortex and stele of lateral roots in the resistant cultivar while ramification to tap roots and hypocotyls of the susceptible cultivar occurred by 8 days. Fewer oogonia and oospores developed in resistant root tissue.

Pre- and postpenetration phenomena reported in this study are consistent with reports of other *Phytophthora* species on plant roots (8,13,14,17,18,25). Zoospores are attracted to, encyst, germinate, and penetrate susceptible, resistant, and tolerant plant roots.

Stössel et al (22,23) observed that zoospores of a compatible race of *Pmg* predominately penetrated between anticlinal walls of epidermal cells in soybean hypocotyls and hyphae ramified deep within hypocotyl tissue. Penetration by an incompatible race occurred between periclinal walls of epidermal cells and hyphal ramification was limited. Differences in the mode of penetration were attributed not to the compatibility of the interaction but to characteristic differences of each race. Our results indicate that penetration by *Pmg* race-1 is primarily between anticlinal walls of epidermal cells in both susceptible and resistant root tissue. At the site of penetration, highly refractive elliptical halos observed may have been host cell wall thickenings or papillae (1).

Penetration of resistant and susceptible soybean roots occurred occasionally via swollen germ tube tips. Lazarovits et al (16) considered these structures to be appressoria and observed them in greater numbers on hypocotyl tissue. According to Emmett and Parbery (6), the criteria used to delimit appressoria are the capacity to adhere to host surfaces and the ability to germinate and

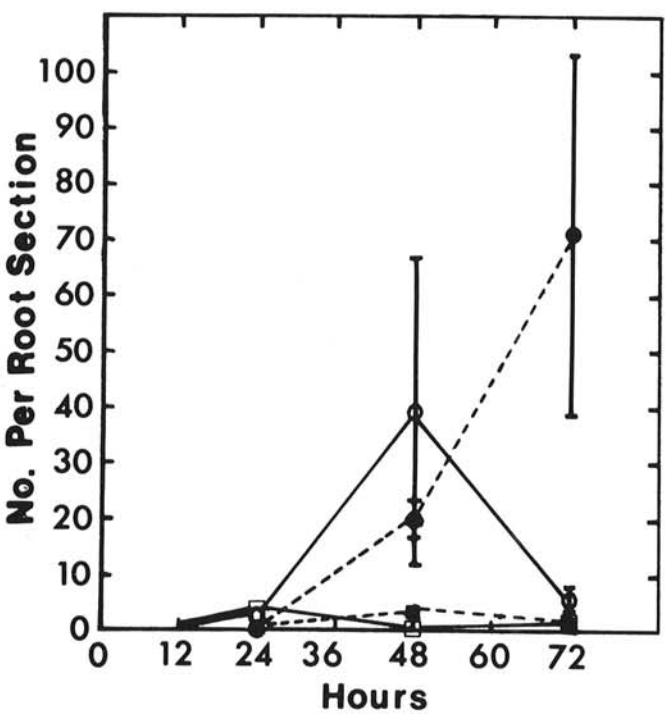


Fig. 12. Mean numbers of oogonia and oospores per root section in Harosoy and Harosoy 63 soybean root tissue from 4.0 to 72 hr after inoculation with zoospores of *Phytophthora megasperma* f. sp. *glycinea*. Mean number of oogonia per root section (H = o——o, H-63 = □——□); mean number of oospores per root section (H = •·····•, H-63 = ■·····■). Vertical bars represent standard errors of the means.

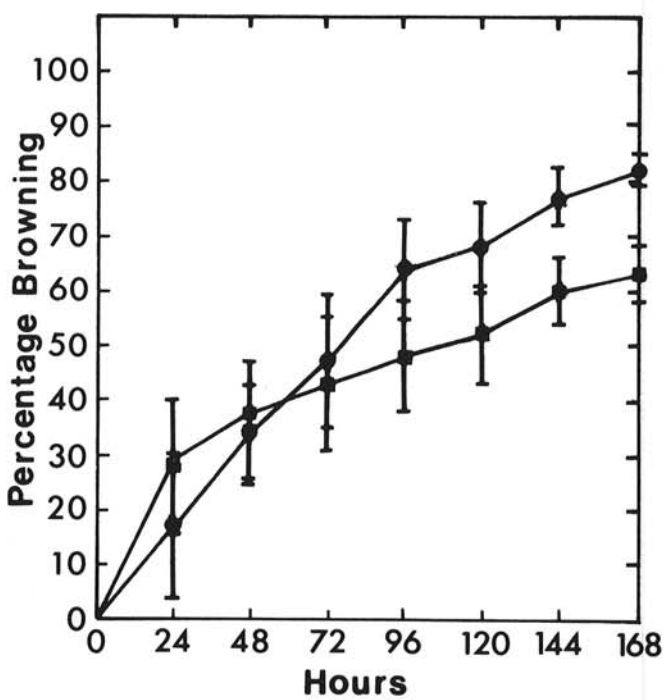


Fig. 13. Percentage browning of lateral roots of cultivars Harosoy and Harosoy 63 plants from 24 to 168 hr after inoculation with zoospores of *Phytophthora megasperma* f. sp. *glycinea*. Percentage browning (H = •——•, H-63 = ■——■). Vertical bars represent standard errors of the means.

penetrate the host. We do not refer to these swollen tips as appressoria since no investigation of their adherence was conducted. In common with other reports (22,25), we consistently observed septa formation between germ tube tips and primary hyphae within root tissue.

The formation of fewer oogonia and oospores in resistant H-63 roots may have been due to the reduced amount of fungal mycelium in this cultivar. Slusher and Sinclair (21) also reported oospore formation in resistant soybean cultivar Amsoy 71 and speculated that a potential inoculum buildup could occur if resistant cultivars are planted in infested fields.

Our study has revealed differences in extent of ramification of *Pmg* in resistant vs. susceptible soybean roots. This information could be useful in directing other research on resistance mechanisms in plant roots and may be eventually useful in resistance breeding programs.

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