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Original Article

Fungicidial Activity of Benthiavalicarb-isopropyl against *Phytophthora infestans* and Its Controlling Activity against Late Blight Diseases

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Benthiavalicarb-isopropyl, a novel member of the amino acid amide carbamate group of fungicides, is effective against all Oomycete fungal plant pathogens except *Pythium* spp. Our results demonstrate that this fungicide effectively controls potato and tomato late blight caused by metalaxyl-sensitive and -resistant strains of *Phytophthora infestans*. Experiments *in vitro* demonstrated that benthiavalicarb-isopropyl was ineffective in stopping the discharge of zoospores from zoosporangia and suppressing their motility; but strongly inhibited mycelial growth, sporulation, and the germination of sporangia and cystospores. Experiments in a greenhouse showed that benthiavalicarb-isopropyl has not only a strong preventive, but also a curative effect; its translaminar properties are effective along with its rainfastness and residual activity. In field trials, it was effective in controlling tomato and potato late blight at 25–75 g a.i./ha.

Keywords: benthiavalicarb-isopropyl, Oomycete, late blight, *Phytophthora*.

INTRODUCTION

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is an important and devastating disease worldwide. *P. infestans* is regarded as a high-risk pathogen spreads rapidly *via* wind and water, and resistance of *P. infestans* to fungicides of the phenylamide group has already been reported.[1–4] The development of fungicides with new modes of action is called for. Benthiavalicarb-isopropyl, isopropyl[(*S*)-1-(6-fluorobenzothiazol-2-yl)ethylcarbamoyl-2-methylpropyl]carbamate (Fig. 1) is a novel amino acid amide carbamate fungicide developed for the control of diseases involving Oomycetes. Benthiavalicarb-isopropyl has favorable toxicological and environmental profiles, and can be used for successfully control disease in various crops without causing any damage when used at the recommended rate. This paper describes the properties of benthiavalicarb-isopropyl, including its biological activity against *P. infestans in vitro* and efficacy in controlling potato and tomato late blight in greenhouse and field trials. Furthermore, it is revealed that benthiavalicarb-isopropyl does not affect respiration, synthesis of nucleic acid, protein and lipid, and or the function of the plasma-membrane of *P. infestans*. Although benthiavalicarb-isopropyl is considered to belong to the same chemical group as iprovalicarb which is proposed to affect the stability of the fungal cell wall,[5] the precise mode of action of this compound still remains to be clarified. Benthiavalicarb-isopropyl has no cross-resistance to existing fungicide groups, and it is a promising product for integrated pest control and resistance management programs.

MATERIALS AND METHODS

1. Pathogens

A standard strain of *P. infestans* obtained from infected tomato leaves was maintained on plants in the Life Science Research Institute of Kumiai (Shizuoka), and was used in greenhouse trials. Strain TK-301 (A2 mating type), a metalaxyl-sensitive strain of *P. infestans* isolated from infected po-

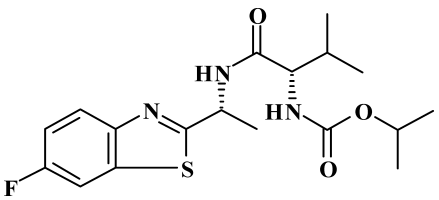

Fig. 1. The chemical structure of benthiavalicarb-isopropyl.

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tato leaves in fields of the National Agricultural Research Center for the Hokkaido Region, was used for all *in vitro* studies and field trials. A metalaxyl-resistant strain, 7-1 (A1 mating type), obtained from the Hokkaido Plant Protection Office, r1234, r1234-2, r0-3, and C-3 (*P. infestans*), IFO-8386 (*P. capsici*), IFO-30285 (*P. palmivora*), IFO-30474 (*P. cactorum*), IFO-30595 (*P. nicotianae*), IFO-30416 (*P. porri*), 30-26 (*P. katsurae*), and 30-27 (*P. megasperma*) were used for experiments *in vitro* on mycelial growth. All isolates were maintained on rye-A agar culture medium[6] at 20°C.

2. Plants

Tomato (*Lycopersicon esculentum* Mill.) cv. Ponderosa seedlings were grown in plastic pots, and used at the fifth to sixth leaf stage. Tomato and potato (*Solanum tuberosum* L.) cv. Danshaku seedlings were transplanted to the field, grown for 45–60 days, and then used in field trials.

3. Chemicals

Benthiavalicarb-isopropyl, technical grade (99.1%), was synthesized by Ihara Chemicals Inc and formulated as a 100 g/kg wettable powder for greenhouse and field trials. For experiments *in vitro*, test compounds were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in assay solutions was lower than 1 g/l. Mancozeb (750 g/kg wettable powder) and metalaxyl (250 g/kg wettable powder) were from Dow AgroSciences and Syngenta, respectively.

4. Effect on Each Life-Stage of P. infestans

4.1. Mycelial growth

Mycelial growth of *Phytophthora* species was tested with rye-A agar culture medium (filtrate of a rye infusion (60 g/l) containing 2 g/l sucrose and 15 g/l agar) which contained the test fungicide at a predetermined concentration. Mycelial discs (4 mm in diameter) were cut from the tip of test fungi which were pre-cultivated on rye-B agar culture medium (supernatant of a rye infusion (60 g/l) containing 2 g/l sucrose and 15 g/l agar), and were transplanted onto the test culture agar and incubated at 20°C for 5–7 days in the dark. Radial growth was measured, the inhibitory activity of the test fungicide was assessed as a percentage of the growth on the untreated medium, and I_{50} (50% inhibitory concentration) values were calculated. The tests were conducted with three replicates.

4.2. Sporulation

The sporulation analysis of *P. infestans* was performed using slices (8 mm in thickness) of potato tuber. A zoosporangial suspension (1.0×10^3 zoosporangia/ml) of test fungi, filtered through two layers of gauze, was prepared by growing the fungi on potato tuber slices at 20°C for 6 days in the dark. The zoosporangial suspension was inoculated onto the surface of a tuber slice with a hand sprayer, and the tuber slice was cultivated for 4 days at 20°C in the dark. After the flushing of the zoosporangia with sterilized water using an ultrasonic syringe, the tuber slices were immersed into the test fungicide fluid; this fluid had a predetermined concentration which had been produced by dilution with sterilized water. Zoosporangia were induced to re-form for 2 days at 20°C in the dark, each tuber slice was then pierced by a 12 mm diameter cork borer, the disk was immersed in sterilized water of a predetermined quantity, and the zoosporangia were separated with an ultrasonic syringe. Re-formed zoosporangia were counted with an optical microscope (Olympus BH-2), and the inhibitory effect of the test fungicide against sporulation was assessed as a percentage of that on the untreated control. The tests were conducted with three replicates.

4.3. Direct germination from zoosporangia and cystospores

Direct germination from zoosporangia was tested in a germination solution,[7,8] (0.2 mM $CaCl_2$, 0.05 mM $MgSO_4$, 0.05 mM KH_2PO_4, and 0.5 mM $NaHCO_3$). Test fungi were grown on potato tuber slices at 20°C for 6 days in the dark, and zoosporangial suspensions were harvested and filtered through two layers of gauze. These suspensions (1.0×10^5 zoosporangia/ml) were then pre-incubated at 33°C for 16 hr, test fungicides were added, and the mixtures were incubated at 10°C for 48 hr. Germination from cystospores was also tested in the germination solution. After zoosporangial suspensions (1.0×10^5 zoosporangia/ml) were pre-incubated at 22°C for 4 hr, they were transferred to 10°C and incubated for 3 hr. After almost all of the zoospores were released and encysted, test fungicides were added and the suspensions were incubated for another 6 hr. The rates of direct germination of zoosporangia and cystospores were determined with an optical microscope, and the inhibitory activity of the test fungicide was assessed as a percentage of the respective germination in the untreated control. The tests were conducted with three replicates.

4.4. Indirect germination from zoosporangia and motility of the zoospores

Indirect germination from zoosporangia was tested in the germination solution. Test fungi were grown on potato tuber slices at 20°C for 6 days in the dark, and zoosporangia were harvested and filtered through two layers of gauze. Zoosporangial suspensions (1.0×10^5 zoosporangia/ml) were pre-incubated at 22°C for 4 hr, test fungicides were added, and the suspensions were incubated at 10°C for 3 hr. The motility of the zoospores was also tested in the germination solution. After the zoosporangial suspensions (1.0×10^5 zoosporangia/ml) were pre-incubated at 22°C for 4 hr, they were transferred into a 10°C incubator for 3 hr. Test fungicides were added and the suspensions were incubated for 3 hr. The rate of indirect zoosporangia germination was determined using an optical microscope, and the inhibitory activity of the test fungicide was assessed as a percentage of the germination of the untreated zoosporangia. The motility of the zoospores was observed with an optical microscope, and the inhibitory activity of the test fungicide was assessed in comparison with that of the untreated zoospores. The tests were conducted with three

replicates.

5. Greenhouse and Field Trials

5.1. Preventive and curative effects

Preventive and curative effects were tested in the greenhouse and each treatment plot consisted of three pots. Tomato seedlings were sprayed with 16 ml per pot of fungicide test solution containing the test compound at a predetermined concentration; spraying was done before or after inoculation with a zoospore suspension of *P. infestans*. Inocula were obtained from fresh sporangia (adjusted to 1.0×10^4 sporangia/ml) which were harvested 3 days after the tomato seedlings were inoculated with the fungus. After the test plants were sprayed with the inoculum, they were transferred to a growth chamber and incubated at 20°C in the dark for 3 days (100% relative humidity). After incubation, the disease intensity on each leaf was expressed using a disease index (Scale of 0 to 4); 0: no lesions, 1: 1–5% lesion area, 2: 6–33% lesion area, 3: 34–66% lesion area, 4: more than 66% lesion area. The disease severity of each pots was calculated with the following formula; Disease severity $=100\times(N_1+2N_2+3N_3+4N_4)/4(N_0+N_1+N_2+N_3+N_4)$, where N_0, N_1, N_2, N_3 and N_4 represent the number of leaves belonging to each disease index. The efficacy of the test fungicides was assessed as a percentage of disease severity in the untreated plot. The tests were conducted with three replicates.

5.2. Translaminar and systemic action

Translaminar and systemic actions were tested in a greenhouse. Each treatment plot consisted of three pots. The translaminar action of the test fungicide was assessed as a means of controlling tomato late blight by spraying the fungicide suspension only on the adaxial surface of the leaves. Test plants were sprayed on the abaxial surface of the leaves with inoculum (1.0×10^4 sporangia/ml) 24 hr after treatment with the fungicide. Systemic action for controlling the diseases development on the upper leaves was assessed by spraying the lower leaves with 16 ml of fungicide suspension per pot; it was also tested in the pot by drenching the soil with 3 liters of fungicide suspension per square meter. Test plants were sprayed with inoculum (1.0×10^4 sporangia/ml) 24 hr after treatment with the fungicide. Then the plants in each test were transferred to a growth chamber and incubated at 20°C in the dark for 3 days (100% relative humidity). The disease severity of each pot was calculated, and the efficacy of the test fungicides was assessed as a percentage of the disease severity in the untreated plot. The tests were conducted with three replicates.

5.3. Rainfastness and residual activity

Rainfastness and residual activity were tested in the greenhouse. Each treatment plot consisted of three pots. Tomato seedlings were sprayed with 16 ml of test solution per pot at predetermined concentrations. Rainfastness of the test fungicide was assessed relative to the plot without artificial rainfall treatment; test plants were exposed to 30 mm/hr rain for 1 hr using artificial rain-fall equipment 24 hr after treatment with the fungicide. The control plants were not exposed. Residual activity of the test fungicide was also assessed relative to a control plot; the test plants and the control plants were retained in the greenhouse for 7 days and 1 day after treatment with the fungicide, respectively. Then the plants in each test were sprayed with inoculum (1.0×10^4 sporangia/ml) and transferred to a growth chamber and incubated at 20°C in the dark for 3 days (100% relative humidity). The severity of disease was calculated for each pot, and the efficacy of the test fungicides was assessed as a percentage of the severity among the untreated plants. The EC_{50} (R1) was calculated from control values for the plot of artificial rain-fall treatment, the EC_{50} (R2) from control values for the plot kept for 7 days in the greenhouse, and the EC_{50} (C) from control values for the plot without these treatments. The EC_{50} ratio was calculated as follows and the value for each treatment was presented as a value which is uninfluential in 1. The tests were conducted with three replicates.

$$EC_{50}\text{ ratio}=EC_{50}(R1)\ \text{ or }\ EC_{50}(R_2)\ /\ EC_{50}(C)$$

5.4. Field trials

All field trials were laid out with a randomized block design and replicated 3 times. Each plot size was 3.75 m^2 (2.5×1.5 m) and consisted of 10 plants for potato, or 2.5 m^2 (2.5×1 m) and consisted of 5 plants for tomato. Test compounds were applied as a spray of a rate of 1000 l/ha. The timing followed the normal practice in the area (7-day intervals). The disease severity of each plot was calculated by observing all test plants in accordance with the standard of the EPPO guidelines on potatoes, or with the same standard as with the pot tests on tomatoes. The efficacy of the test fungicides was assessed as a percentage of the disease control in untreated plants.

RESULTS AND DISCUSSION

1. Effect on Each Life-Stage of P. infestans

1.1. Mycelial growth

Benthiavalicarb-isopropyl strongly inhibited the mycelial growth of *Phytophthora* species (Table 1). The I_{50} and MIC values of this compound against *P. infestans*, *P. capsici*, *P. palmivora*, *P. cactorum*, *P. nicotianae*, *P. porri*, *P. katsurae*, and *P. megasperma* were all within the range of 0.01–0.05 and 0.03–0.3 mg/l, respectively. Benthiavalicarb-isopropyl can control metalaxyl-resistant strains as well as metalaxyl-sensitive strains of *P. infestans*. No cross-resistance was observed. This indicates that the mode of action of benthiavalicarb-isopropyl is different from that of the phenylamide fungicide class. It was reported that phenylamide fungicides strongly inhibit the synthesis of ribosomal RNA in Oomycete fungi.[9] Therefore, it is thought that benthiavalicarb-isopropyl does not inhibit the synthesis of ribosomal RNA in *P. infestans*.

1.2. Sporulation

From the experiments using the slices of potato tuber, it was

Table 1. The inhibitory activity of benthiavalicarb-isopropyl and metalaxyl against mycelial growth of *Phytophthora* spp.

Pathogen	Strain	Benthiavalicarb-isopropyl		Metalaxyl	
		I_{50} [a]	MIC [b]	I_{50} [a]	MIC [b]
Phytophthora infestans	TK-301	0.03	0.03–0.1	nt [d]	nt
	r1234	0.02	0.1–0.3	0.09	0.3–1
	r1234-2	0.03	0.1–0.3	0.05	>10
	r0-3	0.03	0.03–0.1	0.21	>10
	C-3	0.03	0.1–0.3	0.15	3–10
	7-1 [c]	0.04	0.1–0.3	>10	>10
Phytophthora capsici	IFO-8386	0.01	0.03–0.1	0.62	>3
Phytophthora palmivola	IFO-30285	0.02	0.1–0.3	0.54	>3
Phytophthora cactorum	IFO-30474	0.03	0.03–0.1	0.03	0.3–1
Phytophthora nicotianae	IFO-30595	0.05	0.1–0.3	0.08	>3
Phytophthora porri	IFO-30416	0.02	0.1–0.3	0.08	>3
Phytophthora katsurae	—	0.04	0.03–0.1	0.46	>100
Phytophthora megasperma	—	0.02	0.1–0.3	0.34	>100

[a] The 50% inhibitory concentration (mg/l). [b] The minimum inhibitory concentration (mg/l). [c] Phenylamide-resistant strain. [d] Not tested.

clear that the sporulation of *P. infestans* was strongly inhibited by benthiavalicarb-isopropyl at a low concentration. On the other hand, mancozeb was less active: 6.4×10^4 Zoosporangia re-formed in the untreated control. Benthiavalicarb-isopropyl applied at 1 mg/l achieved a 96.8% inhibition of sporulation (Table 2).

1.3. Direct germination from zoosporangia and cystospores

The percent germination of zoosporangia (direct germination) and of cystospores of *P. infestans* in each untreated plot was 63.1% and 90.0%, respectively. Direct germination of zoosporangia and germination of cystospores were completely inhibited by benthiavalicarb-isopropyl at 1 mg/l (Table 2). Mancozeb was less effective than benthiavalicarb-isopropyl.

1.4. Indirect germination from zoosporangia and motility of zoospores

In the untreated zoosporangial suspension of *P. infestans*, 77.3% of the zoosporangia released zoospores (indirect germination). The release was only slightly affected by benthiavalicarb-isopropyl at 100 mg/l (Table 3). No difference was observed in the motility of zoospores of *P. infestans* between the plot treated with 100 mg/l of benthiavalicarb-isopropyl and the untreated plot. On the other hand, motility was completely inhibited by mancozeb at 10 mg/l. *Phytophthora* has

Table 2. The inhibitory activity of benthiavalicarb-isopropyl and mancozeb against sporulation, zoosporangia germination and cystospore germination of *Phytophthora infestans*

Compound	Concentration (μg/ml)	Percent inhibition (±SD)		
		Sporulation	Zoosporangia germination	Cystospore germination
Benthiavalicarb-isopropyl	10	97.6±2.1 [a]	100.0	100.0
	1	96.8±1.5	100.0	100.0
	0.1	45.2±9.1	98.6±2.3	95.5±4.9
Mancozeb	100	66.7±14.0	98.6±2.3	100.0
	10	5.7±9.8	60.8±13.3	100.0
	1	7.3±6.4	47.3±9.6	22.7±11.3
Untreated	—	6.4×10^4 spore/ml [b]	63.1±8.8 [c]	90.0±5.6 [d]

[a] Percent inhibition of spoluration, zoosporangia germination and cystospore germination in each treatment compared to untreated controls. Results are presented as the means and standard deviations (n=3). [b] The amount of re-formed zoospore in untreated control. [c] The percentage of zoosporangia germination in untreated control. [d] The percentage of cystopore germination in untreated control.

Table 3. The inhibitory activity of benthiavalicarb-isopropyl and mancozeb against zoospore release and their effects on zoospore motility of *Phytophthora infestans*

Compound	Concentration (μg/ml)	Percent inhibition (±SD) and effect	
		Zoospore release	Zoospore motility
Benthiavalicarb-isopropyl	100	45.5±11.9[a)]	+++[c)]
	10	0.0	+++
	1	0.0	+++
Mancozeb	100	100.0	−
	10	100.0	−
	1	9.1±8.8	+
Untreated	−	77.3±11.4[b)]	+++

[a)] The percent inhibition of zoospore release in each treatment compared to untreated control. [b)] The percentage of zoospore release in untreated control. [c)] The degree of zoospore motility in each treatment compared with untreated control. +++; large (equal to the untreated control). ++; middle (a little bit less than the untreated control). +; small (much less than the untreated control). −; nothing (stopped).

both a citric acid and a glyoxylate cycle[10] and our experiments showed that benthiavalicarb-isopropyl does not inhibit oxygen consumption in *P. infestans* (Y. Miyake, unpublished data). It was reported that both the release and motility of zoospores are affected by energy generation inhibitors such as famoxadone and cyazofamid.[11,12] Thus, it is suggested that benthiavalicarb-isopropyl does not affect the energy production system of *P. infestans*. But benthiavalicarb-isopropyl prevents zoospores from encysting (data not shown). So the possibility that it had some influence on the synthesis of the cell wall remains.

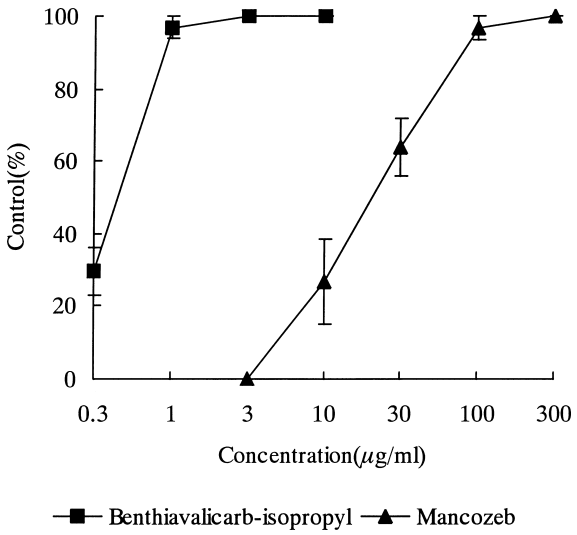

Fig. 2. Preventive effect of benthiavalicarb-isopropyl on tomato late blight. Vertical bars represent the standard deviations of the mean (n=3).

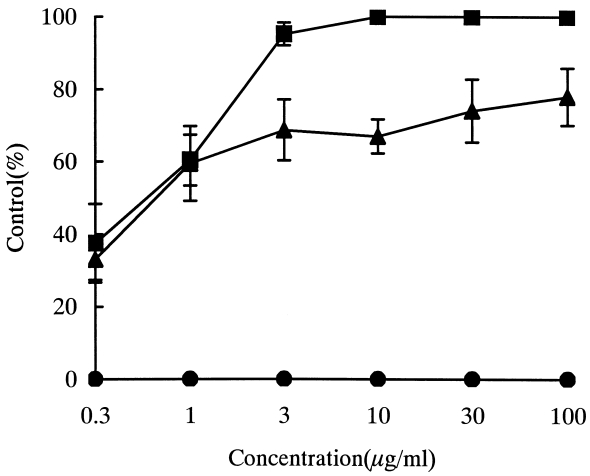

Fig. 3. Curative effect of benthiavalicarb-isopropyl on tomato late blight.
Benthiavalicarb-isopropyl was applied at 12 hr (■) or 24 hr (▲) after inoculation of *Phytophthora infestans*. Mancozeb was applied at 12 hr (●) or 24 hr (◆) after inoculation. Vertical bars represent standard deviations of the mean (n=3).

2. *Greenhouse and Field Trials*

2.1. *Preventive and curative effects*

Benthiavalicarb-isopropyl demonstrated good biological activity in greenhouse trials. Preventive treatments at a low rate of 1–3 mg/l completely controlled tomato late blight (Fig. 2). Mancozeb was less effective than benthiavalicarb-isopropyl. Curative treatments 12 hr after inoculation, at a rate of 10 mg/l, also completely controlled the pathogen, and no symptoms were observed. Following the treatments 24 hr after inoculation, although some slight symptoms of disease were observed even at the high concentration, all of these fungi

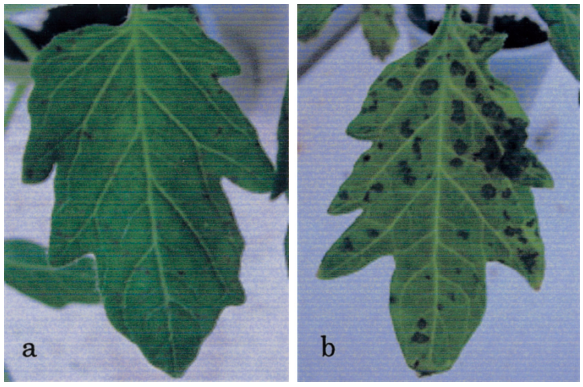

Fig. 4. Disease symptoms on tomato leaves following curative treatments with benthiavalicarb-isopropyl at 12 hr (a) or 24 hr (b) after inoculation at a rate of 10 mg/l.

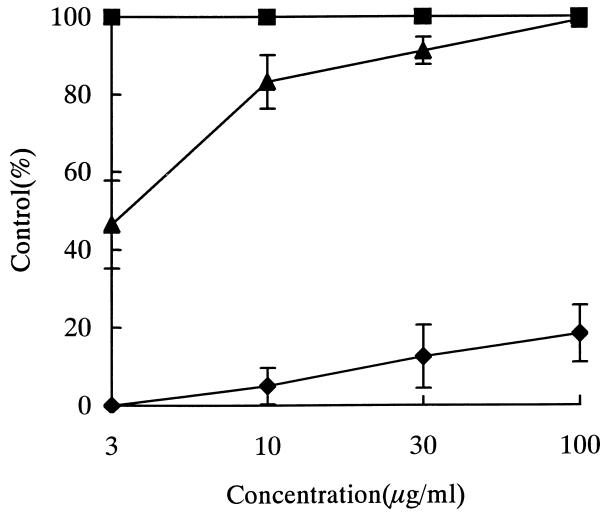

Fig. 5. The translaminar and systemic activitiy of benthiavalicarb-isopropyl against tomato late blight.
Benthiavalicarb-isopropyl was applied only on the adaxial surface of tomato leaves (▲), on lower leaves (◆) or on the soil (■) at 24 hr before inoculation. As for translaminar activity, inoculation was carried out on the abaxial surface of the leaves. Vertical bars represent standard deviations of the mean (n=3).

were inhibited from developing (Figs. 3, 4). It is believed that these results can be attributed to the fact that the treatment with the fungicide took place after the pathogen had started invading the plants. Mancozeb did not have a curative effect on plants infected with tomato late blight.

2.2. Translaminar and systemic action

It was established, by using tomato seedlings in which only the adaxial surface of the leaves had been treated, that benthiavalicarb-isopropyl exhibited good translaminar activity against tomato late blight. Control of fungal activity on the upper leaves through the spraying of the compound on the lower leaves was barely evident even at the highest concentration (100 mg/l). Therefore, it is proposed that the compound had almost no ability to move through the symplast. However, complete control through soil drenching was observed at a concentration of only 3 mg/l, and the compound had good apoplastic mobility (Fig. 5). Mancozeb did not show any translaminar or systemic activity.

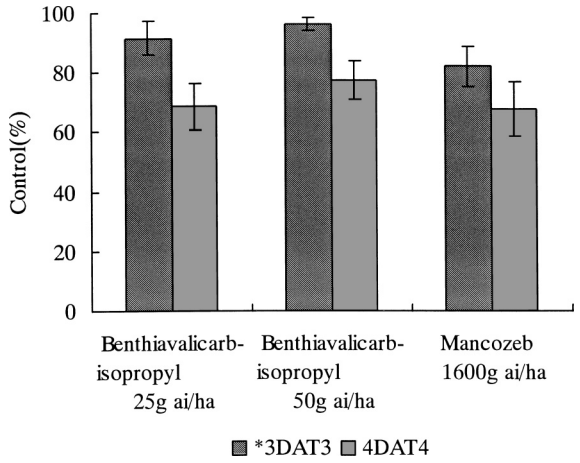

Fig. 7. Control activitiy of benthiavalicarb-isopropyl against tomato late blight.
Vertical bars represent standard deviations of the mean (n=3).
* 3DAT3=3 days after 3rd treatment.

2.3. Rainfastness and residual activity

The rainfastness of benthiavalicarb-isopropyl, established through a ratio of EC_{50} values, was almost equal to that of the standard fungicide mancozeb (Fig. 6). On the other hand, the residual activity of benthiavalicarb-isopropyl surpassed that of mancozeb. It is thought that benthiavalicarb-isopropyl will fully demonstrate control in the field.

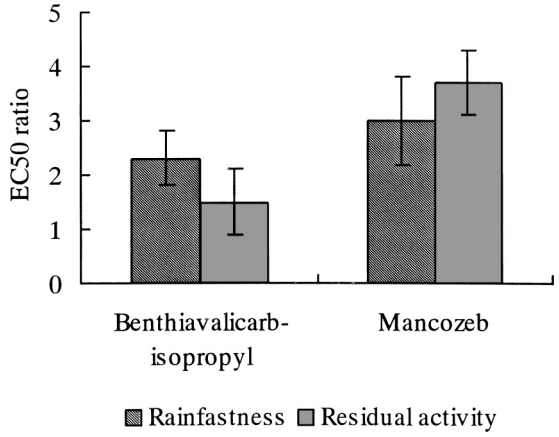

Fig. 6. The rainfastness and residual activitiy of benthiavalicarb-isopropyl and mancozeb against tomato late blight.
The EC_{50} was calculated from control values and the ratio for each treatment was presented as a value which is uninfluential in 1. Vertical bars represent standard deviations of the mean (n=3).

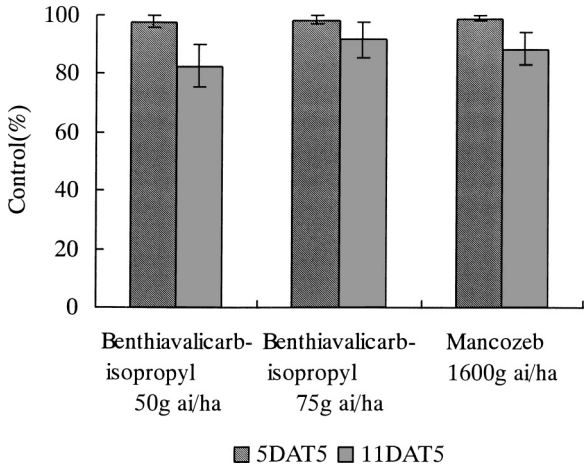

Fig. 8. Control activity of benthiavalicarb-isopropyl against potato late blight.
Vertical bars represent standard deviations of the mean (n=3).

2.4. Field trials

The efficacy of benthiavalicarb-isopropyl was tested for the control of tomato and potato late blight caused by *P. infestans* in the field. The compound was not used until after the first symptoms appeared on the tomatoes and potatoes, and then applied 4 or 5 times at 7-day intervals. The ability of benthiavalicarb-isopropyl at 50 g ai/ha to control tomato late blight was high and equal to or surpassed that of the reference fungicide mancozeb (Fig. 7). The efficacy of benthiavalicarb-isopropyl at 50 or 75 g ai/ha against potato late blight was equal to that of mancozeb (Fig. 8). These results suggest that the biological properties of benthiavalicarb-isopropyl, in addition to its basic antifungal activity, contribute to its good ability to control potato and tomato late blight in the field.

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