Disease Control and Pest Management

Fungitoxicity of Metalaxyl against *Phytophthora parasitica* var. *nicotianae*

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ABSTRACT

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The biological mode of action of metalaxyl (CGA-48988) against *Phytophthora parasitica* var. *nicotianae* was investigated on agar and on tobacco seedlings in petri dishes. The ED_{50} for mycelial growth on V8-juice agar was 0.2 μg/ml. On water agar the formation of sporangia from chlamydospores was completely blocked by a concentration of 1 μg/ml. In the petri dish-tobacco seedling system, 0.2 μg/ml CGA-48988 prevented the formation of sporangia both from chlamydospores and on infected tobacco roots. When sporangia or zoospores were used as inoculum for tobacco seedlings, CGA-48988 did not inhibit germination or penetration processes; however, fungus development within treated roots was completely blocked by a concentration of 1 μg/ml. Protective and therapeutic action against black shank in tobacco seedlings was exhibited at 1.0 μg/ml of CGA-48988.

Additional key words: CGA-48988, *Nicotiana tabacum*, *Phytophthora nicotianae* var. *nicotianae*.

Black shank of tobacco, which is caused by *Phytophthora parasitica* (Dast.) var. *nicotianae* (Breda de Haan) Tucker (= *P. nicotianae* Breda de Haan var. *nicotianae* Waterhouse) (15), continues to be a serious threat to tobacco crops in the United States in spite of efforts to improve disease resistance and cultural practices (6). Chemical treatments for black shank control have depended principally upon the use of preplant soil fumigants (6). Recently, a number of selective fungicides effective for controlling black shank became available for experimentation (1,7–9). One of these compounds, metalaxyl (CGA-48988), is active systemically against air- and soilborne fungi of the order Peronosporales (12,14)

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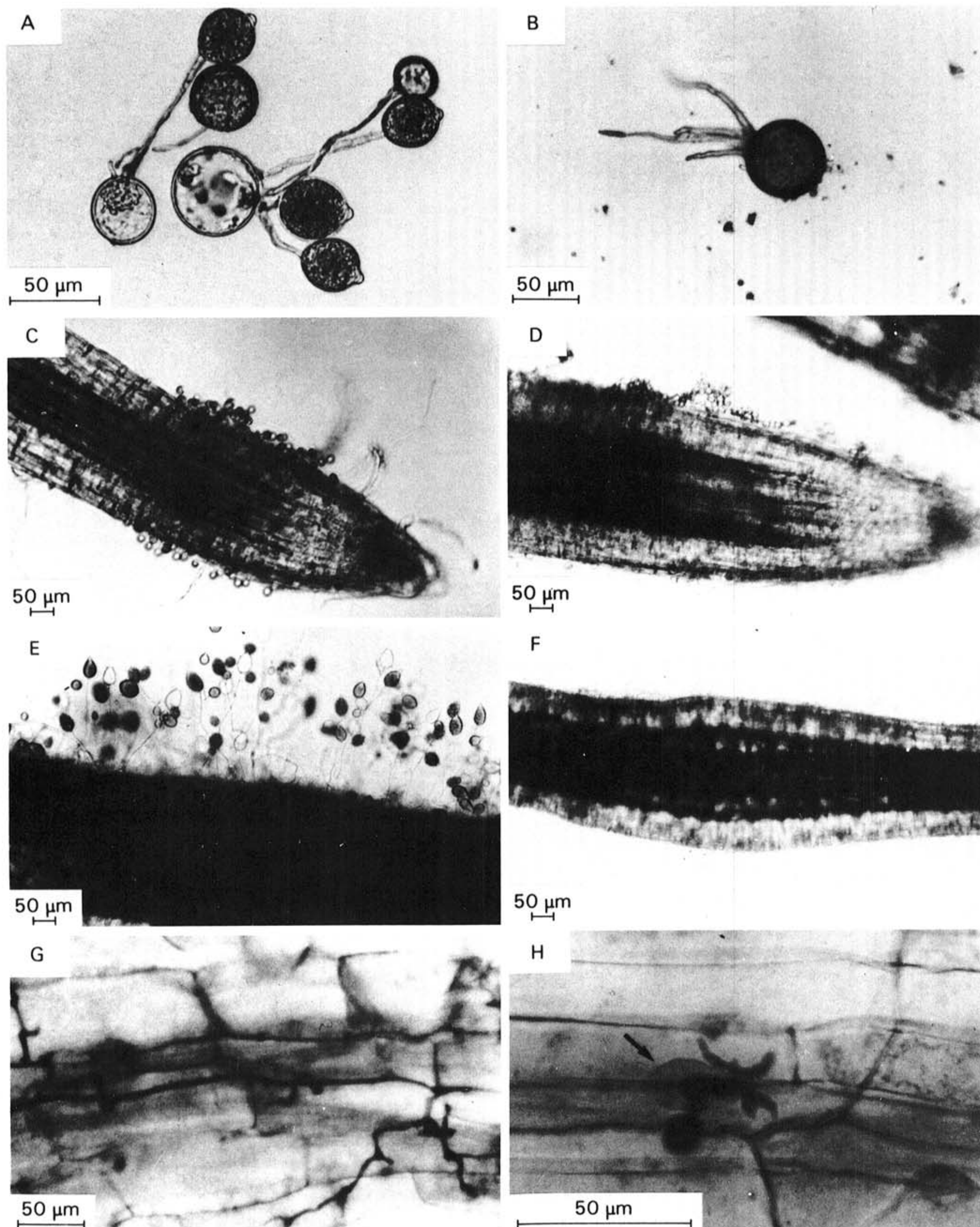

Fig. 1. Photomicrographs of the effects of metalaxyl (CGA-48988) on *Phytophthora parasitica* var. *nicotianae*. (A and B) Germinated chlamydospores on water agar after 24 hr of incubation: **A,** control treatment depicting germ tubes with newly formed sporangia, **B,** 10 ppm CGA-48988 with germ tubes lacking sporangia. (C and D) Tobacco root tips 1 hr after 5,000 zoospores per milliliter were added to the surrounding solution: **C,** Control; **D,** 10 μg/ml CGA-48988. In both C and D the zoospores had attached and encysted behind the root cap. (E and F) Root tips 24 hr after inoculation with zoospores (5,000/ml): **E,** Control, with abundant sporangium formation; **F,** 1 μg/ml CGA-48988 without sporangium formation. Roots that received both treatments appear to be swollen. (G and H) Whole mounts of infected tobacco roots: **G,** Control 48 hr after inoculation with 5,000 zoospores per milliliter, **H,** 10 μg/ml CGA-48988 72 hr after inoculation with zoospores (5,000/ml). Note large callosities in the cells adjacent to the infection site (arrow).

and it provides excellent control of black shank at low rates applied either broadcast or in transplanting water (5,8,9). In this study we determined the effects of CGA-48988 on the in vitro and in vivo development of *P. parasitica* var. *nicotianae*.

MATERIALS AND METHODS

Chemical. The fungicide used in this study was CGA-48988 (*N*-[2,6-dimethylphenyl]-*N*-[2-methoxyacetyl]-alanine methyl ester). It was formulated as a 25% a.i. wettable powder. All concentrations are given as active ingredient (a.i.).

Inoculum. Isolate PPN 5 of *P. parasitica* var. *nicotianae* race O, obtained from D. J. Mitchell (University of Florida, Gainesville, FL) was used for all studies. Stock cultures were maintained on V8-juice agar. For chlamydospore production the fungus was grown for 10 days in 10% (v:v) V8-juice broth at 28 C. Chlamydospore formation was induced by transferring the mycelial mats to sterile distilled water for an additional 7 days. Chlamydospore suspensions were prepared by homogenizing the sporulating mycelial mats in a tissue grinder and passing the homogenate through four layers of cheesecloth to remove mycelial fragments. Spore suspensions prepared in this way usually contained about 10% sporangia and 90% chlamydospores.

Sporangia were produced from chlamydospores by incubation for 48 hr in 6-cm-diameter petri dishes containing sterile water at 25 C. To induce zoospore release the petri plates were kept at 4 C for 30 min, then at 25 C for 1 hr. After this incubation large numbers of swarming zoospores could be collected by pipetting the surface layer of water.

Experimental procedure. The effect of CGA-48988 on the growth and development of the fungus was tested in vitro. Aliquots of stock concentrations of CGA-48988 prepared in sterile distilled water were added to melted V8-agar or water agar to give concentrations ranging 0.1 to 100 μg/ml. Fifteen milliliters of the treated agar was added to each petri dish. Mycelial inoculum was added to the dishes of V8-juice agar to determine the concentration of the chemical which would provide 50% growth inhibition (ED_{50}). Chlamydospore or zoospore inoculum was added to the dishes of water agar to determine the effect of CGA-48988 on spore germination. All in vitro testing was conducted at 28 C and with four replicates per concentration.

In vivo tests were conducted in the lab by using a petri dish-tobacco seedling system. The spore types used in the various experiments were added to 6-cm-diameter petri plates containing 6 ml of sterile distilled water and CGA-48988 at 0.2, 1.0, 4.0, or 20 μg/ml. Tobacco plants, cultivar Hicks (Coker Seed Company, Hartsville, SC 29550), were grown in an organic potting soil. When the plants had three to four true leaves and were about 4 cm in height, they were removed from the potting soil, the roots were washed gently, and they were immersed in nontreated water for 3–4 days to permit production of uninjured root tips. Unless otherwise mentioned, CGA-48988 and the tobacco seedlings were added to petri plates 1 hr prior to the addition of spores.

Fungal development in the treated water and on tobacco roots, and disease progress, could be observed conveniently in seedlings in the petri dishes. Microscopic observations on spore germination were either made directly in the petri plates under low power or by examining 25-μl droplets on slides under the microscope. Zoospore clustering and formation of sporangia on tobacco roots in the petri plates also could be observed directly under low power. For microscopic observations on fungus development inside tobacco roots, whole root pieces were stained and cleared by the technique described by Shipton and Brown (11) and Heath (4) except that we used trypan blue instead of cotton blue to stain the fungus. Stained specimens were cleared in chloral hydrate for 1–5 days.

RESULTS

Agar incorporation tests. The radial growth rate of the fungus was 7.6 mm/day, on nontreated V8-agar. The respective growth rates on V8-agar containing 0.1, 1, 10, and 100 μg/ml CGA-48988 were 5.6, 0.8, 0.4, and 0.4 mm/day, resulting in an ED_{50} value of about 0.2 μg/ml for the inhibition of mycelial growth (determined graphically on semilogarithmic paper).

Chlamydospore germination on water agar was reduced 53% by 1 μg/ml CGA-48988 and 69% by 100 μg/ml CGA-48988 (Table 1). Zoospore germination was unaffected by dosages up to 100 μg/ml, but zoospore germ tube length was reduced about 50% by 10 μg/ml CGA-48988 after 4 hr (Table 1). The most significant activity of CGA-48988 was its effect on the formation of secondary spores from chlamydospores. One μg/ml CGA-48988 completely blocked formation of sporangia and secondary chlamydospores (Table 1, Fig. 1A, B).

Petri dish-tobacco seedling model test. Black shank developed very rapidly on the young tobacco seedlings in water in petri dishes. Nontreated plants usually wilted 2–3 days after inoculation and were dead within 4–5 days. Limited lesion development could be observed on plants treated with 0.2 μg/ml CGA-48988, and no symptoms developed at higher rates (Table 2, Fig. 2).

Control of black shank in seedlings in the petri dishes was similar with CGA-48988 treatments applied at the time of, and 12 hr after, inoculation (Fig. 2). At either time treatments of 1 μg/ml provided virtually complete control. Treatments applied 24 hr after inoculation were slightly less effective. Plants treated 48 hr after inoculation exhibited black shank lesions on roots, but CGA-48988 treatments at 1 μg/ml apparently stopped further lesion progress. Although the infected roots were heavily damaged, plants treated even 48 hr after inoculation survived because adventitious roots grew from healthy root and hypocotyl parts protected by the fungicide.

As on water agar, the formation of sporangia from chlamydospores in water in petri dishes containing seedlings occurred 24 hr after inoculation in nontreated controls (Table 2). The formation of these secondary spores was completely inhibited by CGA-48988 at the lowest concentration tested (0.2 μg/ml). The few free sporangia found in the fungicide treatments possibly could be accounted for by the presence of some sporangia in the initial inoculum.

Large numbers of newly formed sporangia could be observed on infected tobacco roots 24 hr after inoculation. No sporangia formed on the tobacco roots in dishes containing CGA-48988, even at 0.2 μg/ml in which some lesions formed (Table 2). There was a large increase in the number of sporangia in nontreated plants 4 days after inoculation, but this increase was prevented by all

TABLE 1. Effects of metalaxyl (CGA-48988) on germination of chlamydospores and zoospores of *Phytophthora parasitica* var. *nicotianae* on water agar[a]

Concentration of CGA-48988 (μg/ml)	Effects on chlamydospore germination: Germination (%)	Secondary chlamydospores produced (%)	Sporangia produced (%)	Effects on zoospore germination: Germination (%)	Germ tube length (μm)
0	86	11	44	69	30
1	40	0	0	...[b]	...[b]
10	36	0	0	66	18
100	27	0	0	71	15

[a] Each value represents average counts or measurements of 300–400 spores in four plates per treatment incubated at 28 C for 24 hr for chlamydospores and for 4 hr for zoospores.

[b] Not tested.

concentrations of CGA-48988 tested (Table 2).

When sporangia were added to petri dishes containing water, seedlings, and CGA-48988, the fungicide did not affect zoospore release. Sixty minutes after inoculation with sporangia, zoospore counts in dishes treated with 0, 1, or 10 μg/ml CGA-48988 were 54, 58, and 54 zoospores per 25 μl, respectively (Table 3).

When suspensions of swarming zoospores were used as inoculum, they clustered around the tobacco root tips as rapidly and abundantly in a 10 μg/ml solution of CGA-48988 as in nontreated, sterile, distilled water (Table 3). In all cases the zoospores encysted preferentially just behind the root cap (Figs. 1C, D). Microscopic examination of root tips revealed no difference in the abundance of germinated zoospore cysts on roots of control plants versus CGA-48988-treated plants. Twenty-four hours after inoculations with zoospores, an abundance of sporangia could be observed on nontreated root tips whereas no sporangia were present on those of treated plants (compare Figs. 1E, F). In all treatments, however, the area behind the root cap appeared to be swollen.

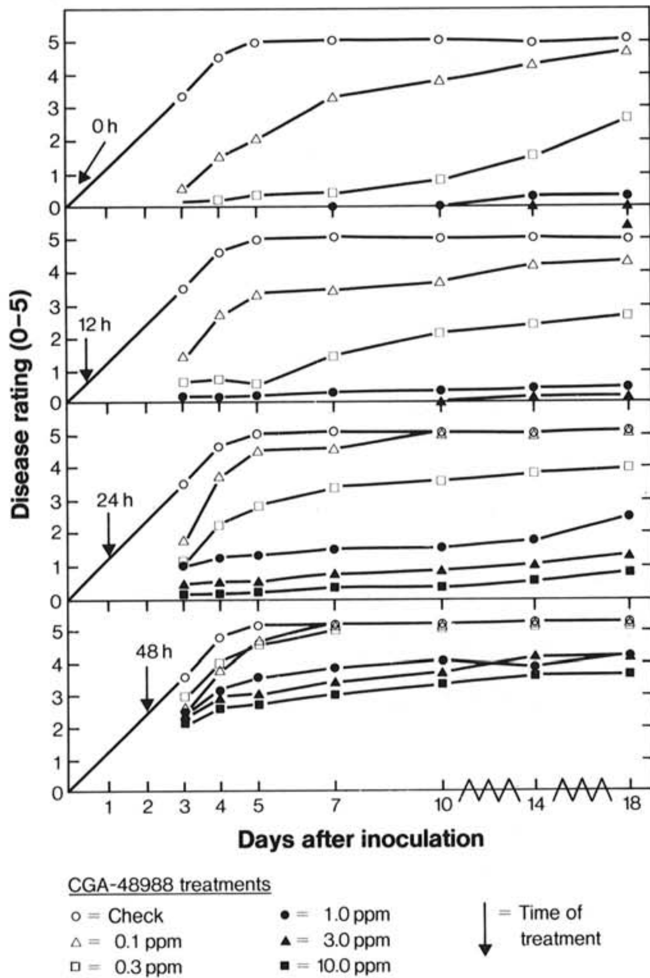

Fig. 2. Activity of pre- and postinfection treatments with metalaxyl (CGA-48988) against tobacco black shank in tobacco seedlings. The ratings represent averages from six seedlings per treatment. Rating scale: 0 = no symptoms, 5 = plants dead.

Histological observations. Microscopic examination of stained whole-root mounts showed that the fungus penetrated the root surface of untreated tobacco plants between 3–5 hr after inoculation with zoospore suspensions. As the fungus grew between and, more rarely, through the epidermal cells, cytoplasm apparently moved out of the zoospore cysts which was correlated with a loss of affinity for trypan blue within 6 hr after inoculation. Root tips from nontreated plants and from plants exposed to 10 μg/ml of CGA-48988 exhibited similar penetration of roots by the fungus. As in the nontreated controls, the fungus penetrated preferentially between the anticlinal walls of epidermal cells. However, 6 hr after inoculation all zoospore cysts on treated roots still contained cytoplasm and stained heavily. The penetration hyphae were still short after 6 hr and had not yet reached the base of the epidermal cell layer which was usual at this time on nontreated roots.

Hyphal growth in nontreated tobacco roots occurred randomly through the cortex. Once the stele was reached, hyphal growth proceeded along the long axis of the roots. Hyphae had often grown 2–3 mm from the point of penetration 24 hr after inoculation. Within 2–3 days after inoculation, infected roots of nontreated plants contained abundant mycelium (Fig. 1G). In roots of plants grown in water containing 4 μg/ml or more of CGA-48988, no hyphae had reached the stele 8 days after inoculation. Apparently they had been stopped between or within the epidermal cells, and large callosities in the adjacent plant cells often were induced (Fig. 1H). In roots treated with 1 μg/ml CGA-48988, hyphae often developed beyond the epidermal cell layer, but further growth was slow and few hyphae had reached the stele after 8 days. In the 0.2 μg/ml CGA-48988 treatment, hyphae often reached the stele and growth progressed slowly during the 8 days of observation after inoculation.

DISCUSSION

Several observations made in this study suggest that a major effect of CGA-48988 on the development of *P. parasitica* var. *nicotianae* is the prevention of sporangium formation both from chlamydospores and from infected tobacco roots. The fungicide prevents the rapid buildup of sporangium inoculum thus reducing the impact of secondary cycles of infection. Nontreated seedlings infected with the fungus had abundant sporangia on their roots 24 hr after inoculation. A similar buildup of inoculum was associated with rapid disease progress in the field (3,5).

Chlamydospores germinated in the presence of CGA-48988 without forming sporangia. In the soil, this might induce early

TABLE 2. Effects of metalaxyl (CGA-48988) on the development of *Phytophthora parasitica* var. *nicotianae* from chlamydospores in relation to control of black shank in tobacco seedlings in petri dishes[a]

Concentration of CGA-48988 (μg/ml)	Chlamydospore[b] germination at 24 hr (%)	Chlamydospores[b] with attached sporangia at 24 hr (%)	Number of free sporangia in 50 μl test solution[b] 4 days after inoculation	Average disease rating[c] 6 days after inoculation
Control				
Without plants	66	43	660	...
With plants	58	30	1,260	5
0.2	25	0	3	1
1	14	0	5	0
4	18	0	4	0
20	24	0	2	0

[a]The inoculum consisted of 1,400 chlamydospores and 180 sporangia per milliliter. Three petri dishes with two tobacco seedlings each per treatment.
[b]Two 25-μl samples were examined per petri dish.
[c]Rating scale: 0 = no symptoms, 5 = plants dead.

selective lysis of germination tubes (2).

When sporangia or zoospores were used as inocula, the release of zoospores from sporangia, zoospore motility and germination, and appressorium formation were not affected by the fungicide. However, CGA-48988 strongly inhibited mycelial growth in tobacco roots immediately after penetration. When the fungicide was applied curatively, there was apparently an immediate effect on further fungus development inside tobacco roots. This pattern of inhibition agrees very closely with the observations made on *Plasmopara viticola* on grape leaves and on *P. infestans* on tomato leaves (13); in both cases the first effects of CGA-48988 on fungus development also were noticed only after penetration of the host tissue. The general pattern of development of *P. parasitica* var. *nicotianae* in tobacco roots described above agrees closely with that described by Rich (10).

In the field, rates of 1–2 μg/ml CGA-48988 provide season-long control of black shank under light-to-moderate disease pressure with moderately resistant tobacco cultivars (9). Similarly, CGA-48988 at 1 μg/ml provided full disease control in tobacco seedlings in petri dishes although the same concentration of the fungicide did not completely inhibit mycelial growth on V8 agar. This suggests that the normal defense reactions of tobacco roots were contributing to inhibition of disease progress. The swellings on treated roots behind the root cap and the appearance of callosities around the fungal penetration sites (Fig. 1H) show that even susceptible plants reacted to infection by *P. parasitica* var. *nicotianae*.

TABLE 3. Effects of metalaxyl (CGA-48988) on the release of zoospores from sporangia and on clustering and germination of zoospores of *Phytophthora parasitica* var. *nicotianae* on tobacco roots in petri plates

Observations	Time after inoculation (min)	Concentration of CGA-48988 (μg/ml)		
		0	1	10
Swarming zoospores in test solution[a] (avg. no./25 μl)	60	54	58	54
Clustering of zoospores around root rips[b]	30	++[c]	++	++
Zoospore germination and appressorium formation on root tips[b]	240	++	++	++

[a]The inoculum consisted of 550 sporangia per milliliter exposed to 4 C for 30 min in the presence of the respective CGA-48988 concentration and then for 1 hr at 25 C. Two samples were examined from each of three plates per treatment.

[b]The inoculum consisted of 5,000 swarming zoospores per milliliter.

[c]++ indicates abundant occurrence of the respective events.

The biological mode of action of CGA-48988 as described above offers new possibilites in studying host-parasite relations with *P. parasitica* var. *nicotianae* and similar fungi since the rate of fungal growth inside the host tissue can be manipulated.

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