Metalaxyl Sensitivity Selection Within *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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After six successive transfers on cornmeal agar amended with 0.1 μg/ml metalaxyl, an isolate of *Phytophthora megasperma* f. sp. *glycinea* displayed reduced sensitivity to metalaxyl and increased virulence on metalaxyl-treated seedlings of Harosoy 63 soybean (*Glycine max*). Analysis of 10 single-zoospore progeny from the isolate, derived before the isolate was grown on metalaxyl-amended medium, demonstrated innate variation in virulence and metalaxyl sensitivity. Virulence of several isolates was compared on Harosoy and Harosoy 63 seedlings pretreated with 0, 0.5, 1.0, 2.5, and 5.0 μg/ml metalaxyl in water. Two isolates, which had been grown on cornmeal agar amended with 0.1 μg/ml metalaxyl, were more virulent on seedlings treated with metalaxyl than were their parental isolates. Exposure to metalaxyl in saprophytic growth can select for both altered virulence and decreased sensitivity to the fungicide in metalaxyl-treated plants.

Phytophthora root rot of soybeans (*Glycine max* (L.) Merr.) is a serious disease caused by *Phytophthora megasperma* Drechs. f. sp. *glycinea* T. Kuan and D. C. Erwin. Breeding for resistance to the pathogen has been a major thrust in disease control efforts. Cultivars resistant to the pathogen have been developed and cultivated, but over 27 races of the pathogen have been identified in areas where soybeans are grown. In fact, races of *P. m. glycinea* are defined by their ability to match compatibility with various host resistance genes, designated *Rps* (1,18). An incompatible response in an inoculated soybean plant is characterized by the development of a localized lesion and restricted growth of the pathogen. Rapid accumulation of the soybean phytoalexin glyceollin may be associated with the incompatible response (25).

Chemical control of Phytophthora root rot of soybeans is also possible. Metalaxyl was registered for use on soybeans in 1986 and is incorporated in soil or placed on seed to prevent Phytophthora root rot. This systemic acylalanine fungicide is effective in limiting growth of *P. m. glycinea* at low concentrations. In early laboratory studies with *P. capsici, P. infestans*, and *P. megasperma* f. sp. *medicaginis*, isolates cultured on metalaxyl-amended media were more sensitive to metalaxyl in parasitic growth within metalaxyl-treated plants than in saprophytic growth on media (3,9). In contrast, some mutants of *P. m. glycinea* selected for reduced sensitivity to metalaxyl actually grew better on metalaxyl-treated plants and in culture medium amended with metalaxyl than without the chemical (4). Pathogen resistance to metalaxyl in the field has led to control problems with late blight of potatoes caused by *P. infestans* (10) and downy mildew of lettuce caused by *Bremia lactucae* (8). The potential exists for development of metalaxyl resistance in *P. m. glycinea*.

Metalaxyl resistant mutants of *P. m. glycinea* were generated by UV irradiation (5) and by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of zoospores (15) followed by selection on media containing metalaxyl. Mutants of *P. m. glycinea* maintained their race phenotype throughout previous studies (16). Metalaxyl resistance was a useful phenotype in a study of parasexuality, in which protoplast fusion led to heterokaryon formation (15). Analysis of the differences in elicitation of glyceollin in susceptible soybean seedlings by metalaxyl-insensitive mutants and their metalaxyl-sensitive parents helps explain how growth of *P. m. glycinea* is restricted at low metalaxyl concentrations in treated plants (4). Metalaxyl appears to cause increased glyceollin accumulation and,

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as a result, changes the response of a compatible soybean to incompatible (2,24). One explanation of these observations is that the phytoalexin acts in concert with RNA polymerase inhibition by metalaxyl to stop fungal growth (23).

P. m. glycinea is exposed to metalaxyl in the soil when commercial formulations, Apron and Ridomil, are used in field applications to control Phytophthora root rot of soybean. Field application of metalaxyl could lead to decreased sensitivity in the *P. m. glycinea* population through exposure to sublethal levels of the fungicide. Bruin and Edgington (3) studied adaptation of many species of *Pythium* and *Phytophthora* to metalaxyl over an 8-mo period. Three strains of *P. capsici* became very insensitive to metalaxyl both in culture medium and in treated pepper (*Capsicum annuum*) plants.

Field populations of *P. m. glycinea* may contain isolates that vary with respect to virulence and sensitivity to metalaxyl. A study of offspring from an individual isolate can reveal diversity that is due to heterokaryosis or heterozygosity present within a population. Hyphae of *Phytophthora* species are multinucleate; a single hypha may contain nuclei with different genotypes that can move within the cytoplasm, unrestricted by cell walls. The production of effective inoculum by zoosporogenesis involves packaging of a nucleus, organelles, and cytoplasm within a membrane, yielding predominantly uninucleate daughter zoospores with a variety of genotypes. From this natural pool of various genotypes, those that best tolerate the fungicide in the rhizosphere and plant host might be selected. This study was undertaken to test the effects of low-level metalaxyl exposure on virulence and metalaxyl sensitivity of *P. m. glycinea*.

MATERIALS AND METHODS

Isolates of *P. m. glycinea* and routine culture. The *P. m. glycinea* isolates used for this study (A10 and A11) were collected before exposure to metalaxyl. Each culture was derived from a single zoospore (determined to be race 10 and race 11, respectively) supplied by Terry Anderson (Agriculture Canada, Harrow, Canada). Single-zoospore colonies derived from A11 are numbered 11-1 through 11-10. After adaptation of A10 and A11 on metalaxyl-amended media, the resulting isolates were called 10M and 11M. Culture history is shown in Figure 1. An isolate recovered from a diseased metalaxyl-treated Harosoy 63 plant inoculated with 11M and subsequently single-zoospored is called 11MZ. Single zoospore colonies of 11MZ were numbered 11MZ-1 through 11MZ-11. Alice Layton (Purdue University, Lafayette, IN) provided a metalaxyl-resistant mutant (1mex6) generated by exposure of zoospores of *P. m. glycinea* isolate R1-5-58 to nitrosoguanidine. All cultures were maintained at room temperature (23–25 C) in soybean broth, which was prepared by autoclaving 15 soybean seeds in 150 ml distilled water (equivalent to 15 g seeds per liter).

Metalaxyl formulations, preparation of media, and treatments of soybean seeds and seedlings. The 25% wettable powder formulation of metalaxyl (Apron) was used in initial tests in culture and in seed treatment tests of all isolates. Standard recommendations for metalaxyl treatment of soybean seeds include application of Apron at 0.25 oz of active ingredient to 100 lb of seed. Experiments comparing the effects of different concentrations of metalaxyl on soybean protection against adapted and untreated isolates were performed using Ridomil, 2 EC (2 lb/gal emulsifiable concentrate). Final concentrations are given as micrograms of active ingredient per milliliter (μg/ml).

Metalaxyl was added to cornmeal agar (Difco) or quarter strength lima bean agar (Difco; LBA/4) (5.75 g LBA and 15 g Bacto agar per liter of distilled water) before autoclaving or filter sterilized and added to the autoclaved medium just before solidification. No difference was detected in growth rates of the fungus when the fungicide was autoclaved or filter sterilized. Plugs of mycelia, 5 mm in diameter, were transferred from the outer margin of colonies actively growing on LBA/4, or from outer margins of colonies on LBA/4 or cornmeal agar containing 0.1 μg/ml metalaxyl during adaptation. Tests for inhibition of growth on LBA/4 containing metalaxyl began with transfer from colonies on LBA/4. The cultures were incubated in the dark at 23–25 C, and the diameter of the colony was measured daily for 7 days. Experiments were repeated three times. Data presented are the average of three replicate plates from one representative experiment. For in vitro selection of *P. m. glycinea*, original (mycelial mass) and single-zoospore colonies were grown on 0.1 μg/ml metalaxyl-amended cornmeal agar for six successive weekly transfers.

Apron seed treatment was used in preliminary tests for in vivo sensitivity of isolates A10 and A11 before and after culture on metalaxyl-amended media and for metalaxyl sensitivity of zoospore colonies. Soybean seeds were dusted with 0.125 g of 25% metalaxyl wettable powder per 100 g seeds (equivalent to 2 oz of Apron per 100 lb of seed). Excess powder was discarded by agitating the seeds in a sieve. Although seeds were well coated, there was no guarantee that each seed carried the same amount of fungicide at the time of treatment or that the concentration of metalaxyl in the treated seedlings was the same. Seven- to 10-day-old seedlings, untreated or from seeds dusted with metalaxyl, were grown in flats of sand or vermiculite in the greenhouse.

In the first test for metalaxyl sensitivity of isolates, 15 seedlings of Harosoy and Harosoy 63, untreated and treated, were placed in cups of water, five plants to a cup, and inoculated with four separate isolates of *P. m. glycinea*, the isolates A10 and A11 unexposed to metalaxyl, and after successive transfers on metalaxyl-amended media (10M and 11M). Harosoy and Harosoy 63 soybean cultivars were used in pathogenicity tests. Harosoy is susceptible to races 10 and 11 of *P. m. glycinea*, whereas Harosoy 63 is resistant to the two races (1). The two near isoline soybean cultivars differ at the *Rps1* locus. Harosoy 63 contains the dominant *Rps1* resistance gene derived from Blackhawk. Each isolate was used to inoculate 60 plants contained in a total of 12 cups. In the tests with zoospore colonies, 10 seedlings were used per cup, with no replicates. Seedlings

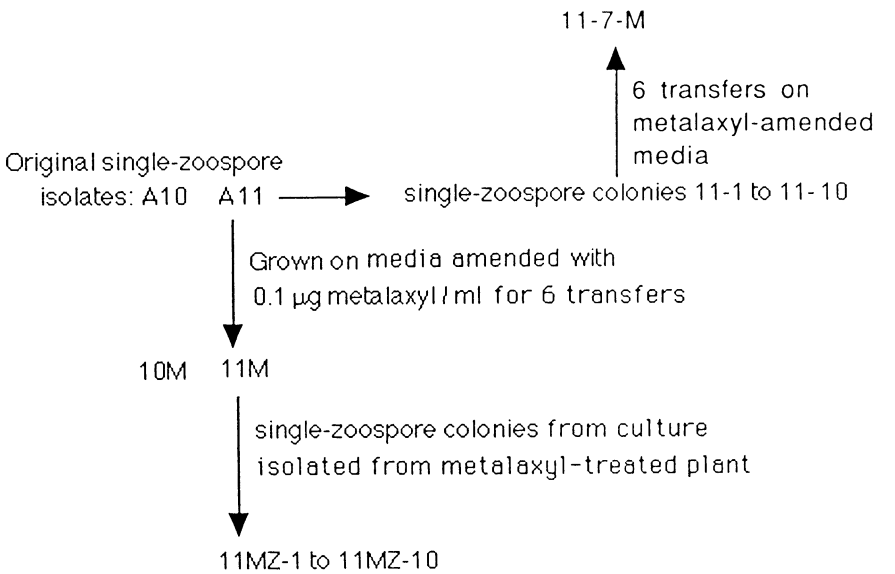

Fig. 1. *Phytophthora megasperma* f. sp. *glycinea* isolate history.

were inoculated by insertion of pieces of mycelium (grown for 2–4 wk in soybean broth) into wounds in the hypocotyl just below the cotyledons. The seedlings were covered with a plastic bag overnight. The cups were arranged randomly in a growth chamber at 24 C, with a 14-hr day length. After 8 days, the number of healthy plants and the number of plants that had collapsed from disease were recorded.

Effect of metalaxyl concentration on disease development was compared quantitatively with the two soybean cultivars Harosoy and Harosoy 63. Metalaxyl treatment before inoculation consisted of exposure of 7- to 9-day-old seedlings for 18 hr in 450-ml beakers containing 250 ml of distilled water and metalaxyl at 0, 0.05, 0.1, 2.5, or 5.0 μg active ingredient per milliliter (80 plants per 250 ml of each Ridomil solution to test eight isolates). The plants were rinsed three times in distilled water before inoculation. Ten plants of each cultivar from each level of metalaxyl treatment were inoculated with each of the isolates to be compared. Five hundred plants were used in a typical experiment.

Zoospore production. A modification of the method of Eye et al (13) was used to produce zoospores. To deplete nutrients from the medium, 4-day-old colonies on LBA/4 were rinsed four times by flooding with 15 ml of sterile, distilled water at half-hour intervals. Zoospore suspension buffer was added (6), and the plates were incubated for 8 hr in the dark. One drop of the zoospore suspension from the flooded agar plate was spread on the surface of LBA/4 with a bent glass rod, and individual colonies were removed from the agar 24 hr later. Ten single zoospore colonies were cultured from those produced from the original isolate A11 and from isolate 11MZ. The twenty colonies were tested for virulence on metalaxyl-treated and untreated Harosoy and Harosoy 63 seedlings. One of the single zoospore colonies from the parental isolate, isolate 11-7, was chosen for repeated transfers on metalaxyl-amended medium and for production of a second generation of zoospores. Each of 30 single-zoospore isolates was used to inoculate 40 plants, 10 each of Harosoy and Harosoy 63, with and without Apron seed treatment.

RESULTS

In preliminary tests, isolates of 12 races of *P. m. glycinea* were transferred to cornmeal agar with 0.1 or 0.01 μg of metalaxyl per milliliter and observed for differences in growth rate. Two isolates, A10 and A11, grew faster on both concentrations than the other isolates and were selected for the adaptation experiment. No sectoring was observed during these experiments. After six transfers on cornmeal agar containing 0.1 μg metalaxyl per milliliter, one isolate (10M) retained virulence on untreated plants but appeared to have reduced virulence on metalaxyl-treated plants (Fig. 2). Inoculation with unadapted isolate A10 resulted in hypocotyl collapse of 7 times more metalaxyl-treated Harosoy plants than inoculation with 10M, which had been cultured in the presence of metalaxyl. The other adapted isolate, 11M derived from isolate A11, was more virulent on Harosoy 63 plants than its unadapted parent and was virulent on metalaxyl-treated Harosoy and Harosoy 63 (Fig. 2). After this test, the fungus was reisolated from the collapsed hypocotyl of a metalaxyl-treated Harosoy 63 plant, and a single zoospore colony was retained and tested further as isolate 11MZ.

Variability in metalaxyl sensitivity of single-zoospore colonies when inoculated onto treated seedlings. The results of testing zoospore colonies indicate a uniform virulence on the susceptible cultivar. Harosoy plants, treated with Apron or untreated, all died when inoculated with mycelium of the 10 zoospore isolates from the parent isolate 11. The response of Harosoy 63 to these isolates varied: each of the 10 zoospore isolates infected 0–40% of inoculated plants. When metalaxyl-treated Harosoy 63 plants were inoculated with parent isolate progeny, 0–40% plants again were killed. In contrast, 99% of all the plants, Harosoy and Harosoy 63, metalaxyl-treated and untreated, died when inoculated with progeny of metalaxyl-adapted 11MZ.

The results of virulence testing of the second generation of zoospores from the unadapted parent showed variability in virulence on Harosoy 63 and lack of sensitivity to Apron seed treatment in colonized Harosoy seedlings. Metalaxyl-treated and untreated Harosoy were susceptible to the zoospore progeny: 98% of the Harosoy and 80% of the metalaxyl-treated Harosoy seedlings died. Forty percent of the untreated Harosoy 63 plants died when inoculated with the 10 zoospore isolates from 11-7 (with a range of 10 to 70% infected). No isolates from 11-7 caused disease on metalaxyl-treated Harosoy 63, when seed dusting with Apron was used as the method of application. Metalaxyl-treated Harosoy 63 plants were completely resistant to isolate 11-7, the parent of the 10 second-generation zoospore isolates, although 40% of the untreated, inoculated Harosoy 63 plants died. (The numbers of plants that collapsed from zoospore isolate inoculations totaled the same percent as when the parental line was used.)

After adaptation by six transfers on metalaxyl-amended agar, isolate 11-7 was designated 11-7-M. Its pathogenicity and metalaxyl tolerance in planta, using seed treatment with Apron, appeared unchanged by exposure to metalaxyl. The metalaxyl mutant of *P. m. glycinea*, 1mex6, displayed lack of sensitivity to metalaxyl but remained a race 1 phenotype. Using Apron seed treatment, the mutant isolate killed 80–100% of the Harosoy plants (treated with metalaxyl or untreated) but less than 30% of the Harosoy 63 plants. The parent, R1-5-58, did not kill metalaxyl-treated plants and was virulent only on Harosoy.

Concentration dependent protection by metalaxyl. A difference existed in the level of protection provided by metalaxyl between Harosoy and Harosoy 63 plants. The unadapted isolate A11 and single zoospore colonies 11-1 and 11-7 were less virulent when inoculated on metalaxyl-treated Harosoy 63 plants than on metalaxyl-treated Harosoy. For example, 11-1 inoculated Harosoy seedlings were protected 100% by 2.5 μg/ml metalaxyl, but only 0.5 μg/ml was required to protect 100% of the Harosoy 63 seedlings (Fig. 3). The adapted isolate 11MZ-11 displays greater tolerance to metalaxyl in Harosoy 63 seedlings than the parent, isolate 11. At 1.0 μg/ml metalaxyl, Harosoy 63 was protected 100% against isolate 11, but 11MZ-11 killed 60% of the treated plants (Fig. 3). At the same metalaxyl concentration, there appears to be a cultivar effect that is characteristic of an isolate. The metalaxyl-adapted isolate 11MZ-11 seems to

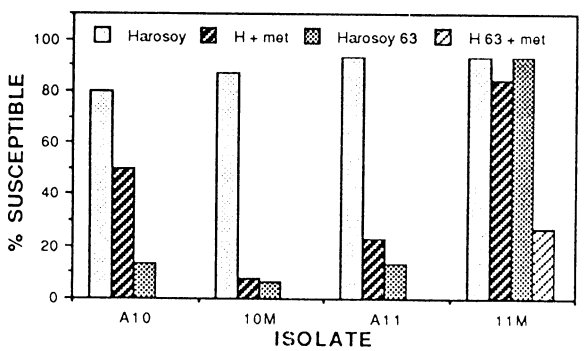

Fig. 2. Reactions of *Phytophthora megasperma* f. sp. *glycinea* isolates A10 and A11, and the isolates derived from them (10M and 11M) by adaptation on cornmeal agar containing 0.1 μg/ml metalaxyl. Harosoy and Harosoy 63 soybean plants with and without Apron seed treatment were inoculated at 7 days after planting. Percent susceptible is number of collapsed plants divided by number inoculated.

be more sensitive to metalaxyl in Harosoy than in Harosoy 63.

The isolate 11-7-M, a zoospore colony of 11-7 that had been grown on LBA/4 with 0.1 μg/ml metalaxyl, killed plants treated with higher concentrations of metalaxyl than its single zoospore parent and isolate 11, from which it was derived (Fig. 4).

Growth rate on solid media. The adapted isolates, 11MZ-11 and 11-7-M, grew on LBA/4 at the same rate as their parental isolates (Fig. 5A and B). However, both adapted isolates grew poorly on LBA/4 with 1 μg/ml metalaxyl. In contrast, metalaxyl mutant 1mex6 grew as well as its parental culture R1-5-58 on LBA/4, but its growth rate was unaltered by addition of metalaxyl at 1 μg/ml (Fig. 5C).

DISCUSSION

Plant pathologists use the results of in vitro fungal growth studies before and after exposure to chemicals to gain information that might have predictive value in the field (7,14,21). Davidse (9) and Erwin (12) recommended in vivo methods for testing metalaxyl sensitivity. The importance of the substrate on which the fungus is grown during testing and development of an in vivo assay was stressed by Shew (21). The results reported here support their recommendations for testing metalaxyl sensitivity of pathogens in treated plants. Our work suggests that there may be differential gene expression by the fungus during saprophytic and parasitic growth phases that affects growth in the presence of metalaxyl.

Variability in *Phytophthora* spp. can be attributed to several processes and characteristics (12). In a diploid organism with gametangia arising from the same mycelium (homothallic), meiosis is a source of recombination. Heterokaryosis, the presence of more than one nuclear genotype, was demonstrated to take place in *P. m. glycinea* by protoplast fusion experiments (15) and by coculture of auxotrophs and of drug resistant mutants of *P. m. glycinea* (17). Other possibilities exist for the appearance of variation. For example, random mutation, mitotic crossing over, and mitochondrial genomic differences may

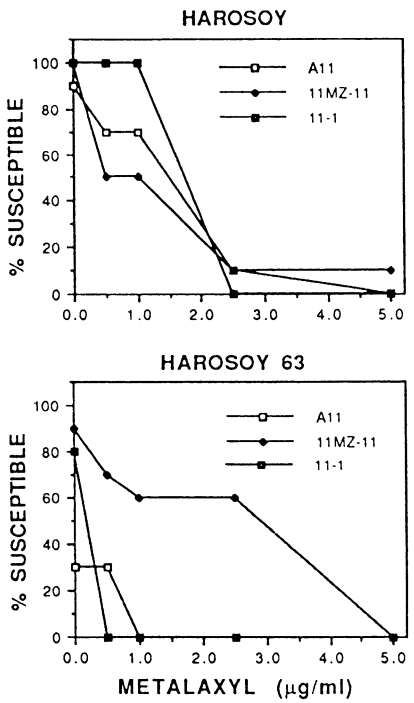

Fig. 3. Metalaxyl protection (Ridomil, 2 EC) of Harosoy and Harosoy 63 plants against disease caused by inoculation with three isolates of *Phytophthora megasperma* f. sp. *glycinea*, A11, 11MZ-11, and 11-1. Although the trends were the same each time the experiment was done, data from replicate experiments were not combined. Each data point represents the results with 10 plants contained in one pot. See Figure 1 for the history of each isolate.

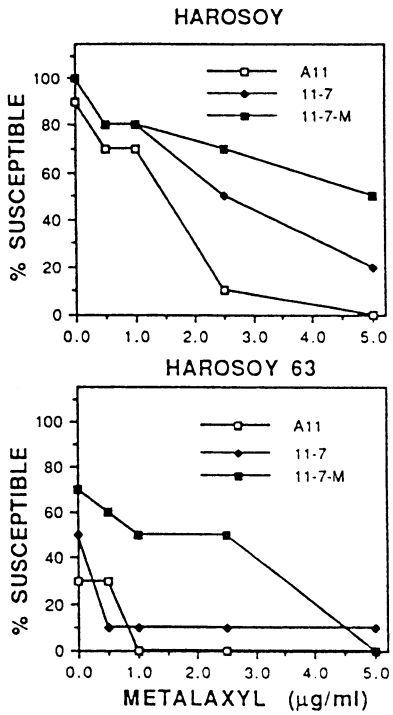

Fig. 4. Metalaxyl protection (Ridomil, 2 EC) of Harosoy and Harosoy 63 plants against disease caused by inoculation with three isolates of *Phytophthora megasperma* f. sp. *glycinea*, A11, 11MZ-11, and 11-7-M. The experimental design was the same as in Figure 3. See Figure 1 for the history of each isolate.

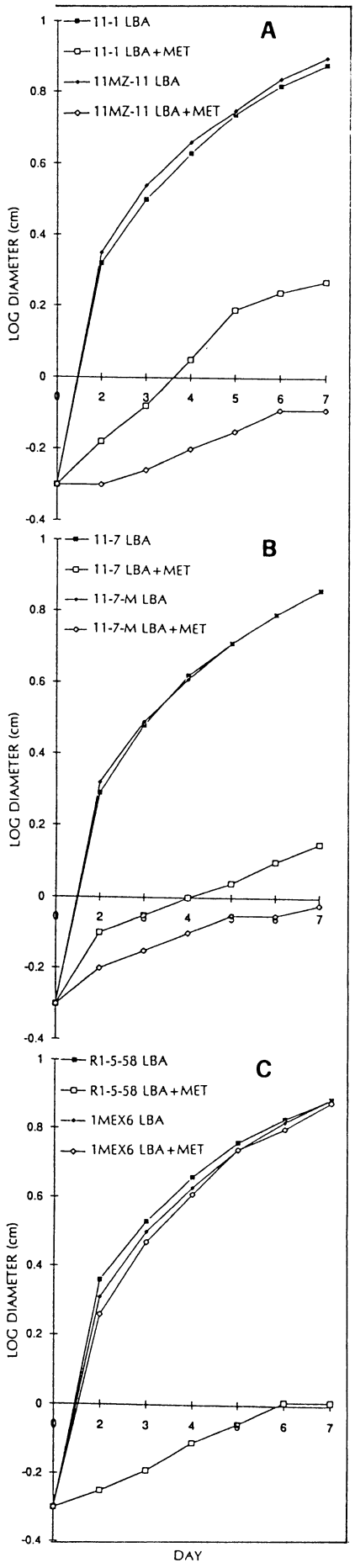

Fig. 5. Growth of isolates of *Phytophthora megasperma* f. sp. *glycinea* on lima bean agar with 0.1 μg/ml metalaxyl (Ridomil, 2 EC) (LBA + met) and without metalaxyl (LBA). **(A)** Isolate 11-1 and metalaxyl-adapted 11MZ-11. **(B)** Isolate 11-7 and metalaxyl-adapted 11-7-M. Note that these adapted isolates grow more slowly on the metalaxyl-amended medium than the unadapted isolates. **(C)** Mutant 1mex6 and parental culture R1-5-58. Each data point is the mean of three measurements, diameters that were nearly identical.

contribute to the natural pool for selection. Zoosporogenesis is a process that may also yield colonies with new races, altered virulence (19), or variable metalaxyl sensitivity (9,14). Another factor contributing to variability can be differences in parts of a mycelial mass. The most rapid growth takes place at the hyphal tips of mycelia or germinating zoospores. The specific local environment can induce changes in gene expression.

Adaptation to low levels of a fungicide in the saprophytic phase may coincidentally select for traits advantageous in parasitic growth, as is shown here in the results with 11MZ-11 and as has been reported with other species of *Phytophthora* (3). Reduced elicitation of phytoalexins by metalaxyl-resistant mutants of *P. m. glycinea* in a normally resistant, metalaxyl-treated plant has been observed (4). Changes in gene expression of the fungus grown in the presence of metalaxyl are suggested by the mechanism of inhibition: different regulation of the activity of ribosomal RNA polymerase, reduced degradation of its product (ribosomal RNA), or a new isozyme of this enzyme, the direct target of metalaxyl inhibition (11).

A quantitative assay for in vivo tolerance of metalaxyl is important in analyzing the single zoospore colonies, their parents, and adapted isolates. None of the adapted isolates in our study displayed the degree of metalaxyl tolerance observed in the induced mutants selected for metalaxyl tolerance. Some metalaxyl mutants reportedly grow better in treated plants or in culture medium containing the fungicide than without metalaxyl (4), but that is not so for the adapted isolates 11MZ-11 and 11-7-M described in these studies. A screening for tolerance of 2 μg/ml metalaxyl in culture medium would not have picked up these metalaxyl-adapted isolates, which grow very poorly on lima bean agar containing metalaxyl but were virulent on metalaxyl-treated plants.

This work with metalaxyl, *P. m. glycinea*, and soybean differential cultivars Harosoy and Harosoy 63 resulted in two significant findings. First, after metalaxyl exposure in vitro, a new phenotype appeared, as characterized by virulence on Harosoy 63. We suggest that this occurred as selection pressure acting upon a preexisting mixed population even though the culture was derived from a single zoospore. Zoosporogenesis followed by analysis of single-spore progeny indicated that a potential for variation existed in the original isolate A11. Not all isolates showed a virulence change. The innate variability that exists in a *P. m. glycinea* isolate includes virulence or race specificity and variability with respect to sensitivity to metalaxyl on treated soybean cultivars. Furthermore, the cultivar has an impact on the level of the isolate's sensitivity to metalaxyl. Race change in *P. m. glycinea* following exposure to a chemical has not been reported. We defined race change here for simplicity as change in virulence on the differential cultivars Harosoy and Harosoy 63. Such a change was demonstrated here at a sublethal level of metalaxyl that can commonly occur in treated fields. The phenotype change was similar to that which is observed after zoosporogenesis. Second, a metalaxyl-adapted isolate with low in vitro tolerance and a very slow growth rate on culture media with metalaxyl colonized metalaxyl-treated plants. These results imply differential gene expression by the fungus during saprophytic and parasitic growth phases. Nutrient source has a major effect on the physiology of an organism. Metalaxyl probably has an effect on *P. m. glycinea* other than or in addition to the inhibition of a ribosomal RNA polymerase and reported increased elicitation of phytoalexins. This work suggests a need for more study of the physiological effects of metalaxyl, especially in the area of regulation of gene expression in planta and in vitro.

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