

# Effect of Metalaxyl on Sporulation and Growth of Metalaxyl-Resistant and Metalaxyl-Sensitive Isolates of *Phytophthora parasitica* In Vitro

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## ABSTRACT

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The in vitro production of sporangia by two metalaxyl-resistant isolates of *Phytophthora parasitica* in the absence of the fungicide was within the range of that by five sensitive isolates. Sporangium production by the resistant isolates was not affected by metalaxyl at 1, 10, or 100  $\mu\text{g/ml}$ , whereas that by three sensitive isolates was inhibited by as little as 1  $\mu\text{g/ml}$  of the fungicide. The in vitro production of chlamydospores by one of the resistant isolates (P-015F) was greater than that by three sensitive isolates in the absence of the fungicide, but chlamydospore production by the second resistant isolate (P-014F) was less than that by the sensitive isolates. Chlamydospore production by P-014F was not affected by metalaxyl at 1, 10, or 100  $\mu\text{g/ml}$ , whereas that by P-015F was inhibited at these concentrations. Chlamydospore production was inhibited by as little as 1  $\mu\text{g/ml}$  for the three sensitive isolates, but the degree of inhibition was generally greater than for P-015F. Linear growth of P-015F was stimulated by metalaxyl at concentrations up to 100  $\mu\text{g/ml}$  but was inhibited at 1000  $\mu\text{g/ml}$ . In contrast, mycelial dry weight in metalaxyl-amended liquid media was not affected at 1 or 10  $\mu\text{g/ml}$  but was inhibited at 100 and 1,000  $\mu\text{g/ml}$ .

Two isolates of *Phytophthora parasitica* Dastur from *Catharanthus roseus* (L.) G. Don in southern California were recently demonstrated to be resistant to metalaxyl in vitro (13) and in vivo (14). The mean  $\text{EC}_{50}$  values for inhibition of linear growth of these isolates exceeded 700  $\mu\text{g/ml}$  as compared to  $\text{EC}_{50}$  values ranging from 0.26 to 3.08  $\mu\text{g/ml}$  for 24 sensitive isolates (13). Metalaxyl has been shown to be highly inhibitory to various processes in the life cycles of sensitive isolates of *Phytophthora* spp. (2,5,8,10,12,17,25). In addition to inhibition of mycelial growth, the formation of sporangia, chlamydospores, and oospores is generally inhibited by low concentrations of the fungicide. However, insensitivity of mycelial growth to metalaxyl has not always been associated with insensitivity of sporangium production. For sensitive and tolerant isolates of *P. megasperma* Drechs. f. sp. *medicaginis* T. Kuan & D. C. Erwin, no relationship was observed between the levels of sensitivity to metalaxyl of mycelial growth and sporangium production (16). For *P. infestans* (Mont.) de Bary, inhibition of sporangium production was as great for two resistant isolates as for a sensitive isolate (9). The objective of this research was to further characterize the resistance to metalaxyl of the two isolates of *P. parasitica* by comparing the in vitro sporangium and chlamydospore produc-

tion by resistant and sensitive isolates in the presence and absence of metalaxyl and by comparing the sensitivity to metalaxyl of one of the resistant isolates on solid versus liquid media. A preliminary report of this research was published (15).

## MATERIALS AND METHODS

**Isolates.** Two metalaxyl-resistant isolates (P-014F and P-015F) and five metalaxyl-sensitive isolates (P-012F, P-048F, P-068F, P-075F, and P-076F) of *P. parasitica* from ornamental hosts from southern California were used in this study. Specific information on mating type, year of isolation, and hosts from which they were recovered is presented elsewhere (13).

**Sporangium production.** Sporangia were produced in liquid culture following the method of Chen and Zentmyer (6). For each concentration of metalaxyl (Subdue 2E, 25.1% a.i.), 20 15-cm-diameter plates containing 15 ml of clear, V8 juice-CaCO<sub>3</sub> (V8C) broth (20%) were inoculated with three 4-mm-diameter plugs from the leading edges of 5-day-old cultures on V8C agar. Plates were incubated in the dark at 25 C for 48 hr. At that time the broth was siphoned from the plates, which were then flooded with sterile deionized water (SDW) and allowed to stand for 15 min. The mycelial mats were washed four times in this manner. Following the final rinse, 10 ml of SDW or a solution of metalaxyl was added to each plate, and the plates were incubated under constant illumination at 25 C for an additional

48 hr. Mycelial mats bearing sporangia were rinsed once with SDW prior to further use, and mats from two plates were combined to give 10 replicates per treatment. Half of these mats were dried overnight in an oven at 80 C. The remaining mats were blended in 25 ml of SDW in a microblender for 30 sec. Sporangia were counted with hemacytometers; 20 counts were made for each suspension. The number of sporangia per milligram of dry weight of tissue was calculated.

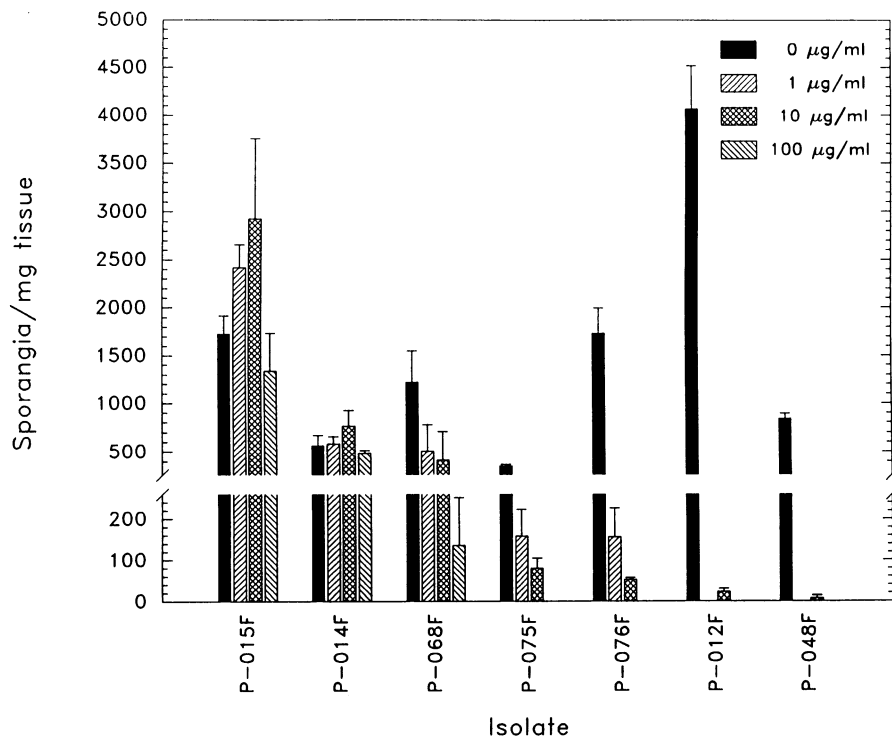
**Chlamydospore production.** Chlamydospores were produced by the method of Tsao (26). For each metalaxyl concentration tested, 10 500-ml prescription bottles containing 25 ml of V8C broth were inoculated with three 4-mm-diameter plugs from the leading edge of 5-day-old cultures on V8C agar. Following incubation in the dark at 25 C for 24 hr, the bottles were shaken vigorously to fragment the mycelium. The cultures were then incubated horizontally under the same conditions for an additional 6 days. The resulting mycelial mats were then submerged by the addition of 174 ml of SDW and 1 ml of a metalaxyl solution to give final metalaxyl concentrations of 0, 1, 10, or 100  $\mu\text{g/ml}$ . Following further incubation in the dark at 18 C for 14 days, the dry weight of each of the mats from five bottles per concentration was determined as described for sporangia. Chlamydospores were harvested from the remaining bottles and counted as described for sporangia. The number of chlamydospores per milligram of dry weight of tissue was calculated.

**Mycelial growth.** Mycelial growth of one of the metalaxyl-resistant isolates (P-015F) was examined on V8C agar and in V8C broth at metalaxyl concentrations of 0, 1, 10, 100, and 1,000  $\mu\text{g/ml}$ . The center of each of five V8C agar plates per concentration was inoculated with a 4-mm-diameter plug from the leading edge of a 5-day-old culture on V8C agar. Each of ten plates containing 15 ml of V8C broth amended with metalaxyl were inoculated with three 4-mm-diameter plugs. All plates were incubated in the dark at 25 C for 5 days. Three colony diameters were measured for each of the colonies on solid medium, and radial growth was calculated. The mycelial mats from the liquid cultures were dried overnight in an oven at 80 C, and mycelial dry weights were determined.

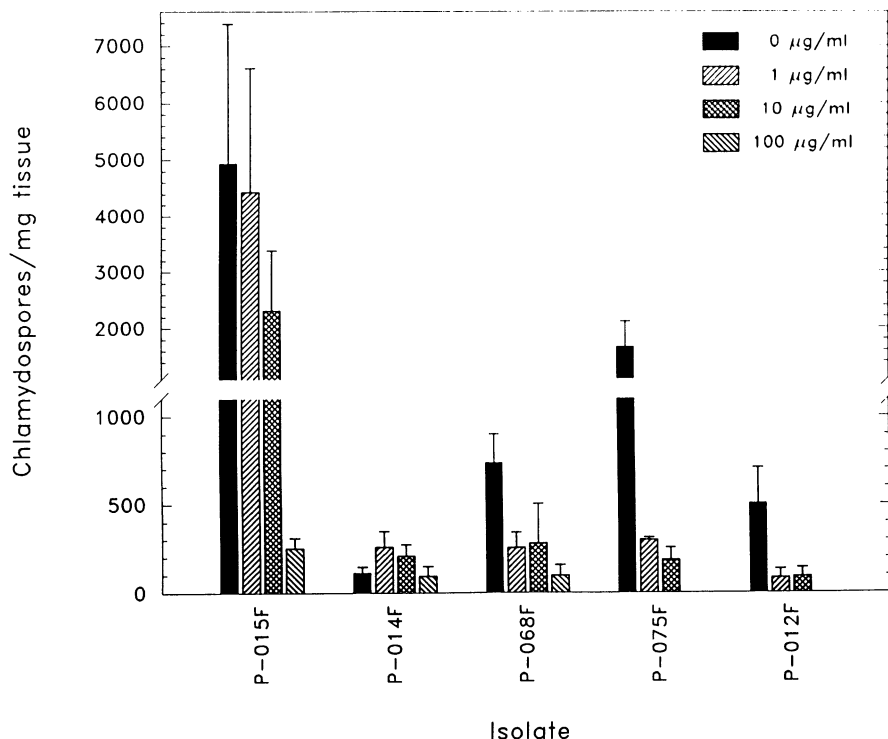
## RESULTS

**Sporangium production.** The mean numbers of sporangia produced per milligram of dry weight of mycelium in the presence and absence of metalaxyl by the seven isolates of *P. parasitica*

tested are shown in Figure 1. In the absence of the fungicide, the number of sporangia produced by the two metalaxyl-resistant isolates (P-014F and P-015F) was within the range of the number produced by the five metalaxyl-



**Fig. 1.** Effect of metalaxyl on the numbers of sporangia produced by two metalaxyl-resistant (P-014F and P-015F) and five metalaxyl-sensitive isolates of *Phytophthora parasitica* in vitro. The vertical lines represent the standard errors of the means of three to five experiments per isolate. Isolates P-012F and P-048F were not tested at 1 µg/ml, and isolates P-012F, P-048F, P-075F and P-076F were not tested at 100 µg/ml.



**Fig. 2.** Effect of metalaxyl on the number of chlamydo-spores produced by two metalaxyl-resistant (P-014F and P-015F) and three metalaxyl-sensitive isolates of *Phytophthora parasitica* in vitro. The vertical lines represent the standard errors of the means of three to five experiments per isolate. Isolates P-075F and P-012F were not tested at 100 µg/ml.

sensitive isolates. Although not statistically significant ( $P < 0.05$ ), mean sporangium production by the two resistant isolates in the presence of metalaxyl was 9.3 and 46% greater than that of the control at 1 µg/ml and 47 and 62% greater than that of the control at 10 µg/ml, but it was 4.6 and 22% less than the control at 100 µg/ml. Mean sporangium production by the sensitive isolates was reduced 49–92% and 62–99% by metalaxyl at 1 and 10 µg/ml, respectively.

**Chlamydo-spore production.** The mean numbers of chlamydo-spores produced per milligram of dry weight of tissue in the presence and absence of metalaxyl by the five isolates of *P. parasitica* tested are shown in Figure 2. In the absence of the fungicide, one of the resistant isolates (P-015F) produced significantly ( $P < 0.05$ ) more chlamydo-spores than the three sensitive isolates, whereas the second resistant isolate (P-014F) produced significantly fewer chlamydo-spores than the sensitive isolates. Mean chlamydo-spore production by P-014F was 168 and 106% greater than the control at metalaxyl concentrations of 1 and 10 µg/ml, respectively, but was 21% less than the control at 100 µg/ml. Mean chlamydo-spore production by P-015F and the three sensitive isolates was inhibited by metalaxyl at all concentrations tested. However, mean inhibition of chlamydo-spore production by P-015F was 10 and 52% of that of the control at 1 and 10 µg/ml, respectively, whereas for the sensitive isolates it ranged from 66 to 83% at 1 µg/ml and from 71 to 86% at 10 µg/ml.

**Mycelial growth.** Mycelial growth of the metalaxyl-resistant isolate P-015F was more sensitive to the fungicide in liquid than on solid media. Linear growth on solid media increased as the metalaxyl concentration was increased from 0 to 100 µg/ml but decreased at 1,000 µg/ml. Linear growth was 6.1, 15, and 36% greater than that of the control at concentrations of 1, 10, and 100 µg/ml, respectively, but was 60% less than the control at 1,000 µg/ml (Table 1). In contrast, growth of this isolate in liquid culture, as measured by mycelial dry weight, was not affected by metalaxyl at concentrations of 1 or 10 µg/ml but was 77 and 98% less than the control at 100 and 1,000 µg/ml, respectively.

## DISCUSSION

The in vitro sensitivity to metalaxyl among isolates and species of *Phytophthora* has been studied extensively (2,5,7,8,9,10,11,12,13,17,20,23,25). Intraspecific variation in response to metalaxyl has been observed among sensitive isolates of many species of *Phytophthora* (7,8,13,16,23). Farhi et al (12) demonstrated that metalaxyl inhibited processes at virtually all stages of the life cycle of an isolate of *P. para-*

*sitica* from citrus. Linear growth of this isolate was inhibited by 92 and 94%, sporangium production was inhibited by 93 and 93%, and chlamyospore production was inhibited by 66 and 67% at 1 and 10  $\mu\text{g/ml}$ , respectively. However, linear growth of an isolate of *P. parasitica* from tomato was inhibited by 53 and 85%, and sporangium production was inhibited by 59 and 86%, at 1 and 10  $\mu\text{g/ml}$ , respectively (17).

Studies on the assessment of resistance of soilborne species of *Phytophthora* and *Pythium* to metalaxyl have primarily addressed the lack of inhibition of mycelial growth in vitro and the failure of the fungicide to control disease in vivo (4,6,11,13,14,18,21,22). Relatively few studies have addressed the in vitro effects of metalaxyl on sporulation by resistant isolates. For *P. m. medicaginis*, Stack and Millar (24) reported that a metalaxyl-insensitive isolate did not differ from wild type-sensitive isolates in total growth, growth rate, sporulation, propagule germination, or pathogenicity. However, insensitivity to metalaxyl, as measured by linear growth on fungicide-amended media, has not always been associated with insensitivity of sporangium production. No relationship was found between the tolerance levels of isolates of *P. m. medicaginis* to metalaxyl and inhibition of sporangium formation in the presence of the fungicide (16). For *P. infestans*, linear growth of a sensitive isolate was inhibited by 69% at 1  $\mu\text{g/ml}$ , and that of the resistant isolates was inhibited by only 31 and 36% at 150  $\mu\text{g/ml}$ ; however, sporangium production by the sensitive isolate was inhibited by 76% at 1  $\mu\text{g/ml}$ , and that by the resistant isolates was inhibited by 69 and 85% at this concentration (9). In contrast, insensitivity to metalaxyl of mycelial growth of the two resistant isolates of *P. parasitica* from *C. roseus* was also observed for in vitro production of sporangia. Inhibition of linear growth of three sensitive isolates was 57, 45, and 70% at 1  $\mu\text{g/ml}$ , whereas the two resistant isolates were not inhibited at this concentration. Likewise, sporangium production at 1  $\mu\text{g/ml}$  was 49, 58, and 92% less than the controls for three

sensitive isolates but was 9.3 and 46% greater than the controls for the two resistant isolates.

In this study, chlamyospore production by one of the resistant isolates of *P. parasitica* (P-015F) was not affected by metalaxyl at 1, 10, or 100  $\mu\text{g/ml}$ , whereas that by the second isolate (P-014F) was inhibited at these concentrations. However, the degree of inhibition for the latter isolate was generally less than for the sensitive isolates.

Observed changes in colony morphology of the resistant isolates in the presence of metalaxyl, along with the stimulation of growth, prompted examination of the sensitivity of one of these isolates to metalaxyl in liquid media. This isolate was found to be more sensitive to the fungicide in liquid than on solid media at concentrations of 10, 100, and 1000  $\mu\text{g/ml}$ , although a high level of resistance was still expressed. The increase in linear growth observed on metalaxyl-amended agar media was not associated with an increase in biomass of the fungus in liquid culture. Thus, although determination of the  $\text{EC}_{50}$  for linear growth on solid media may be useful for the identification of resistance, it may not provide an accurate assessment of the true level of resistance expressed in vivo.

Given the levels of production of sporangia and chlamyospores in the absence of the fungicide, the metalaxyl-resistant isolates of *P. parasitica* appear to be as fit as the sensitive isolates tested. However, sporulation capacity is only one aspect of the fitness of resistant isolates relative to sensitive ones. Additional factors affecting the fitness of metalaxyl-resistant isolates have been observed. In one case, a metalaxyl-resistant isolate of *P. capsici* Leonian was found to survive better in soil than a sensitive isolate (3). Additionally, zoospore release by metalaxyl-resistant isolates of *P. infestans* was faster than for sensitive isolates (1). For these same isolates, no difference in sporulation capacity was observed, although the resistant isolates produced larger lesions than the sensitive isolates (19). Thus, factors contributing to the fitness of

metalaxyl-resistant isolates can occur at many different stages of the life cycle of the pathogen or the disease cycle. Studies are currently underway to assess the in vivo fitness and competitiveness of one of the metalaxyl-resistant isolates of *P. parasitica*.

#### ACKNOWLEDGMENT

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**Table 1.** Effect of metalaxyl on mycelial growth of a resistant isolate of *Phytophthora parasitica* (P-015F) on solid and in liquid media<sup>a</sup>

Concentration ( $\mu\text{g/ml}$ )	Linear growth		Biomass	
	Radius (mm) <sup>b</sup>	Percent of control	Dry weight (mg) <sup>b</sup>	Percent of control
0	23.1	...	84.7	...
1	24.5	106	85.0	100
10	26.5	115	85.9	101
100	31.2	135	20.0	24
1,000	9.2	40	2.0	2.4

<sup>a</sup>Cultures were grown for 5 days in the dark at 25 C on metalaxyl-amended clear V8 juice-CaCO<sub>3</sub> (V8C) agar and in metalaxyl-amended V8C broth.

<sup>b</sup>Values are the means of three experiments each with five and 10 replicate plates per concentration for determination of the mean radius and dry weight of colonies, respectively.

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