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In Vitro Anti-Oomycete Activity and *In Vivo* Control Efficacy of Phenylacetic Acid Against *Phytophthora capsici*

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Phenylacetic acid (PAA) was evaluated for *in vitro* anti-oomycete activity and *in vivo* control efficacy against *Phytophthora capsici*. Microscopic observation revealed that the high level of anti-oomycete activity of PAA (10 μg/ml) against *P. capsici* is mainly due to the lytic effect on zoospores. Zoospore lysis began in the presence of 5 μg/ml of PAA and most of the zoospores were collapsed at 10 μg/ml. PAA showed inhibitory activity against the zoospore germination and hyphal growth of *P. capsici* at the concentration of 50 μg/ml. In the glasshouse, the protective effect of PAA against Phytophthora blight was high on pepper plants when treated just before inoculation with *P. capsici*. In the artificially infested field, protection of pepper plants against the Phytophthora epidemic was achieved at a considerable level by PAA treatment.

Keywords **:** Anti-oomycete activity, Phenylacetic acid, Phytophthora blight, *Phytophthora capsici*.

Oomycetes resemble fungi, both morphologically and physiologically, but are actually phylogenetic relatives of diatoms and brown algae within the kingdom Stramenopiles (Tyler, 1997; Govers, 2001). They include many destructive pathogens of plants, animals and humans. The synthetic fungicides, such as prothiocarb, propamocarb, phosphate and acyanilide including metalaxyl have practically been used to control the plant diseases caused by oomycetes (Cohen and Coffey, 1986). However, a number of synthetic fungicides for the control of fungal plant diseases are not effective when confronted by oomycetes, because many fungicide targets are absent in oomycetes and our knowledge of their distinct physiology is limited (Tyler, 1997).

Phytophthora is grouped in the class of oomycetes. Among the oomycetes, *Phytophthora* species cause the most destructive diseases in plants worldwide (Kamoun, 2001). Phytophthora blight of pepper, which is caused by *Phytophthora capsici* Leonian, is one of the most devastating soilborne diseases of pepper in Korea (Kim, 1993). Intensive studies have been concentrated on the biology of *P. capsici*, evaluation of pepper germplasm for disease resistance, yield-loss assessment, and the testing of chemical, biological, and cultural measures of control (Hwang and Kim, 1995).

Plants and microorganisms can derive phenylacetic acid from phenylalanine using phenylalanine ammonia lyase (Sarwar and Frankenberger, 1995). Phenylacetic acid, a deamination product of phenylalanine, has been known as a growth- and development-promoting compound in maize (Sarwar and Frankenberger, 1995). Wightman and Lighty (1982) and Leuba and LeTourneau (1990) reported that phenylacetic acid acts as a natural auxin in the shoots of higher plants, such as barley, corn, tobacco and tomato. Phenylacetic acid was also found to affect the growth of sunflower hypocotyls and wheat coleoptile segments (Dubouchet and Zouzou, 1992). Kawazu et al. (1996a; 1996b) demonstrated that phenylacetic acid from *Bacillus subtilis* strain HY-16, *B. cereus* strain HY-3 and *B. megaterium* strain HY-17 has *in vitro* toxic effect against pine wood nematode *Barsaphelenchus xylophilusi*.

In our previous search program for microorganisms producing anti-oomycete and antifungal compounds useful for the control of plant diseases, *Streptomyces humidus* strain S5-55, which showed substantial antagonistic activity against plant pathogens, was isolated from soils in Korea (Lim et al., 2000). The anti-oomycete and antifungal compounds active against *P. capsici* and *Magnaporthe grisea* were purified from the culture filtrates of *S. humidus* strain S5-55. They were identified as phenylacetic acid and sodium phenylacetate by analyzing NMR, EI-Mass and ICP mass spectral data (Hwang et al., 2001). Burkhead et al. (1998) previously provided preliminary evidence for antifungal activity of phenylacetic acid against *Gibberella pulicaris*. Recently, we firstly demonstrated that phenylacetic acid and sodium phenylacetate had not only strong *in vitro* anti-oomycete and antifungal activities against some plant pathogens, but also *in vivo* control efficacy against *P. capsici* infection on pepper plants under glasshouse conditions (Hwang et al., 2001).

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In the present study, *in vitro* anti-oomycete activity of phenylacetic acid was examined against *P. capsici* by evaluating zoospore lysis and inhibition of zoospore germination, and inhibition of hyphal and mycelial growth. Herewith, *in vivo* control efficacies of phenylacetic acid against Phytophthora blight were also compared with those of the commercial fungicide metalaxyl under glasshouse conditions. We further examined the protective effect of phenylacetic acid against Phytophthora blight in pepper plants in the field.

Materials and Methods

Chemical compounds. The compound phenylacetic acid was purchased from Sigma Chemical Co. The oomycete fungicide metalaxyl [methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-DL-alaninate], which was obtained as technical grade material (95% active ingredients) from Sungbo Chemical Co, Korea, was used to compare anti-oomycete activity with phenylacetic acid in all the *in vitro* and *in vivo* assays. Both phenylacetic acid and metalaxyl were dissolved in methanol as stock solutions to use in this study.

Zoospore lytic activity of phenylacetic acid. The activity of phenylacetic acid to lyze zoospores of *P. capsici* was evaluated by counting the number of zoospores collapsed after treatment with phenylacetic acid at various concentrations. Zoospore suspensions were prepared from *P. capsici* cultures grown on oatmeal agar for 10 days at 28°C (Kim et al., 1989). The 0.1-ml aliquots of zoospore suspension (1×10^6 zoospores/ml) were mixed in micro-tubes with 0.4 ml volume of various concentrations of phenylacetic acid and metalaxyl. The number of zoospores lyzed at each concentration was determined on a haemocytometer under a light microscope after incubation for 5 min at 28°C. The experiments were repeated three times with four replicates.

Inhibition of zoospore germination by phenylacetic acid. Zoospore cysts of *P. capsici* were prepared from the zoospore suspensions. To induce encystment, zoospore suspensions were vigorously shaken for 2 min. A 0.1-ml aliquot of zoospore cyst suspension was dispensed in the micro-tube containing potato dextrose broth (Difco, 0.4 ml, 0.5%) amended with phenylacetic acid and metalaxyl at each concentration. After incubation of zoospore cysts for 6 h at 28°C, the zoospores germinated were counted on a haemocytometer under a light microscope. The experiment was repeated three times with four replicates.

Effect of phenylacetic acid on hyphal growth. The zoospore cyst suspension (0.1 ml) in potato dextrose broth was incubated in the micro-tubes at 28°C, until the hyphae of the germlings had an average length of 30 μm. Each of 0.4 ml of phenylacetic acid and metalaxyl at various concentrations was added onto the germlings. The mixtures were then incubated at 28°C, until the control germlings attained an average length of 400 μm. The length of 50 individual hyphae was determined under a light microscope. Percentage of inhibition of the hyphal growth was determined by comparison of the hyphal length of the germlings treated with phenylacetic acid and metalaxyl with that of control germlings. Experiments with 4 replicates were repeated three times with similar results.

TLC bioautography of phenylacetic acid effect against *P. capsici*. To evaluate inhibitory effect of phenylacetic acid against mycelial growth of *P. capsici*, direct bioautography was carried out on TLC plate (Homans and Fuchs, 1970; Lazarovits et al., 1982). A series of amounts of phenylacetic acid and metalaxyl, both of which were dissolved in methanol, were applied as a spot on a silica gel TLC plates (60 F_{254}, 0.2 mm in thickness, Merck). After air-drying to remove the remaining solvents, the TLC plate was transferred onto the water agar (1.8% agar in a 15 cm-diameter Petri dish). Molten V8 agar (20% V8-juice, 20 g agar, 1 L H_2O, pH 6.4) was seeded with the zoospore suspensions of *P. capsici* (10^6 zoospores/ml) and then uniformly spread onto the TLC plate as a layer of 0.5 mm in thickness. After incubation for 2 days at 28°C, the assay plate was stained with naphthol blue black solution (1 g/l) in acetic acid (50 ml/l) for 2-3 min, and then destained with acetic acid (50 ml/l) for 3-4 h. Inhibition of mycelial growth was clearly visualized on the blue-black-colored background.

***In vivo* evaluation of anti-oomycete activity.** The inhibitory activity of phenylacetic acid against *P. capsici* infection on pepper plants was examined under the controlled environmental conditions. Pepper seeds (*Capsicum annuum* L., cv. Nokkwang) were sown in a plastic tray containing a steam-sterilized soil mix (peat moss, perlite and vermiculite, 5:3:2, v/v/v). Seedlings at the four-leaf stage were transplanted into plastic pots ($5 \times 15 \times 10$ cm) containing the same soil mixture. Pepper plants were raised at 28 ± 2°C with approximately 80 μmol photons m^{-2} s^{-1} (white fluorescent lamps) for 16 hr a day. Phenylacetic acid and metalaxyl, both dissolved in methanol, were diluted with water containing Tween 20 (0.5 g/l) to give the concentrations of 10, 100 and 1,000 μg/ml. Each of the two chemicals was soil-drenched at a volume equivalent to 500 L/ha into each pot at different time intervals before or after inoculation with *P. capsici*. Control plants were treated with Tween 20 solution only. Plants were wounded by vertically making 1 cm-longitudinal slits in the stems 1 cm from the soil surface. Sterile cotton wool dipped in zoospore suspension was placed on the wounded sites and covered with plastic tape to maintain a moist condition. The pepper plants were also inoculated by soil-drench with zoospore suspension (10^5 zoospores/ml). Disease severity was rated daily after inoculation, based on a scale 0-5: 0 = no visible disease symptoms, 1 = leaves slightly wilted with brownish lesions beginning to appear on stems, 2 = 30-50% of entire plant diseased, 3 = 50-70% of entire plant diseased, 4 = 70-90% of entire plant diseased, 5 = plant dead. Data are the means of 6 plants per treatment.

Evaluation of control efficacy of phenylacetic acid against *Phytophthora* disease in the field. The control efficacy of phenylacetic acid against Phytophthora blight of pepper (cv. Nokkwang) plants was evaluated in the artificially *P. capsici*-infected field in Dukso, Korea. Pepper seeds were sown on March 25 in 2001 and 2002 and the seedling plants in pots were transplanted in the field on May 20 in 2001 and 2002. Insecticides or herbicides were sprayed onto the pepper field, if necessary. Phenylacetic acid-treated, metalaxyl-treated and untreated control plots were placed with 3 replicates in a randomized block design.

The size of each plot was 24 m^2 in which 70 to75 plants were grown. Each of 100 ml of phenylacetic acid (1,000 μg/ml) and metalaxyl (2.5 g/l) were soil-drenched for each of pepper plants on July 23 and August 2 in 2001 and 2002. Ten ml of zoospore suspension (10^4 zoospores/ml) were artificially soil-drenched for each of pepper plants in the fields on July 27 in 2001 and 2002. Disease incidence and severity of Phytophthora blight in plots were rated daily from August 2 to August 23 in 2001 and 2002.

Statistical analyses. Statistical analyses of the experimental data were conducted using the Statistical Analysis System (SAS institute, Cary, NC, USA). Fishers protected least significant difference (LSD) at $P = 0.05$ was applied to determine whether or not differences between the treatments were significant.

Results and Discussion

To evaluate zoospore-lytic activity, zoospore suspensions of *P. capsici* treated with phenylacetic acid and metalaxyl was examined under a light microscope at a time sequence. Zoospores were rendered immotile within a few seconds following exposure to phenylacetic acid (10 μg/ml) and subsequent lysis began to occurr within 30 seconds (Fig. 1A). The zoospores started to swell and eventually rupture. The complete lysis of zoospores of *P. capsici* occurred at 60 s after treatment with phenylacetic acid. The concentration of 10 μg/ml of phenylacetic acid was sufficient to lyze most of *P. capsici* zoospores (Fig. 1B). At the concentration of 1 μg/ml of phenylacetic acid, zoospore motility was distinctly reduced, compared with the untreated control, although no lytic activity was observed. However, metalaxyl of over the concentration of 100 μg/ml did not show any significant zoospore-lytic activity.

Many oomycetes reproduce asexually, forming mobile flagellate zoospores under moist conditions. Since zoospores do not have cell walls but only have plasma membranes, zoospores may not be strong enough to bear osmotic pressure of water under conditions where active transport is inhibited, thus resulting in the plasma membrance rupture (Mitani et al., 2001). In our previous study, rhamnolipid B, produced by *Pseudomonas aeruginosa* strain B5, has been demonstrated to exhibit not only lytic effects on zoospores, but also inhibitory activity against the zoospore germination and hyphal growth of *P. capsici* (Kim et al., 2000). Purified rhanmnolipids caused the cessation of zoospore motility and subsequently the lysis of entire zoospore population within 1 min (Stanghellini and Miller, 1997). Some natural products, such as anacardic acid from *Ginkgo biloba* (Begum et al., 2002) and phytuberin from potato tubers (Harris and Dennis, 1976), showed anti-oomycete activity against *P. infestans*, resulting in the lysis of the zoospores.

Phenylacetic acid completely inhibited zoospore germination of *P. capsici* at 50 μg/ml, whereas metalaxyl did not show significant effectiveness against zoospore germination up to 100 μg/ml (Fig. 2A). There was no difference between phenylacetic acid and metalaxyl in inhibiting zoospore germination up to 5 μg/ml. Phenylacetic acid strongly inhibited the hyphal growth of *P. capsici* at 50 and 100 μg/ml (Fig. 2B). The average hyphal length of germlings was reduced at 50 μg/ml by 66% of that in the untreated control. However, the inhibitory effect of metalaxyl against hyphal growth was not significantly as great as that of its counterpart. Mycelial growth of *P. capsici* was strongly

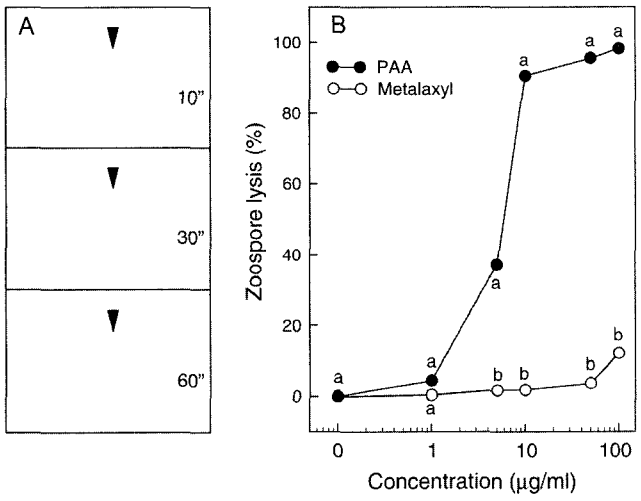

Fig. 1. The lytic activity of phenylacetic acid (PAA) and metalaxyl against zoospores of *Phytophthora capsici* at various concentrations. (A) Time-course of zoospore lysis after exposure to PAA (10 μg/ml), examined under a light microscope (magnification, 100×). Means at each concentration followed by the same letters are not significantly different ($P = 0.05$) according to the least significant difference (LSD) test.

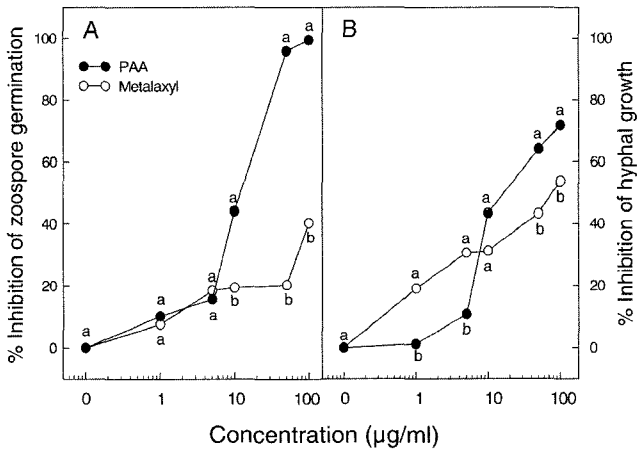

Fig. 2. Inhibitory effects of phenylacetic acid (PAA) and metalaxyl on (A) zoospore germination and (B) hyphal growth of *Phytophthora capsici* at various concentrations. Means at each concentration followed by the same letters are not significantly different ($P = 0.05$) according to the least significant difference (LSD) test.

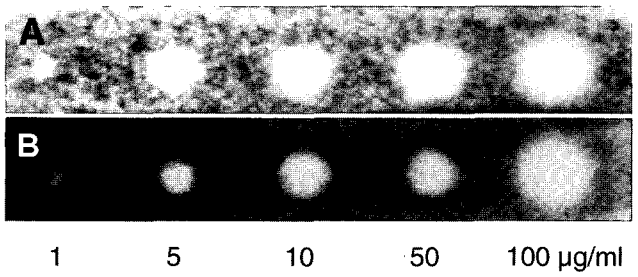

Fig. 3. TLC bioautography of (A) phenylacetic acid and (B) metalaxyl against mycelial growth of *Phytophthora capsici* at various concentrations.

inhibited on the TLC plate supplemented with phenylacetic acid at various concentrations (Fig. 3). Metalaxyl was highly as active as phenylacetic acid against inhibition of mycelial growth of *P. capsici.*

In this study, phenylacetic acid at low concentrations inhibited all stages of the life cycle of *P. capsici,* including zoospore motility, zoospore germination, and mycelial growth. It is well known that zoospore motility and release are affected by the inhibition of the energy supply (Ziogas and Davidse, 1987). Energy generation inhibitors, such as fluazinam and famoxadone inhibit zoospore motility and release of *P. infestans* (Cooke, et al., 1998; Jordan et al., 1999). On the other hand, non-respiratory inhibitors such as metalaxyl and dimethomorph exhibit little or no inhibition of zoospore motility and release of *P. infestans* (Bruck et al, 1980; Cohen et al., 1995). These results suggest that the mode of action of phenylacetic acid may be connected with impairment of the energy generation system.

In vivo efficacy of phenylacetic acid and the commercial fungicide metalaxyl against Phytophthora blight development in pepper plants is shown in Figures 4 and 5. Treatment with phenylacetic acid and metalaxyl provided substantial control of Phytophthora blight in soils infested with the pathogen. As the concentration of the two compounds increased, the Phytophthora disease was gradually inhibited on the pepper plants at the first-branch stage. Regardless of the inoculation methods, treatments with phenylacetic acid 3 days and just before inoculation were more effective than those 3 days after inoculation in controlling Phytophthora disease in pepper plants (Figs. 4 and 5), suggesting that phenylacetic acid has preventive rather than curative effects. In the pepper plants inoculated by soil-drench, control efficacy of phenylacetic acid against was somewhat higher than those inoculated by stem-wound. In general, more significant protective and curative activity against *P. capsici* infection was observed in metalaxyl.

The control efficacy of phenylacetic acid and metalaxyl against of pepper plants was evaluated in the pepper field artificially inoculated by *P. capsici* in the year of 2001 and 2002. Since natural infection by *P. capsici* has not been found in pepper-growing field in Dukso, zoospore suspension

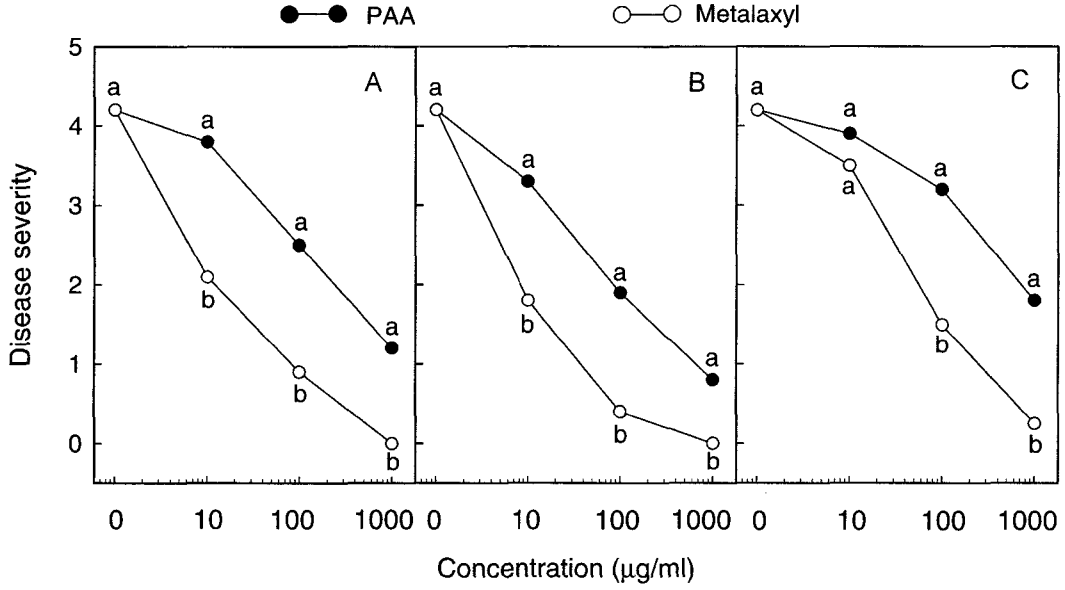

Fig. 4. *In vivo* control efficacy of phenylacetic acid (PAA) and metalaxyl against *Phytophthora capsici* infection on pepper plants inoculated by soil drench at the first-branch stage. Each of the two chemicals was soil-drenched into pots at different time intervals before or after soil-drench inoculation of *P. capsici.* Disease severity (0: no disease, 5: plant dead) was rated on day 8 after inoculation. (A) Treatment with phenylacetic acid and metalaxyl 3 days before inoculation, (B) Treatment with phenylacetic acid and metalaxyl just before inoculation, (C) Treatment with phenylacetic acid and metalaxyl 3 days after inoculation. Means at each concentration followed by the same letters are not significantly different ($P = 0.05$) according to the least significant difference (LSD) test.

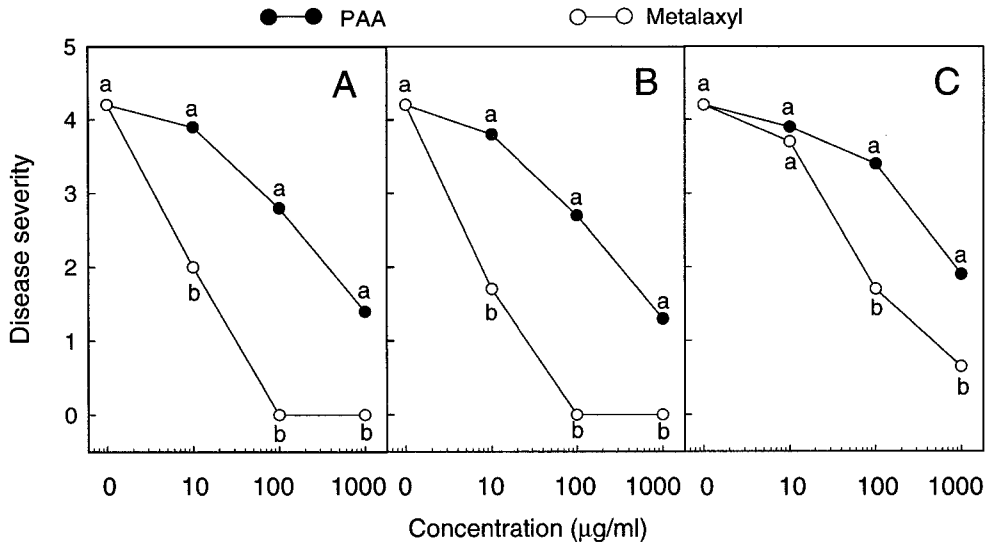

Fig. 5. *In vivo* control efficacy of phenylacetic acid (PAA) and metalaxyl against *Phytophthora capsici* infection on pepper plants inoculated by stem-wounding at the first-branch stage. Each of the two chemicals was soil-drenched into pots at different time intervals before or after stem-wound inoculation of *P. capsici*. Disease severity (0: no disease, 5: plant dead) was rated on day 8 after inoculation. (A) Treatment with phenylacetic acid and metalaxyl 3 days before inoculation, (B) Treatment with phenylacetic acid and metalaxyl just before inoculation, (C) Treatment with phenylacetic acid and metalaxyl 3 days after inoculation. Means at each concentration followed by the same letters are not significantly different ($P = 0.05$) according to the least significant difference (LSD) test.

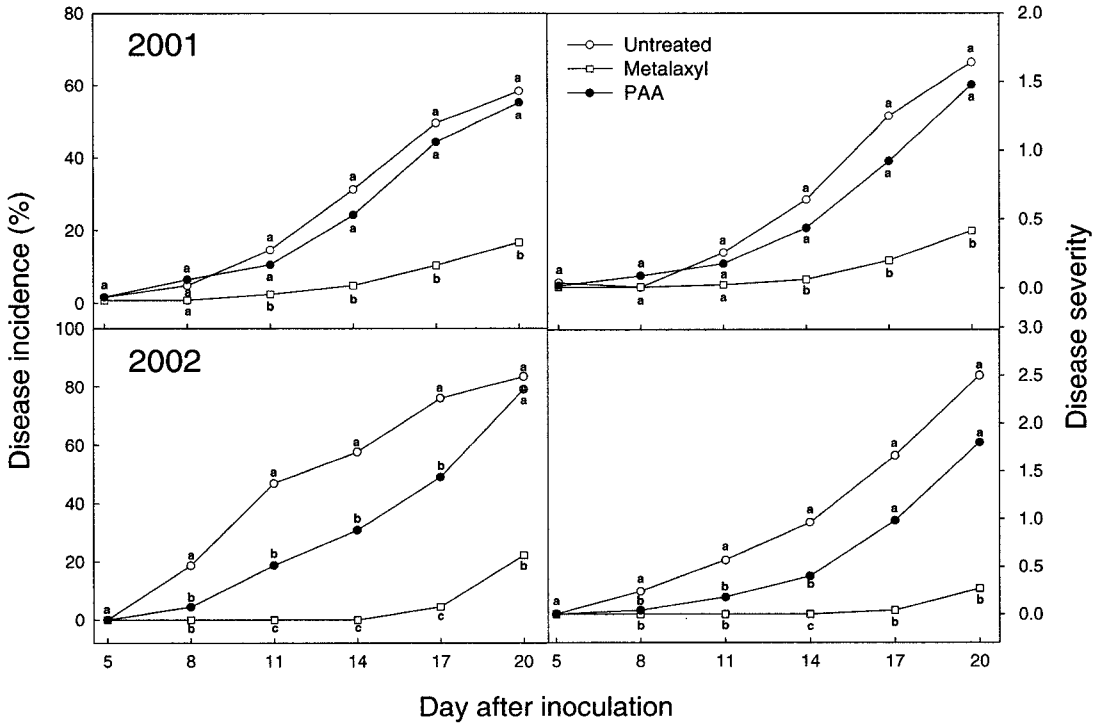

Fig. 6. Effects of phenylacetic acid (PAA) and metalaxyl on incidence and severity of Phytophthora blight in the pepper field in Dukso, Korea, in 2001 and 2002. The two chemical compounds were soil-drenched in the pepper field. The zoospore suspension (10^4 zoospores/ml) of *Phytophthora capsici* was inoculated by soil-drench in the field. Disease incidence was rated daily after appearance of Phytophthora blight on pepper plants. Means at each concentration followed by the same letters are not significantly different ($P = 0.05$) according to the least significant difference (LSD) test.

of *P. capsici* was artificially inoculated by soil drench in the tested plots. The symptom of the Phytophthora blight began to appear on pepper plants 6 days after inoculation of *P. capsici* in all the plots, except for metalaxyl-treated plots. Brownish lesions occurred on the pepper stems and extended rapidly into the upper part of plants, accompanied by a wilt of the entire plants, leaf defoliation, and damping-off. Protection of pepper plants against the Phytophthora epidemic by phenylacetic acid treatment was maintained at a considerable level by 14 days after inoculation in 2001 and 2002 (Fig. 6). Metalaxyl significantly and consistently controlled Phytophthora blight on pepper plants. The high level of Phytophthora disease control was observed 17 days or longer after inoculation in 2001 and 2002, following application of metalaxyl on pepper plants which indicates a good persistence of metalaxyl in the soil. The control efficacy of phenylacetic acid against Phytophthora blight was somewhat higher in 2002 than in 2001, possibly due to the lower incidence of Phytophthora disease in the year 2002. These results led us to conclude that *in vitro* anti-oomycete activity and *in vivo* control efficacy of phenylacetic acid against *P. capsici* infections may offer a possible usefulness of phenylacetic acid for the control of the oomycete pathogens in crop plants. To our knowledge, this is the first study to demonstrate zoosporicidal activity of phenylacetic acid against *P. capsici* and its control efficacy of Phytophthora diseases in pepper plants in the field.

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