Inoculation of Soybean Plants by Injection with Zoospores of *Phytophthora megasperma* var. *sojae*

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ABSTRACT

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Suspensions of *Phytophthora megasperma* var. *sojae* zoospores were injected into the hypocotyls of soybean plants. This method took one-third to one-quarter the time required for hypocotyl inoculation with agar pieces bearing mycelium. When 10^5 zoospores per milliliter were used, the results of the two methods were nearly identical, and intervals between inoculation and reaction determination were similar under greenhouse conditions. Plants grown at 28 C under continuous light in controlled environment chambers could be inoculated as early as 4 days after planting in vermiculite, and the disease reaction determined 3 days later.

Additional key words: disease screening, Phytophthora root and stem rot.

Phytophthora megasperma (Drechs.) var. *sojae,* a pathogen of soybeans, occurs as several races in much of the soybean growing region of the USA and Canada (4,5,8–13). Resistance to the fungus is an important objective in cultivar development, and the process of screening experimental lines and cultivars has received considerable attention (1,6–8). The method of Kaufmann and Gerdemann ([5], method 8), in which fungus mycelium is inserted into a wound in the hypocotyl, has given the clearest and most consistent results. We describe here an inoculation method that gives results comparable to those of that widely used method, but with less time and effort. In our method, a zoospore suspension is injected into soybean seedling hypocotyls. When used with plants grown in a growth chamber, it reduces screening time to 7 days from planting to final reaction determination.

MATERIALS, METHODS, AND RESULTS

Zoospores for the trials were produced by the method of Eye et al (2). Zoospore suspensions were injected into hypocotyls of soybean plants which had been grown in the greenhouse, by using 0.26–0.18 mm (24–26 ga) needles on 3-ml syringes. Plants were used when the unifoliolate leaves had expanded, usually at 10–14 days. Best results (comparable to those from the mycelium insertion method) were obtained by touching the beveled side of the needle tip to the hypocotyl, forcing out a small droplet, then pushing the needle through this droplet and into the stem at an angle of 45 degrees from vertical. A small droplet was again ejected, and the needle withdrawn, leaving liquid on both ends of the opening in the plant. In preliminary tests, one to four injections per hypocotyl were used, with zoospore concentrations ranging from 1 to 400×10^3 zoospores per milliliter. A minimum of three injections with a suspension of 10^5 zoospores per milliliter gave consistent results, provided the injections were at least 1 cm apart. One injection of 3×10^5 zoospores per milliliter did not give the same results as did three injections of 10^5 zoospores per milliliter. With the single injection, up to 50% of the plants of susceptible cultivars did not die, and the other plants died several days later than did comparable plants injected several times. "False positives" (ie, plants that were killed, even though they were of cultivars assumed to be resistant) were rare with this technique, even with three injections at 8×10^5 zoospores per milliliter. Unless otherwise specified, all subsequent tests were done using $1.0–1.5 \times 10^5$ zoospores per milliliter, with three injections per hypocotyl. Disease incidence was not affected by placing plants in plastic bags for 12 or 24 hr after inoculation, or by shading plants in the greenhouse following inoculation. A mycelium insertion method similar to that of Kaufmann and Gerdemann ([5], method 8) was used as a standard; we grew the fungus on lima bean agar (Difco), inserted fungus and agar together into a slit in the hypocotyl, and covered the wound with petrolatum.

With the zoospore injection technique, we inoculated eight to ten 10-day-old plants of 420 experimental lines and cultivars per race with *P. megasperma* var. *sojae*, races 1–6. An additional 4,039 experimental lines and cultivars were tested for reaction to race 1 only. Every 10th pot contained a check cultivar of known race reaction (4). Students and technicians did the inoculations using this method, as they have for the past several years using the mycelium insertion method. Inoculation time varied; the most efficient person did 90–100 pots (more than 700 plants) per hour, whereas the same person could do only 25–30 pots per hour when using the mycelium insertion method. Plants in the 656 check pots responded as expected to inoculation with the different races. The zoospore injection method produced more uniform reactions within plants in a pot than the mycelium insertion method used in the past.

For tests under controlled environment, a chamber was set for $22–27 \times 10^3$ lux of continuous fluorescent/incandescent light and 28 C. Seeds were planted in fine vermiculite, eight seeds per 7.6 cm-square plastic pot, covered with about 0.5 cm of fine vermiculite, set in 5-cm-deep trays, and watered with tap water containing 1 gm 20-20-20 (N-P-K) fertilizer per liter. The plants were watered with tap water again 4–5 days later, then at 2-day intervals, alternating fertilizer solution (1 gm/L) with tap water. Water and fertilizer solutions were added to the tray rather than poured over the plants. Plants grown for 6–7 days under these conditions were comparable in development to plants grown 10–14 days in the greenhouse. Plants were inoculated at 4, 5, 6, 8, 10, or 14 days after planting. Plants 4, 5, and 6 days old gave results consistent with those from 10- to 14-day-old plants grown in the greenhouse and inoculated with mycelium. The reactions of plants inoculated at 4 days could be determined 3 days later. This 7-day cycle was comparable to a cycle of 14–28 days (depending upon the environment) in the greenhouse by the conventional method.

In another test, Columbus (susceptible) and Calland and Mack (resistant) seedlings were grown in vermiculite as described, then placed in the growth chamber. Plants were inoculated with race 1 4 days later, when the cotyledons were still close together, but pointing up. Approximately eight plants of each cultivar were inoculated with 2, 5, 10, 20, or 40×10^4 zoospores per milliliter. Symptoms were apparent within 48 hr, and by 72 hr after

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inoculation all Columbus plants were dead regardless of inoculum level used. All uninoculated Columbus plants and all Calland and Mack plants were healthy. In another test, eight 6-day-old plants of Columbus, Calland, and Mack were inoculated with races 1–6 at 1 to 4×10^5 zoospores per milliliter. The reaction pattern fit the expected (4) perfectly at 48 hr after inoculation; at 5 days after inoculation there were no false positives.

DISCUSSION

The zoospore inoculation method proposed by Eye et al (3) seems to be much easier to use than the mycelium insertion method (referred to in their paper as the hypocotyl inoculation method), or than the zoospore injection method proposed here. However, their method resulted in a relatively high number of escapes, and the numbers of zoospores used had to be monitored quite closely; high numbers gave false positives with some cultivars. Their method apparently can be used to measure field tolerance. While this tolerance is useful, it varies with environment. By contrast, plants of cultivars that are resistant to the fungus in the laboratory, as measured by the mycelium insertion method, are resistant to the same race(s) of the fungus under field conditions. Therefore, we have used this method during cultivar development testing, and our purpose here was to develop a faster, easier method that gave results comparable to those obtained by the mycelium insertion method.

During inoculation, plants with thin hypocotyls are much less likely to be broken by a hypodermic needle than by a scalpel. Another potential advantage of this system over the standard method is that zoospores of several races can be mixed and injected simultaneously. In preliminary studies, we have found no synergistic or antagonistic effects among races, which agrees with the results of Kilen and Keeling (7). Further studies on this aspect are in progress.

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