# Hypocotyl Reactions and Glyceollin in Soybeans Inoculated with Zoospores of *Phytophthora megasperma* var. *sojae*

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We thank Peter Albersheim for supplying a sample of glyceollin.
Accepted for publication 14 February 1979.

## ABSTRACT

WARD, E. W. B., G. LAZAROVITS, C. H. UNWIN, and R. I. BUZZELL. 1979. Hypocotyl reactions and glyceollin in soybeans inoculated with zoospores of *Phytophthora megasperma* var. *sojae*. Phytopathology 69:951-955.

Soybean (*Glycine max* L.) cultivars were tested for resistance or susceptibility to races of *Phytophthora megasperma* var. *sojae* by placing droplets of zoospore suspensions on hypocotyls of etiolated seedlings. Within 24 hr, resistant, hypersensitive interactions produced brown necrotic lesions, whereas susceptible tissues were soft, water-soaked, and rotted. Up to four races were evaluated on a single hypocotyl, and the method could be of value for screening cultivars and typing races and, especially, for genetic studies. The concentration of the phytoalexin glyceollin (determined from absorption measurements at 285 nm of ethyl acetate extracts of diffusates) was consistently higher in resistant reactions than in susceptible ones. However, because of considerable quantitative variation and the fact that visual ratings were entirely reliable, glyceollin determination did not appear to provide information of critical value for screening purposes.

New races of *Phytophthora megasperma* Dresch. var. *sojae* Hildeb. (*Pms*) virulent on various cultivars of soybean (*Glycine max* [L.] Merr.) have been described in recent publications (3,10–13). If this trend continues, screening cultivars for resistance and typing isolates of the fungus will become increasingly more complex and time-consuming. Procedures in general use involve either root inoculation, in which plants are grown in soil or nutrient solutions infested with *Pms* (5,9,12), or hypocotyl inoculation, in which mycelium is inserted into artificial wounds (3,5). Keeling (6) concluded that both methods are satisfactory, although results from hypocotyl inoculation tend to be more consistent. Presumably, because of convenience, mycelium was used for inoculum in these procedures. With the exception of Eye and Sneh's study (2), inoculation with zoospore suspensions does not appear to have been examined extensively. Theoretically, it could provide some advantages. Because zoospore suspensions can be prepared relatively free of medium constituents and mycelial fragments, they can be standardized numerically. Also, zoospores readily germinate on and penetrate host tissues without the necessity for wounding. Methods for zoospore production by *Pms* have been described (2,4), and in this paper we examine the possibility that zoospore suspensions could be used for hypocotyl inoculation in testing cultivars for resistance or susceptibility to *Pms*.

Several detailed studies have shown the consistent production of the phytoalexin glyceollin in incompatible soybean *Pms* interactions (7,8,14). Glyceollin can be monitored by relatively simple and rapid procedures (1), and the present study provided an opportunity to determine if it can be used as a marker in testing soybean cultivars for disease resistance.

00031-949X/79000170$03.00/0


## MATERIALS AND METHODS

Isolates of races 1–6 of *Pms* used in this study were those described previously by Haas and Buzzell (3). They were grown routinely and also for zoospore production on V8 agar at 25 C in the dark. Procedures for zoospore production followed those of Ho and Hickman (4) and Eye and Sneh (2). Zoospores in suspensions were counted with a hemacytometer and adjusted to a suitable concentration (usually $1 \times 10^5$/ml) with sterile tap water.

Soybean cultivars used are listed in the caption for Fig. 1; their reactions to *Pms* races 1–6 determined by mycelial inoculation of hypocotyl wounds are given by Haas and Buzzell (3). Seeds were surface-sterilized in 0.6% sodium hypochlorite for 3–5 min, rinsed under running tap water for 10 min, and air-dried. After soaking in water for 24 hr at room temperature, seeds were planted in vermiculite in greenhouse flats. The flats were steeped overnight in 15-30-15 fertilizer solution (2.25 g/L) and placed in a dark growth chamber on a temperature cycle of 7 hr at 16 C, 5 hr increasing at 2.2 C/hr, 7 hr at 27 C, and 5 hr decreasing at 2.2 C/hr. The plants were fertilized again on the fifth day (4.5 g/L).

On the sixth day after planting, the seedlings were washed in running tap water, blotted dry, and transferred to glass trays (35 ×

21 cm). Up to 20 seedlings were placed horizontally in each tray, held in position by two slotted Plexiglas racks (1.2 cm wide), one just above the roots and the other immediately below the cotyledons. The roots were covered with a layer of Cellucotton soaked in water. Seedlings were inoculated by placing 1–4 droplets (about 10 $\mu$l each) of zoospore suspension 5–10 mm apart on the upper one-third of the hypocotyl using a 1-ml glass syringe fitted with a 26-gauge needle (o.d. 0.457 mm, i.d. 0.254 mm). The trays were sealed with plastic film and incubated for 20–24 hr at 25 C in the dark. The type of response was then recorded, and material was collected for glyceollin assay. The trays were returned to the incubator, and further visual observations were made 24 hr later if required.

For glyceollin determination, the droplets were removed by suction and replaced briefly with droplets of distilled water to wash the inoculation sites. The total volume of the droplets (diffusate) collected in each cultivar-race interaction was measured and, after being combined with the washings, thoroughly mixed with 2 ml of ethyl acetate in a 15-ml conical test tube. Then, 1.5 ml was removed from the ethyl acetate layer and dried under $N_2$ gas at 35 C, and the residue was redissolved in absolute ethanol. Glyceollin in ethyl acetate extracts was confirmed by reference to a sample supplied by P. Albersheim, using thin-layer chromatography (silica gel and ethyl acetate-tertiary butanol, 95:10, v/v), benzene and methanol (95:8, v/v), and gas-liquid chromatography of the trimethylsilylated derivative (flame ionization; column, 180 cm × 1.5 mm packed with 3% SE 30 on Gas Chrom Q 80-100; column temperature, 225 C; $N_2$ flow rate, 40 ml/min). Retention times of the three isomers of glyceollin were 354, 409, and 495 sec. Glyceollin in the ethanol solution was measured by its absorbance at 285 nm as described by Ayers et al (1). Data were calculated as absorbance units per milliliter of diffusate to allow for differences in diffusate volumes.

TABLE 1. Comparison of reaction and glyceollin production in the soybean cultivars Harosoy 63 and Altona after hypocotyl inoculation with zoospores of six races of *Phytophthora megasperma* var. *sojae*

| | Harosoy 63 | | Altona | |
|---|---|---|---|---|
| Race | Reaction[a] | Glyceollin[b] | Reaction | Glyceollin |
| 1 | R | 1.76 | R | 0.93 |
| 2 | R | 0.98 | R | 1.96 |
| 3 | S | 0.18 | R | 0.57 |
| 4 | S | 0.20 | R | 1.40 |
| 5 | S | 0.12 | S | 0.20 |
| 6 | S | 0.40 | S | 0.16 |

[a] S = susceptible, spreading lesion, water-soaked; R = resistant, hypersensitive, necrotic.
[b] Absorbance values (285 nm) of the ethyl acetate soluble fraction of the diffusate collected from three inoculum droplets on each of 20 hypocotyls inoculated with zoospores ($1 \times 10^5$/ml) and incubated for 24 hr at 25 C in the dark.

TABLE 2. Comparison of reaction and glyceollin production in eight cultivars of soybeans after hypocotyl inoculation with zoospores of races 4 and 6 of *Phytophthora megasperma* var. *sojae*

| | Race 4 | | Race 6 | |
|---|---|---|---|---|
| Cultivar | Reaction[a] | Glyceollin[b] | Reaction | Glyceollin |
| Harosoy | S | 1.36 | S | 0.54 |
| Harosoy 63 | S | 0.97 | S | 0.37 |
| Mack | S | 0.11 | R | 0.70 |
| Altona | R | 1.71 | S | 0.68 |
| PI 103.091 | R | 3.08 | S | 1.33 |
| PI 171.442 | R | 3.46 | S | 0.54 |
| Sanga | R | 2.10 | R | 2.48 |
| Tracy | R | 3.33 | R | 1.77 |

[a] S = susceptible, spreading lesion, water-soaked; R = resistant, hypersensitive, necrotic.
[b] Absorbance values (285 nm) of the ethyl acetate soluble fraction of the diffusate collected from three inoculum droplets on each of 20 hypocotyls inoculated with zoospores ($1 \times 10^5$/ml) and incubated for 24 hr at 25 C in the dark.

## RESULTS

Resistant and susceptible reactions were sharply contrasted (Figs. 1–3). Typically, a resistant, hypersensitive response was characterized by dark-brown necrosis of the tissue beneath the droplet and red pigmentation of the droplet itself. The necrosis spread only marginally beyond the area covered by the droplet, and the surrounding tissue remained firm and healthy. In susceptible reactions, there was little or no browning of the tissue and the red pigment did not appear in the droplet. The tissue beneath and around the droplet was soft and water-soaked, and the limits of the reaction zone were not clearly defined, as in incompatible combinations. By the second day after inoculation, rotting was so extensive that the hypocotyl usually collapsed. Resistant hypocotyls continued to elongate above the inoculation site, but susceptible hypocotyls stopped growing.

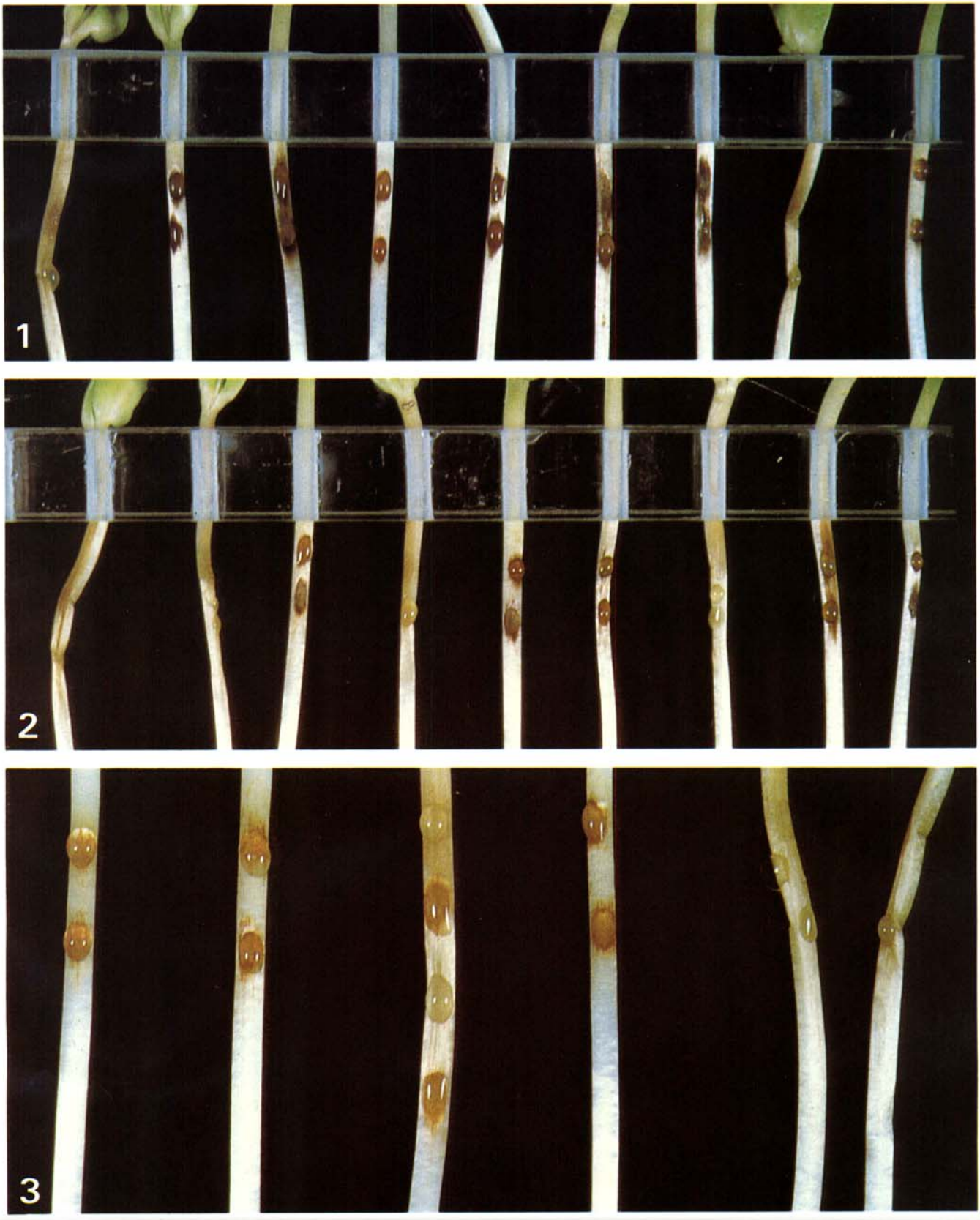
To assess the accuracy of the method with reference to results obtained by others using established procedures, two representative cultivars were inoculated with races 1–6 of the fungus (Table 1, Fig. 3) and nine differential cultivars were inoculated with two races of the fungus (Table 2, Figs. 1 and 2). With one exception (PI 103.091), the results were in agreement with those reported by Haas and Buzzell (3) using mycelium and hypocotyl wounding. The response of PI 103.091 to race 6 was variable; both resistant (Fig. 2) and susceptible reactions (Table 2) were obtained. This was confirmed in an additional test in which zoospore inoculum at two concentrations ($2 \times 10^4$ and $1.2 \times 10^5$ zoospores/ml) was used. Four of 19 (21%) hypocotyls were resistant at the higher inoculum level and 11 of 19 (58%) at the lower inoculum level.

Two other aspects examined were the effect of zoospore concentrations on the development of the response and the feasibility of testing several races on a single hypocotyl. Zoospore concentrations as low as $25 \times 10^3$/ml of race 2 and 4 gave readily distinguishable resistant and susceptible responses on Harosoy 63, and similar results were obtained with races 4 and 5 on Altona (Fig. 4). Droplets of races 2, 4, 5, and 6 were placed in four different sequences on Altona hypocotyls (Table 3, Fig. 3). Regardless of their positions relative to one another, races 2 and 4 induced typical

TABLE 3. Comparison of reaction and glyceollin production after inoculation of soybean hypocotyls (cultivar Altona) with zoospores of four races of *Phytophthora megasperma* var. *sojae* placed at adjacent sites on each hypocotyl

| | Race 2 | Race 4 | Race 5 | Race 6 |
|---|---|---|---|---|
| Position[a] | 1 | 2 | 3 | 4 |
| Reaction[b] | R | R | S | S |
| Glyceollin[c] | 2.53 | 2.92 | 1.47 | 0.77 |
| Position | 3 | 4 | 2 | 1 |
| Reaction | R | R | S | S |
| Glyceollin | 4.20 | 1.97 | 0.51 | 0.40 |
| Position | 3 | 1 | 2 | 4 |
| Reaction | R | R | S | S |
| Glyceollin | 4.73 | 2.50 | 0.86 | 1.50 |
| Position | 4 | 2 | 1 | 3 |
| Reaction | R | R | S | S |
| Glyceollin | 3.43 | 2.07 | 0.47 | 0.48 |

[a] Position on hypocotyl of droplet of zoospore inoculum of each race approximately 1 cm between sites from the top downward.
[b] S = susceptible, spreading lesion, water-soaked; R = resistant, hypersensitive, necrotic.
[c] Absorbance values (285 nm) of the ethyl acetate soluble fraction of the diffusate collected for each race at each position on 20 hypocotyls inoculated with zoospores ($1 \times 10^5$/ml) and incubated for 24 hr at 25 C in the dark.




**Figs. 1–3. 1,** Response of hypocotyls of plants of nine soybean cultivars to inoculation with zoospore suspensions of race 2 of *Phytophthora megasperma* var. *sojae*. Cultivars (left to right): Harosoy (S), Harosoy 63 (R), Mack (R), Altona (R), PI 86.050 (R), PI 103.091 (R), PI 171.442 (R), Sanga (S), Tracy (R). S = susceptible, R = resistant. **2,** Response of hypocotyls of plants of nine soybean cultivars to inoculation with zoospore suspensions of race 6 of *P. megasperma* var. *sojae*. Cultivars (left to right): Harosoy (S), Harosoy 63 (S), Mack (R), Altona (S), PI 86.050 (R), PI 103.091 (R), PI 171.442 (S), Sanga (R), Tracy (R). S = susceptible, R = resistant. **3,** Response of hypocotyls of plants of the soybean cultivar Altona to inoculation with zoospore suspensions of races 2, 4, 5, and 6 of *P. megasperma* var. *sojae*. Hypocotyls inoculated with (left to right): race 2 (R); race 4 (R); in order from the top, race 6 (S), race 4 (R), race 5 (S), race 2 (R); race 4 (R); race 5 (S); race 6 (S). S = susceptible, R = resistant.

resistant responses and races 5 and 6 induced typical susceptible responses. The reaction at any one inoculation site was not influenced by that at adjacent sites.

Values for glyceollin production were in general agreement with the visual ratings in that resistant responses invariably resulted in higher absorbance values than susceptible responses for any one cultivar (Tables 1–3, Fig. 3). The absorbance values from resistant reactions were 2–10 times higher than those from susceptible reactions. These differences were also found when hypocotyls were inoculated with four races of the fungus and, as with the visual symptoms, were unaffected by reactions at neighboring sites (Table 3). Glyceollin production increased with increasing zoospore concentration, especially in incompatible combinations, and levels were consistently higher in incompatible than in compatible interactions (Fig. 4). In contrast, there was considerable variation among the values obtained for a single race having the same reaction type on different cultivars (Table 2) and for races giving the same reaction type on a single cultivar (Table 1).

## DISCUSSION

The evidence shows that zoospore inoculation of soybean hypocotyls can be used to distinguish resistant and susceptible cultivars. Not only were results generally consistent with earlier reports (3,6,12,13) but the method has advantages over hypocotyl wounding or root inoculation procedures. For breeding and, especially, for genetic studies the most valuable feature of the use of zoospore inoculum is that more than one race can be evaluated on a single hypocotyl. Up to four races were compared on one hypocotyl, and the responses were typical and not influenced by interactions at neighboring sites. Only the upper one-third of the hypocotyl was inoculated, as responses to inoculations of the lower part were atypical.

For general screening purposes, zoospore inoculation apparently has the disadvantage that producing zoospore suspension is somewhat more complicated than growing mycelium. However, this is offset, at least partly, by the need for only small volumes of zoospore suspension. Thus, about 15 ml of zoospore suspension of $1 \times 10^5$ spores per milliliter can be obtained from a single culture plate, sufficient to inoculate about 1,500 hypocotyls. In addition, inoculation is simplified, as wounding is not required and, where diffusates are not needed for glyceollin determination, droplets can be placed on hypocotyls without removing plants from flats or pots.

The method has distinct advantages for studies of the physiology of the host-parasite interaction and phytoalexin production. It permits use of the drop-diffusion technique, avoids complications due to wounding tissue, reduces to a minimum the transfer of medium constituents and cell wall fragments that may influence the interaction, and facilitates inoculum standardization, permitting the application of varying inoculum loads.

There was considerable variation in the response of individual plants of PI 103.091 to race 6 compared with the uniform and unambiguous response of other cultivars. Seeds of PI 103.091 germinated poorly and seedlings were spindly. Possibly, the cultivar is basically resistant but tends to become susceptible because of poor vigor. The greater susceptibility observed at the higher of the two inoculum levels tested would be consistent with this, as well as the observation that even when the reaction appeared to be susceptible, glyceollin levels were relatively high (Table 2).

The glyceollin levels determined were consistent with the visual ratings in that levels were higher in resistant than in comparable susceptible interactions. However, because of the variability in absorbance values within a reaction type, these values by themselves could not serve as a reliable basis for evaluation. Differences in absorbance for the same reaction type were frequently as great as those between opposite types. Possibly, some of the variation is due to materials other than glyceollin that absorb at the same wavelength. Ayers et al (1) concluded that glyceollin in diffusates from elicitor-treated cotyledons accounted for 24% of the absorption at 285 nm. In our procedures, ethyl acetate extraction provides a measure of purification, and the contribution of glyceollin to the absorbance value may have been higher. Nevertheless, different race and cultivar combinations may yield different proportions of material other than glyceollin absorbing at 285 nm. Whereas this could be resolved by careful purification of the glyceollin in each of the ethyl acetate fractions, it would be too complex and time-consuming for routine screening purposes.

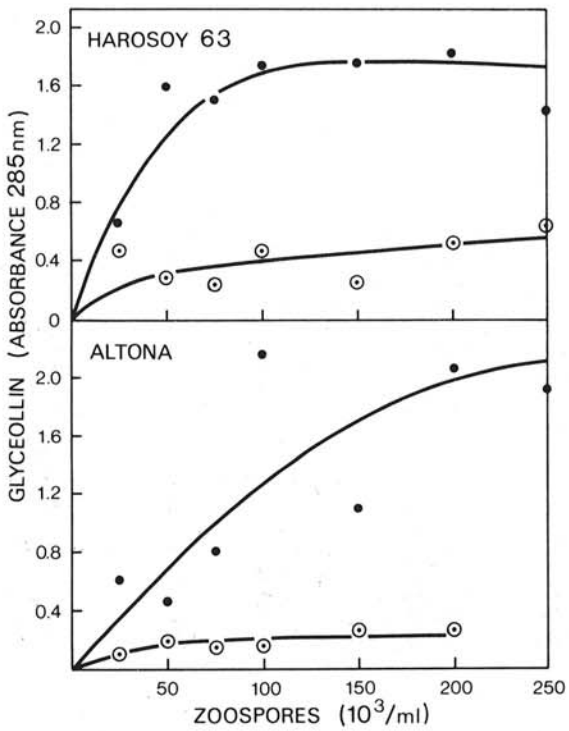



**Fig. 4.** Influence of zoospore concentration on the production of glyceollin by soybean hypocotyls inoculated with zoospore suspensions of races of *Phytophthora megasperma* var. *sojae*. Harosoy 63 inoculated with race 2 (resistant -•-) and race 4 (susceptible -⊙-); Altona inoculated with race 4 (resistant -•-) and race 5 (susceptible -⊙-).

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