# In Vitro Effects of Metalaxyl on Growth, Sporulation, and Germination of *Phytophthora parasitica* and *P. citrophthora*

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**ABSTRACT**

Farih, A., Tsao, P. H., and Menge, J. A. 1981. In vitro effects of metalaxyl on growth, sporulation, and germination of *Phytophthora parasitica* and *P. citrophthora*. Plant Disease 65:651-654.

Metalaxyl at low concentrations was highly inhibitory to mycelial growth and formation of sporangia, chlamydospores, and oospores of *Phytophthora parasitica* and/or *P. citrophthora*. Chlamydospore germination was sensitive to low concentrations of metalaxyl, but zoospore germination was not greatly reduced. With both spore types, however, germ tube growth was highly sensitive to metalaxyl. Metalaxyl therefore controls the diseases caused by these *Phytophthora* fungi by affecting the pathogens at all stages of their life cycle.

Additional key words: spore germination

The new systemic fungicide metalaxyl (Ridomil, Subdue) has been effective in the control of diseases caused by *Phytophthora* spp. and certain other oomycetes under field or greenhouse conditions (8,14–16). The biological and chemical mechanisms for its efficacy have not yet been determined, however. Studies on its in vitro toxicity toward target oomycetous fungi were generally incomplete (3,6,7,16), except those on *Phytophthora cinnamomi* (1) and *P. parasitica* var. *nicotianae* (T. R. Young, *personal communication*).

Metalaxyl controls fruit, stem, and/or root infections of citrus by *P. parasitica* and *P. citrophthora* (4,7,9) and drastically reduces or eliminates *P. parasitica* populations in treated soils (4). Our studies were undertaken to investigate the in vitro effects of this new fungicide on the different stages in the life cycles of these two *Phytophthora* spp. in an attempt to elucidate the biological mechanism of control.

## MATERIALS AND METHODS

**Fungi.** *P. parasitica* Dast., isolate T131, and *P. citrophthora* (R. E. Sm. & E. H. Sm.) Leonian, isolate T544, both pathogenic on citrus, were used in this study.

**Fungicide solutions.** We used the liquid metalaxyl formulation 2EC (240 g a.i./L). Stock solutions of the fungicide were prepared by dissolving metalaxyl in sterile distilled water in appropriate concentrations and then adding each to water, broth, or agar medium to reach the final test concentrations. Metalaxyl did not appreciably alter the pH of V8 agar medium (pH 5.9) even at 1,000 mg/L. Metalaxyl at 10 and 100 mg/L lowered the pH of water from 6.7 to 5.3 and 5.0, respectively.

**Mycelial growth.** Toxicity of the fungicide toward linear mycelial extension was measured by adding metalaxyl to cleared V8-$CaCO_3$ (V8) agar medium (Campbell V8 juice, 100 ml; 2% $CaCO_3$, 100 ml; agar, 15 g; deionized water, 800 ml) after autoclaving. Inoculum plugs (4 mm diameter) were taken from the margin of a 4- to 5-day-old colony grown on V8 agar, and each was placed at the edge of a plate with a fungicide incorporated. Plates were incubated at 25 C in the dark, and linear measurements were taken from the edge of the inoculum plug.

**Sporangium formation.** The previously described method (12) for producing *P. parasitica* sporangia was used and applied also to *P. citrophthora* with some modifications. Briefly, mycelial mat colonies were established in V8 broth (11) on nylon mesh squares; then each colony was placed in a 60-mm glass petri dish containing 2 ml of water (control) or fungicide solution and then incubated for 2–3 days under light (for *P. parasitica*) or in the dark (for *P. citrophthora*) for sporangium formation.

**Sporangium germination.** Sporangia were produced by the method similar to that used for sporangium formation. One-day-old nylon mesh square cultures were covered with 0.3 ml of mineral salt solution (2) instead of water (to prevent premature zoospore release) and incubated for 2–3 days as described. Each nylon mesh square supporting a sporangium-bearing mat was briefly rinsed in water and immediately placed in a 60-mm glass petri dish containing 2 ml of water or fungicide solution. Petri dishes were incubated at 25 ± 1 C for 30 min to allow toxicants to act on the sporangia (containing undifferentiated protoplasm) and then at 18 C for 90 min to induce indirect sporangium germination, ie, zoospore formation and release.

**Zoospore germination.** Zoospores were obtained from sporangia produced as described for sporangium formation but without the nylon mesh squares. Concentrations of zoospores were adjusted to about 3,000/ml and 0.3 ml was transferred, in drops, onto each of three replicate V8 agar plates containing the fungicide or water. After 7–8 hr of incubation in the dark at 25 C, germination was terminated by placing plates at 1 C before reading. A spore was considered germinated if the germ tube length exceeded its width. One-hundred spores from each replicate plate were counted.

**Chlamydospore formation.** For chlamydospore formation of *P. parasitica*, the mycelium was first grown at 25 C for 7 days in 16-oz prescription bottles, each containing 25 ml of cleared V8 broth. To each bottle, 125 ml of water or 110 ml of water and 15 ml of fungicide (×10 final concentration) were added, and bottles were incubated vertically in the dark at 18 C for 3 wk (11). The chlamydospore-bearing mycelial mats were blended for 3 min in 50 ml of water in a Sorvall Omnimixer, and the blended suspensions were kept at 1 C before chlamydospores were counted. *P. citrophthora* could not be induced to form chlamydospores by this method and was therefore not included in this phase of our study.

**Chlamydospore germination.** Holdaway and Tsao's method (5) as modified

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Accepted for publication 18 November 1980.

0191-2917/81/08065104/$03.00/0

**Table 1.** Effect of metalaxyl incorporated in V8 agar medium on linear mycelial growth of *Phytophthora parasitica* and *P. citrophthora*

| Metalaxyl (mg/L) | Linear mycelial growth (mm)[z] | | | |
|---|---|---|---|---|
| | *P. parasitica* | | *P. citrophthora* | |
| | At 3 days | At 7 days | At 3 days | At 7 days |
| 0 | 12.4 a | 36.8 a | 24.7 a | 63.1 a |
| 0.1 | 8.5 b | 11.5 b | 17.1 b | 53 b |
| 0.2 | 4.2 c | 6.1 c | 11.7 c | 45.9 c |
| 0.4 | 2.4 d | 3.7 d | 5.8 d | 33.3 d |
| 0.8 | 1.3 def | 2.7 de | 2.6 e | 26.8 e |
| 1 | 1.7 de | 3 de | 1.4 f | 24.8 e |
| 10 | 1.3 def | 2.3 de | 1 fg | 16.8 f |
| 100 | 1 ef | 2 e | 1 fg | 10.8 g |
| 1,000 | 0 f | 0 f | 0 g | 0 h |

[z] Each number is an average of three replicates. Figures with same letter in each column are not significantly different ($P = 0.05$, Duncan's multiple range test).

**Table 2.** Effect of metalaxyl on sporangium formation, indirect sporangium germination (zoospore formation and release), and zoospore germination of *Phytophthora parasitica* (*Pp*) and *P. citrophthora* (*Pc*)

| Metalaxyl (mg/L) | (*Pp*) | (*Pc*) |
|---|---|---|
| | Sporangia (no./mm$^2$)[w,x] | |
| 0 | 67.7 a | 113.2 a |
| 0.1 | 10 b | 37.3 b |
| 1 | 4.5 bc | 1.8 c |
| 10 | 4.6 bc | 1.6 c |
| 100 | 6.9 bc | 0.8 c |
| 500 | 0 c | 0 c |
| 1,000 | 0 c | 0 c |
| | Indirect sporangium germination (%)[y] | |
| 0 | 100 a | 100 a |
| 1 | 106.2 a | 107 a |
| 10 | 92.3 a | 124.5 a |
| 100 | 61 b | 62.1 ab |
| 1,000 | 2.3 c | 18.4 b |
| | Zoospore germination (%)[x,z] | |
| 0 | 100 a | 100 a |
| 1 | 88 bc | 91 a |
| 10 | 81.8 c | 100 a |
| 100 | 93.1 ab | 48.5 b |
| 1,000 | 0 d | 0 c |

[w] Each number is an average of three replicates.
[x] Figures with same letter in each column are not significantly different ($P = 0.01$, Duncan's multiple range test).
[y] Each number is an average of three replicates and is expressed as percent of the appropriate water control (0 mg/L). The actual percent germination of control was 73.7 for *Pp* and 34.3 for *Pc*. The percent germination at 0 hr was 6.3 for *Pp* and 4.3 for *Pc*. Figures with same letter in each column are not significantly different ($P = 0.05$, Duncan's multiple range test).
[z] Each number is an average of three replicates and is expressed as percent of the control (0 mg/L). The actual percent germination of control was 97.7 for *Pp* and 88.7 for *Pc*.

by Tsao and Oster (12) was used for chlamydospore harvest and germination. The procedure described for zoospore germination was also used in chlamydospore germination tests except that the incubation period was 16–18 hr. Viability was generally 90–95%.

**Oospore formation.** A 12-mo-old, highly oosporogenic culture (13) of *P. parasitica* isolate T131 was first grown for 7 days on V8 agar medium to produce inoculum. By this time the entire agar plate area was covered by oospores. Inoculum plugs (4 mm diameter) were taken from areas 10 mm away from the center of the colony and placed in the center of each test plate of V8 agar containing the fungicide. Plates were incubated in the dark at 25 C for 7 days before oospores were counted. *P. citrophthora* does not form oospores and was not included in this phase of the study.

**Methods for reading spore formation.** *Sporangium formation.* Sporangia were counted in five ×125 microscope fields (2.57 mm$^2$ per field) and are reported as number per square millimeter.

*Chlamydospore formation.* A portion of the blended suspension of the chlamydospore-bearing mat was diluted to a known volume and an aliquot was used for counting spores on a Hawksley counting slide (Gelman-Hawksley, Lansing, Sussex, England). Data are reported as number of chlamydospores formed per culture.

*Oospore formation.* Oospore formation was assessed by two methods: 1) The margins of mycelial growth of the colony and the area of oospore formation were delineated with a pen marker on each plate under the stereoscopic microscope and then traced on transparent paper. The area size was determined by following the traced outline with a polar-compensating planimeter (Salmoiraghi 236A, Lietz No. 3651-00, Filotecnica Salmoiraghi, S.P.A., Milano, Italy), which gives direct measurements in square centimeters. Data are reported as the percentage of the area of mycelial growth containing oospores. 2) Three fields representative of the oospore formation area were chosen under a stereoscopic microscope. In each field, 10 (258 × 258 μm) squares on the ocular reticle, which contains 100 squares, were chosen randomly and oospores counted. Data are reported as number of oospores formed per square millimeter.

All experiments were repeated at least once, with or without modifications in treatments.

## RESULTS

**Mycelial growth.** Metalaxyl was highly toxic to the mycelial growth of both *Phytophthora* spp. (Table 1). The $ED_{50}$ values were 0.15 and 0.18 mg/L after 3 days and 0.04 and 0.56 mg/L after 7 days for *P. parasitica* and *P. citrophthora*, respectively.

**Sporangium formation.** Metalaxyl at 0.1 mg/L resulted in 85 and 67% inhibition of sporangium formation of *P. parasitica* and *P. citrophthora*, respectively (Table 2). Inhibition was complete only at concentrations above 100 mg/L. In a repeat experiment, metalaxyl at 0.1 mg/L reduced sporangium formation by 51 and 42% for *P. parasitica* and *P. citrophthora*, respectively.

To test the possibility that inhibition of sporangium formation might be due to a pH effect, an experiment using *P. parasitica* sporangia was conducted in which the water control and the metalaxyl solutions of 10, 100, and 500 mg/L were prepared each with and without the solutions adjusted to pH 6.5 by adding KOH or HCl. When pH was not adjusted, the inhibition in the three concentrations was 59, 93, and 100%, respectively, and that of the pH-adjusted series was 75, 83, and 100%, respectively. Inhibition of sporangium formation by metalaxyl, therefore, was probably a direct fungitoxic effect and not an indirect pH effect.

**Sporangium germination.** Metalaxyl at 1–10 mg/L did not inhibit indirect sporangium germination (zoospore formation and release) of *P. parasitica* and *P. citrophthora*. At higher concentrations, sporangium germination was reduced by more than 37 and 81% at 100 and 1,000 mg/L, respectively (Table 2). In a repeat experiment, metalaxyl at 1–10 mg/L also did not inhibit sporangium germination, but 100 mg/L or higher concentrations resulted in 46% or greater inhibition.

**Zoospore germination.** Metalaxyl at 100 mg/L or lower concentrations did not greatly inhibit zoospore germination of *P. parasitica*. Zoospore germination of *P. citrophthora* was not inhibited at 10 mg/L but was reduced by more than 50% at 100 mg/L. At 1,000 mg/L inhibition of zoospore germination was complete in both fungi (Table 2). Germinated zoospores on fungicide-free agar produced 330 and 116 μm germ tubes for *P. parasitica* and *P. citrophthora*, respectively, after 8-hr incubation. Although lower concentrations of metalaxyl did not inhibit zoospore germination, germ tube length was reduced by 80 and 57% for *P. parasitica* and *P. citrophthora*, respectively, even at 1 mg/L. In a repeat experiment, results were similar for *P. parasitica*, but 100 mg/L of metalaxyl reduced zoospore germination of *P.*

**Table 3.** Effect of metalaxyl on the formation and germination of chlamydospores of *Phytophthora parasitica*

| Metalaxyl (mg/L) | Formation (no. of chlamydospores $\times 10^{-3}$/culture)[x,y] | Germination (%)[y,z] |
|---|---|---|
| 0 | 875 a | 100 a |
| 0.1 | 440 b | 79.4 b |
| 1 | 294 c | 42.2 c |
| 10 | 291 c | 39 c |
| 50 | ··· | 21 d |
| 100 | 0 d | 8.6 de |
| 1,000 | 0 d | 0 e |

[x] Each number is an average of readings from three replicate bottles of liquid culture.
[y] Figures with same letter in each column are not significantly different ($P = 0.01$, Duncan's multiple range test).
[z] Each number is an average of readings from three replicate plates of V8 agar medium and is expressed as percent germination of control and of viable spores. Viability of spores used was 93%. The actual percent germination of control was 83.5.

**Table 4.** Effect of metalaxyl on oospore formation of *Phytophthora parasitica*[y]

| Metalaxyl (mg/L) | Area ($cm^2$) | | Mycelial growth area containing oospores (%)[z] | Oospores (no./$mm^2$)[z] |
|---|---|---|---|---|
| | Mycelial growth | Oospore formation | | |
| 0 | 23.5 | 11.9 | 50.8 a | 19.4 a |
| 0.01 | 31.5 | 15 | 47.5 a | 16.8 ab |
| 0.02 | 27.3 | 4.5 | 17.3 b | 15.2 ab |
| 0.04 | 16.1 | 1.3 | 8.9 b | 4.9 c |

[y] Results 7 days after growing an oosporogenic culture on V8 agar medium in the dark at 25 C.
[z] Figures with same letter in each column are not significantly different ($P = 0.05$, Duncan's multiple range test).

*citrophthora* by only 29%.

**Chlamydospore formation.** Metalaxyl at 0.1 mg/L reduced *P. parasitica* chlamydospore formation in liquid culture by approximately 50%. At 100 mg/L, complete inhibition of chlamydospore formation occurred (Table 3). Results were similar in a repeat experiment.

**Chlamydospore germination.** On fungicide-free agar medium, *P. parasitica* chlamydospores germinated and produced a germ tube 1,600 μm or longer after 16–18 hr of incubation. Chlamydospore germination was inhibited by 58% and germ tube length was reduced by 71% with metalaxyl at 1 mg/L. However, inhibition of chlamydospore germination was 91% at 100 mg/L (Table 3). In a repeat experiment, metalaxyl at 1 mg/L reduced chlamydospore germination by 44%.

**Oospore formation.** The oosporogenic culture of *P. parasitica* grown on fungicide-free agar produced oospores that covered 50% of mycelial growth area. Metalaxyl at 0.02 and 0.04 mg/L reduced the area containing oospores to 17.3 and 8.9%, respectively (Table 4). Oospore density (number per unit area) was also greatly reduced at 0.04 mg/L (Table 4). In a repeat experiment, metalaxyl at 0.04 mg/L completely inhibited oospore formation.

## DISCUSSION

Our studies have confirmed the reports of various workers that low concentrations of metalaxyl inhibit mycelial growth of *Phytophthora* spp. The $ED_{50}$ value for linear mycelial growth of *P. cinnamomi* is 0.11 mg/L (1) and for *P. parasitica* var. *nicotianae*, 0.2 mg/L (T. R. Young, *personal communication*). Kellili (7) found that metalaxyl at 1 mg/L completely inhibited linear mycelial growth of *P. citrophthora*. At 7 days, metalaxyl was 13 times more inhibitory to linear extension of *P. parasitica* than to that of *P. citrophthora*. However, *P. parasitica* appeared to become more sensitive to metalaxyl, but *P. citrophthora* became less sensitive to the fungicide as the experiment progressed.

As reported for other *Phytophthora* spp. (1; T. R. Young, *personal communication*), metalaxyl at very low concentrations was highly inhibitory to sporangium formation of *P. parasitica* and *P. citrophthora*. However, metalaxyl was not highly inhibitory to sporangium germination of *P. parasitica* and *P. citrophthora*, a finding similar to other results obtained with *P. parasitica* var. *nicotianae* (T. R. Young, *personal communication*). Cohen et al (3), however, found that metalaxyl at 50 mg/L reduced zoospore release of *P. infestans* by about 80%.

Although metalaxyl inhibits zoospore germination only slightly, zoospore germ tube growth was highly sensitive to the fungicide even at low concentration (1 mg/L). These results are similar to the findings on *P. parasitica* var. *nicotianae* (T. R. Young, *personal communication*) and *P. infestans* (3).

As with sporangium formation, chlamydospore formation of *P. parasitica* was highly sensitive to metalaxyl at low concentrations. Benson (1) reported similar results with *P. cinnamomi*. As with *P. parasitica* var. *nicotianae* (T. R. Young, *personal communication*), chlamydospore germination of *P. parasitica* was highly sensitive to metalaxyl even at 1 mg/L.

Before our study, there had not been any report on the effect of metalaxyl on oospore formation. Our tests with *P. parasitica* showed for the first time that metalaxyl at very low concentration (0.04 mg/L) reduced both the area of oospore formation and the density of oospores produced.

Our studies also showed that metalaxyl can control Phytophthora diseases by affecting the pathogens at any or all stages of their life cycle. It may affect the germination of chlamydospores, one of the primary inocula in soil, and their germ tube development, thus reducing the potential of initial infection. After infection, metalaxyl may inhibit mycelial growth in infected tissues, thus reducing the rate of colonization. It may also inhibit sporangium formation from infected tissues or infested debris, thus reducing or preventing the production of secondary inoculum and the rate of dissemination. Finally, it may reduce or prevent the formation of chlamydospores and oospores, generally considered as the propagules responsible for long-term survival (10), and thus decrease the inoculum density of *Phytophthora* in the plant tissue, debris, or soil.


### ACKNOWLEDGMENTS

We thank J. W. Eckert for advice, J. J. Oster for assistance, and CIBA-GEIGY Corp. for supplying metalaxyl.


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