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**Original Article**

# Biochemical basis of selective disease controlling activity of mepanipyrim

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Mepanipyrim exhibited excellent disease control activity against *Botrytis cinerea*, but poor activity against *Cochliobolus miyabeanus*; however, the mycelial growth of *C. miyabeanus* was inhibited more strongly than that of *B. cinerea*. Therefore, disease control efficacy by mepanipyrim *in vivo* is not correlated with mycelial growth inhibition *in vitro*. While mepanipyrim prevented pectinase secretion in *B. cinerea* at 0.1–1 μg/ml, it did not interfere with secretion in *C. miyabeanus*, even at 100 μg/ml, indicating that its action is an important mechanism in disease control. Mepanipyrim affected the uptake of glucose and phenylalanine in the mycelia of both pathogens at higher doses. Thus, a secondary action of mepanipyrim may bring about mycelial growth inhibition *in vitro*. © Pesticide Science Society of Japan

*Keywords*: anilinopyrimidine, mepanipyrim, fungicide, *Botrytis cinerea*, mode of action.

## Introduction

Mepanipyrim, *N*-(4-methyl-6-prop-1-ynylpyrimidin-2-yl) aniline, is an anilinopyrimidine fungicide with a broad spectrum of activity that can control fungal diseases caused by *Botrytis cinerea* (gray mold), *Venturia* spp. (scab) and *Monilinia fructicola* (brown rot). It also has good disease control activity against the *Alternaria alternata* apple pathotype (leaf spot) and the powdery mildew genera *Erysiphe* and *Podosphaera*.[1,2]

Mepanipyrim inhibits the uptake of growth substrates such as amino acids and glucose by *B. cinerea* at an effective dose of 10–100 μg/ml[3] and blocks the secretion of host-cell wall-degrading enzymes at 1 μg/ml. The inhibition of protein secretion is the mode of action considered the most important mechanism in disease control.[4]

While mepanipyrim markedly affects the infection process of *B. cinerea* at 1–10 μg/ml, especially germ tube elongation, secondary appressorium formation and penetration, it hardly inhibited spore germination and did not fully inhibit the mycelial growth of *B. cinerea*.[1,3] Taking advantage of its strong inhibition against germ tube elongation instead of weakly mycelial growth inhibition, the FGA-paper disc method[5] can evaluate the sensitivity of *B. cinerea* to mepanipyrim. In addition, assay qualities of the FGA-paper disc method were improved to use gelatin in the medium, resulting in utilizing the inhibitory activity of mepanipyrim in protease secretion.

The *in vitro* fungicidal activities of many compounds correspond to disease control *in vivo*. Polyoxin interferes with cell wall chitin biosynthesis[6] but does not affect Oomycete fungi, which do not have chitin as a cell wall component. Although the selective mechanism that determines which fungi are sensitive has not been fully characterized, mepronil can control Basidiomycete diseases, and its inhibitory activity *in vitro* is restricted to the Basidiomycetes.[7] Benthiavalicarb-isopropyl is effective against all Oomycete plant pathogens except *Pythium* spp. both *in vitro* and *in vivo*.[8] With the notable exception of non-fungicidal protectants such as probenazole[9,10] and tricyclazole,[11] the disease control efficacy of a fungicide can generally be predicted from its inhibitory activity against mycelia or spores of plant pathogens. Mepanipyrim, however, strongly affects fungal diseases caused by *B. cinerea* despite the weak inhibition of spore germination and mycelial growth *in vitro*.[1,3] The *in vitro* antifungal activity of mepanipyrim thus does not accurately reflect disease control efficacy *in vivo*.

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This paper provides the first suggestion of the selectivity of mepanipyrim for various plant pathogens in disease control and gives some evidence of the possible modes of action.

## Materials and Methods

### 1. *Chemicals and fungal cultures*

Mepanipyrim was provided as a reagent grade product by Ihara Chemical Industry Co., Ltd. (Tokyo, Japan). A stock solution of mepanipyrim (2000–20,000 $\mu$g/ml) was prepared in methanol and diluted appropriately for each assay. A 40% suspension concentrate formulation (Frupica®) of mepanipyrim was also used for *in vivo* assays. *B. cinerea* (Kumiai stock culture No. 50–01) and *Colletotrichum lagenarium* (Kumiai stock culture No. 53–03) were grown and maintained on a potato-sucrose agar (PSA) plate at 20°C. *Cochliobolus miyabeanus* (Kumiai stock culture No. 22–01) was grown and maintained on a V-8 juice agar plate at 27°C. The culture of *B. cinerea* was illuminated with near-ultraviolet light (FL15B, Toshiba Lighting & Technology Co., Tokyo, Japan) for 2–3 days to induce spore formation.

### 2. *Labeled materials*

[U-$^{14}$C]L-Phenylalanine (13.43 GBq/mmol) and [U-$^{14}$C]D-glucose (0.089 GBq/mmol) were obtained from NEN Research Products (Boston, MA, USA). [U-$^{14}$C]Mepanipyrim ($^{14}$C-Be-mepanipyrim, 0.562 GBq/mmol) was synthesized from [U-$^{14}$C]–aniline by Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan).

### 3. *In vivo assays*

Cucumber gray mold or cucumber anthracnose (caused by *C. lagenarium*) was evaluated with cucumber plants (*Cucumis sativus* cv. Sagami-hanjiro) at the cotyledon stage grown in 9 cm pots. Test compounds were applied as a spray at 10 ml/pot. After the spray deposit had dried, the plants were inoculated with spores of the pathogens. For cucumber gray mold assay, paper discs (6 mm dia., thick type; Toyo Roshi Kaisya, Ltd., Tokyo, Japan) dipped in the spore suspension ($1\times10^6$ spores/ml, containing 0.3% yeast extract and 2% glucose) were placed on the center of cotyledons to prevent the inoculated zone from drying. The inoculated plants were transferred to a growth chamber and incubated at 20°C for 3 days (100% relative humidity). For cucumber anthracnose assay, the treated plants were sprayed with a spore suspension ($2\times10^5$ spores/ml). They were transferred to a growth chamber and incubated at 20°C for 1 day (100% relative humidity). Cucumber gray mold was assayed for lesion diameter after 3 days of inoculation, and anthracnose was assayed for the number of lesions after 6 days of inoculation. The disease control efficacy of mepanipyrim was assessed as a percentage of the means of diameters or lesion numbers in treated and untreated plants. Rice brown spot (caused by *C. miyabeanus*) was evaluated on rice plants (*Oryza sativa* Japonicum cv. Aichi-asahi) in the fourth leaf stage grown in 9 cm pots. Fungicide application, fungal inoculation ($5\times10^4$ spores/ml, incubated at 27°C for 1 day) and disease evaluation (after 5 days of inoculation) were the same as for cucumber anthracnose. Three replications were made for each treatment.

### 4. *Mycelial growth assays*

Mycelial discs (4 mm in diameter) were cut from the growing edge of fungal cultures and transplanted onto PSA media containing mepanipyrim. Cultures were incubated at 20°C (*B. cinerea* and *C. lagenarium*) or 27°C (*C. miyabeanus*), and radial growth was measured after 3 days, 8 days, and 5 days of incubation, respectively. The inhibitory activity of mepanipyrim was assessed as a percentage of growth on control media. The reported results are the means of three replications.

### 5. *Uptake of radioactive substrates*

Fungal spores were suspended in Czapek liquid medium (0.3% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% $MgSO_4\cdot7H_2O$, 0.05% KCl and 0.001% $FeSO_4\cdot7H_2O$, W/V) supplemented with 0.3% yeast extract and 2% glucose (C-medium), and incubated with reciprocal shaking in the absence of mepanipyrim for 24 hr at 20°C (*B. cinerea* and *C. lagenarium*) or 27°C (*C. miyabeanus*). Mycelia were washed three times with distilled water by centrifugation. Washed mycelia (24 mg or 26.5 mg wet weight) were suspended in Czapek medium with or without mepanipyrim. After 1 hr pre-incubation, radioactive precursors were added to give 3.7 KBq/ml. Incubation was stopped after 30 min by adding cold distilled water with the corresponding non-labeled substrates (NLS-solution) at 2.5 mM. The labeled mycelia were washed three times with NLS solution by centrifugation. They were suspended in cold 5% trichloroacetic acid (TCA) and kept on ice overnight. Major radioactivities extracted by TCA were regarded as substrates taken up by the cells. Radioactivity was measured with a liquid scintillation system (LSC-700, Aloka Co., Ltd., Tokyo, Japan) in a cocktail AQUASOL-2 (NEN Research Products). Results indicate the means of three replications.

### 6. *Uptake of mepanipyrim*

Fungal spores were incubated in C-medium for 24 hr at 20°C (*B. cinerea* and *C. lagenarium*) or 27°C (*C. miyabeanus*). Mycelia were washed three times with distilled water by centrifugation. The washed mycelia (10 mg wet weight) were suspended in Czapek liquid medium supplemented with 0.5% glucose (Czapek G medium). Radioactive isotope-labeled mepanipyrim was added to give a final concentration of 3.7 KBq/ml (1.47 $\mu$g/ml). After 60 min, incubation was stopped by adding cold distilled water and cold chased with non-labeled mepanipyrim (NLM-solution) at 10 $\mu$g/ml. The labeled mycelia were then washed three times with NLM solution by centrifugation, suspended in cold 5%TCA and kept on ice overnight. Major radioisotopic signals in TCA extracts were regarded as mepanipyrim taken up by the cells. Radioactivity

**Table 1.** Effect of mepanipyrim on mycelial growth and disease control activity

| Test fungi | % Inhibition of mycelial growth[a] | | | Protective value (%)[b] | | |
|---|---|---|---|---|---|---|
| | 300 μg/ml | 100 μg/ml | 10 μg/ml[c] | 300 μg/ml | 100 μg/ml | 10 μg/ml |
| *B. cinerea* | 84.2 | 73.1 | 65.5 | 100.0 | 100.0 | 100.0 |
| *C. miyabeanus* | 98.8 | 98.6 | 98.2 | 55.9 | 36.8 | 0 |
| *C. lagenarium* | 33.3 | 25.6 | 2.1 | 47.5 | 22.4 | 0 |

[a] Means ($n$=3). [b] Means ($n$=3). [c] Concentration of mepanipyrim.

was measured with a liquid scintillation system in a cocktail AQUASOL-2. Data are the means of two replications.

*7. Pectinase secretion assays*

Spores were inoculated into Czapek liquid medium supplemented with 0.5% sodium polypectate as the sole carbon source (Czapek P medium) and incubated with reciprocal shaking in the absence of mepanipyrim at 20°C (*B. cinerea* and *C. lagenarium* ) or 27°C (*C. miyabeanus*) for 45.5, 48 and 47 hr, respectively.

After incubation, mycelia were separated by centrifugation, and an extracellular crude enzyme solution was prepared by filtering the supernatant through a 0.45 μm cellulose membrane filter of sterilized mixed esters (Millipore Corp., Bedford, MA, USA). The assay methods of pectinase secretion were carried out according to our previous report.[4] Three replications were made for each treatment.

## Results and Discussion

*1. In vivo and in vitro activity of mepanipyrim*

The *in vivo* disease control activity of many fungicidal compounds can be more or less accurately extrapolated from fungicidal or fungistatic assays under controlled conditions *in vitro*. Thus, the first aim of this study was to determine if there was any disparity between the efficacy of mepanipyrim *in vivo* and mycelial growth inhibition *in vitro* toward several plant pathogens.

Mepanipyrim completely protected plants from cucumber gray mold down to a concentration of 10 μg/ml in pot trials (Table 1); however, mepanipyrim was not effective in controlling cucumber anthracnose (*C. lagenarium*) or rice brown spot (*C. miyabeanus*), even at 300 μg/ml. The protective values against *C. lagenarium* or *C. miyabeanus* are too low to be practical for disease control. Although the mycelial growth of *B. cinerea* (16% of control) or *C. lagenarium* (77% of control) was inhibited to some degree by mepanipyrim, the growth of *C. miyabeanus* was essentially prevented at 300 μg/ml.

There are three evident responses of plant pathogenic fungi to mepanipyrim: high efficacy *in vivo* with weak inhibition *in vitro* (*e.g.*, *B. cinerea*), low efficacy *in vivo* with weak inhibition *in vitro* (*e.g.*, *C. lagenarium*), and low efficacy *in vivo* with strong inhibition *in vitro* (*C. miyabeanus*). A consistently high level of efficacy both *in vivo* and *in vitro* was not observed for any of the plant pathogens in our studies.[1]

As the effectiveness of many fungicides, such as benthiavalicarb-isopropyl and mepronil, can generally be predicted from inhibitory activity *in vitro*, it is interesting that mepanipyrim could have unique mechanisms for mycelial growth inhibition *in vitro* and disease control efficacy *in vivo*.

*2. Effect on uptake of radioactive substrates*

Since mepanipyrim inhibits the uptake of growth substrates such as amino acids and glucose by *B. cinerea,*[3] the next attempt examined the uptake of these substrates to demonstrate whether uptake inhibition is related with the difference of mycelial inhibitory activity among these pathogens.

In the untreated control, there were uptake differences of amino acids and glucose among the three tested pathogens; however, preincubation with mepanipyrim resulted in the dose-responsive inhibition of glucose and phenylalanine uptake by each of the three fungi at higher doses of 10–100 μg/ml (Tables 2 and 3), which did not correspond to either mycelial growth inhibition *in vitro* and disease control efficacy *in vivo* by mepanipyrim. Differences in uptake at higher doses suggest that the effects of mepanipyrim are nonspecific and thus unrelated to disease control modes of action.

Inhibition of nutrient import at some level could bring about chronic nutritional deficiency, resulting in mycelial growth retardation. Therefore, these inhibitions are suggested to relate to mycelial growth inhibition by mepanipyrim at a higher concentration. Mepanipyrim at 10 μg/ml exhibited 98.2% inhibition against the mycelial growth of *C. miyabeanus*, although the inhibition rates of the uptake of glucose and phenylalanine are 45.0% and 33.2%, respectively. Thus, the question as to why mepanipyrim more strongly inhibited mycelial growth of *C. miyabeanus* remained.

*3. Uptake of mepanipyrim*

Inhibiting the uptake of growth substrates by mepanipyrim did not necessarily relate to efficacy differences *in vitro* and *in vivo* among the tested pathogens; however, it remained a possibility that the amounts of mepanipyrim taken into the mycelia differed among these pathogens. To address this possibility, the uptake of $^{14}C$-mepanipyrim into the mycelia was examined.

**Table 2.** Effect of mepanipyrim on uptake of $^{14}$C-glucose into mycelia by *B. cinerea*, *C. miyabeanus* and *C. lagenarium*[a)]

| Concentration (μg/ml) | Radioactivity (dpm)[b)] | | | | | |
|---|---|---|---|---|---|---|
| | *B. cinerea* | | *C. miyabeanus* | | *C. lagenarium* | |
| 100 | 93,691 d | (48.9) | 39,027 d | (41.0) | 15,377 d | (13.8) |
| 10 | 179,866 c | (93.8) | 52,326 c | (55.0) | 79,564 c | (71.4) |
| 1 | 193,220 a | (100.8) | 99,573 a | (104.7) | 100,607 b | (90.3) |
| 0.1 | 189,167 b | (98.7) | 89,672 b | (94.3) | 111,526 a | (100.2) |
| 0 | 191,679 a | (100.0) | 95,138 ab | (100.0) | 111,358 a | (100.0) |

[a)] Spores of *B. cinerea*, *C. miyabeanus* and *C. lagenarium* were incubated in C-medium with shaking for 24 hr at 20°C (*B. cinerea* and *C. lagenarium*) or 27°C (*C. miyabeanus*). Harvested mycelia were washed three times with distilled water. Washed mycelia (24 mg wet weight) were incubated in Czapek medium with radioactive precursors for 30 min after 1 hr pre-incubation with or without mepanipyrim. Incorporation of the precursor into 5% TCA soluble fraction was measured. Figures (dpm) followed by a common letter do not differ significantly according to Tukey's multiple range test ($p<0.05$). [b)] Values in parentheses indicate % of the control.

No difference was evident in the uptake of labeled mepanipyrim among *B. cinerea*, *C. miyabeanus* and *C. lagenarium* for 60 min incubation (Table 4). The uptake of mepanipyrim in *C. miyabeanus* according to the incubation time from 15 min to 90 min was similar to that in *B. cinerea* (data not shown). The data suggested that mycelial uptake is not a critical requirement for mepanipyrim action *in vivo* or *in vitro*, while it remains to be clarified whether the difference in fungal metabolism or detoxification could affect its disease control efficacy.

### 4. *Effect on pectinase secretion*

Since mepanipyrim strongly inhibited pectinase secretion in *B. cinerea*,[4)] the next attempt examined the pectinase secretion of these pathogens to demonstrate whether secretory inhibition is related with selective activity *in vivo* or *in vitro* among these pathogens.

Although mepanipyrim strongly inhibited pectinase secretion by *B. cinerea*, pectinase secretion by the other two fungi was either not affected, or changes were not detected because the secretion levels were near the baseline levels in the control cultures (Table 5).

This fungicide provided good protection against *Botrytis*, but not against *Cochliobolus* or *Colletotrichum*, a trend which is not consistent with the possible modes of action related to the uptake of either the compound itself or the nutrients glucose and phenylalanine. Only the inhibitory activity against pectinase secretion distinguished sensitive fungal pathogens from insensitive fungal pathogens *in vivo*.

Since *B. cinerea* has long been known to produce and secrete pectinase, and cause the maceration of infected tissue,[12–14)] it might produce and secrete more pectinases than other pathogens. As shown in Table 5, secreted pectinases of the untreated control by *C. miyabeanus* and *C. lagenarium* were lower than those by *B. cinerea*. The data suggested that pectinases and their secretion are more important for *B.*

**Table 3.** Effect of mepanipyrim on uptake of $^{14}$C-phenylalanine into mycelia by *B. cinerea*, *C. miyabeanus* and *C. lagenarium*[a)]

| Concentration (μg/ml) | Radioactivity (dpm)[b)] | | | | | |
|---|---|---|---|---|---|---|
| | *B. cinerea* | | *C. miyabeanus* | | *C. lagenarium* | |
| 100 | 3,924 c | (43.9) | 5,746 d | (34.6) | 1,793 d | (15.6) |
| 10 | 7,539 b | (84.3) | 11,093 c | (66.8) | 7,223 c | (63.0) |
| 1 | 8,865 b | (99.1) | 14,879 b | (89.6) | 12,545 a | (109.3) |
| 0.1 | 10,750 a | (120.1) | 16,585 a | (99.9) | 10,500 b | (91.5) |
| 0 | 8,947 b | (100.0) | 16,600 a | (100.0) | 11,473 ab | (100.0) |

[a)] Spores of *B. cinerea*, *C. miyabeanus* and *C. lagenarium* were incubated in C-medium with shaking for 24 hr at 20°C (*B. cinerea* and *C. lagenarium*) or 27°C (*C. miyabeanus*). Harvested mycelia were washed three times with distilled water. Washed mycelia (26.5 mg wet weight) were incubated in Czapek medium with radioactive precursors for 30 min after 1 hr pre-incubation with or without mepanipyrim. Incorporation of the precursor into 5% TCA soluble fraction was measured. Figures (dpm) followed by a common letter do not differ significantly according to Tukey's multiple range test ($p<0.05$). [b)] Values in parentheses indicate % of the control.

**Table 4.** Uptake of $^{14}$C-mepanipyrim by mycelia of *B. cinerea*, *C. miyabeanus* and *C. lagenarium*

| Test fungi | Radioactivity (dpm)[a] |
|---|---|
| *B. cinerea* | 15,828±212[b] |
| *C. miyabeanus* | 12,804±331 |
| *C. lagenarium* | 22,264±212 |

[a] Spores of *B. cinerea*, *C. miyabeanus* and *C. lagenarium* were incubated in C-medium with shaking for 24 hr at 20°C (*B. cinerea* and *C. lagenarium*) or 27°C (*C. miyabeanus*). Harvested mycelia were washed three times with distilled water. Washed mycelia (10 mg wet weight) were incubated in Czapek G medium with radioactive precursors for 60 min. Incorporation of $^{14}$C-mepanipyrim into 5% TCA soluble fraction was measured. [b] Means±SD ($n=2$).

*cinerea* than other pathogens.

In our previous study,[4] mepanipyrim significantly inhibited the secretion of protease and lipase by *B. cinerea*. It also influenced pectinase secretion by *A. alternata* apple pathotype, an applicable disease for mepanipyrim. Cumulatively, it is possible that the secretory repression of proteins which contain host-cell wall-degrading enzymes for pathogenicity is an important mechanism utilized by mepanipyrim in disease control. A further investigation of the secretory inhibition of several proteins by other sensitive pathogens *in vivo* may shed light on the selectivity of target diseases by mepanipyrim.

Mepanipyrim specifically inhibited the secretion of pectinase in sensitive pathogens *in vivo* and exhibited non-specific action on various fungal pathogens by inhibiting the uptake of growth substrates. Thus, there are two possible reasons why antifungal activities *in vitro* are not predictive of disease control efficacy.

**Table 5.** Effect of mepanipyrim on pectinase secretion by *B. cinerea*, *C. miyabeanus* and *C. lagenarium*[a]

| Concentration ($\mu$g/ml) | Secreted pectinase activity (units / mg protein) | | |
|---|---|---|---|
| | *B. cinerea* | *C. miyabeanus* | *C. lagenarium* |
| 100 | nt | 0.44 | 0.22 |
| 10 | nt | 0.57 | 0.26 |
| 1 | 0.13 b | 0.41 | 0.28 |
| 0.1 | 0.23 b | nt | nt |
| 0 | 4.00 a | 0.40 | 0.27 |

[a] Spores of *B. cinerea*, *C. miyabeanus* and *C. lagenarium* were incubated in Czapek P medium with shaking at 20°C (*B. cinerea* and *C. lagenarium*) or 27°C (*C. miyabeanus*) for 45.5, 48 and 47 hr, respectively. Pectinase activity was measured using the culture filtrate. Lower case letters indicate values that are either significantly different or not significantly different according to Tukey's multiple range test ($p<0.05$). There was no significant difference in pectinase secretion by *C. miyabeanus* and *C. lagenarium*. "nt": Not tested.

However, mepanipyrim hardly protected against rice brown spot (caused by *C. miyabeanus*) at 10–1 $\mu$g/ml, while it strongly inhibited the mycelial growth of the pathogen. The above possible reasons are assumed to not be applicable to the difference between *in vitro* and *in vivo* efficacy of *C. miyabeanus*; therefore, another action mechanism of mepanipyrim against inhibition in *C. miyabeanus* is required.

Many fungicides for gray mold, such as benzimidazole and dicarboximide fungicides, can control the diseases caused by *Sclerotinia sclerotiorum*,[15] but mepanipyrim did not effectively control these diseases.[1] Although mepanipyrim prevented peach scab caused by *Cladosporium carpophilum*, it failed to control tomato leaf mold caused by a related fungus (*C. fulvum* is now *Fulvia fulva*).[1] Mepanipyrim can thus be used to identify differences in these fungi by its controlling activity *in vivo*. Since the processes of protein secretion are shown to be conserved among eukaryotes,[16,17] a mepanipyrim binding site would likely be quite specific. The next goal of our investigation is to clarify the selective mechanisms by mepanipyrim in the process of protein secretion.

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**References**

1) S. Maeno, I. Miura, K. Masuda and T. Nagata: *Proc.; Brighton Crop Protection Conference-Pests and Diseases 1990* **2**, 415–422 (1990).
2) T. Nagata, K. Masuda, S. Maeno and I. Miura: *Pest Manag. Sci*. **60**, 399–407 (2003).
3) I. Miura, T. Kamakura, S. Maeno, T. Nagata, S. Hayashi and I. Yamaguchi: *J. Pestic. Sci*. **19**, 103–109 (1994).
4) I. Miura, T. Kamakura, S. Maeno, S. Hayashi and I. Yamaguchi: *Pestic. Biochem. Physiol*. **48**, 222–228 (1994).
5) M. Takagaki, I. Miura and K. Nagayama: *J. Pestic. Sci*. **29**, 369–371 (2004).
6) M. Hori and K. Kakiki: *J. Pestic. Sci*. **2**, 345–355 (1997) (in Japanese).
7) T. Shimizu, T. Nakao, Y. Suda and H. Abe: *J. Pestic. Sci*. **17**, 39–46 (1992).
8) Y. Miyake, J. Sakai, M. Shibata, N. Yonekura, I. Miura, K. Kumakura and K. Nagayama: *J. Pestic. Sci.* **30**, 390–396 (2005).
9) Y. Sekizawa and T. Watanabe: *J. Pestic. Sci*. **6**, 247–255 (1981) (in Japanese).
10) K. Yoshioka, H. Nakashita, D. F. Klessig and I. Yamaguchi: *Plant J*. **25**, 149 –157 (2001).
11) I. Yamaguchi, S. Sekido and T. Misato: *J. Pestic. Sci*. **7**, 523–529 (1982).
12) P. DI Lenna and A. H. Fielding: *J. Gen. Microbiol*. **129**, 3015–3018 (1983).
13) K. Verhoeff and J. M. Warren: *Neth. J. Path*. **78**, 179–185

(1972).
14) K. Verhoeff: *Ann. Phytopathol*. **10**, 137–144 (1978).
15) Y. Hisada and Y. Kawase: *Ann. Phytopath. Soc. Japan* **43**, 151–158 (1977).
16) S. Ferro-Novick and R. Jahn: *Nature* **370**, 191–193 (1993).
17) Y. Misumi, Y. Misumi, K. Miki, A. Takatsuki, G. Tamura and Y. Ikehara: *J. Biol. Chem*. **261**, 11398–11403 (1986).