Mepanipyrim, \(N\)-(4-methyl-6-prop-1-ynylpyrimidin-2-yl) aniline, is a broad spectrum anilinopyrimidine fungicide that controls various plant diseases caused by *Botrytis cinerea*, *Venturia* spp., *Monilinia fructicola*, *Alternaria alternata*, *Erysiphe* spp. and *Podosphaera* spp.\(^1,2\) Studies on its action against *B. cinerea* have found that mepanipyrim at 1 \(\mu g/ml\) inhibited the secretion of host-cell wall-degrading enzymes, leading to their accumulation in the cell.\(^3\) Many fungal pathogens would be prevented from invading plant tissues if the secretion of host-cell wall-degrading enzymes were blocked. Mepanipyrim also prevented the secretion of lipase, protease and invertase in *B. cinerea*,\(^3\) and is thought to affect the intracellular transport process of secretory proteins. Brefeldin A is a typical inhibitor of intracellular transport of secretory proteins,\(^4,5\) causing disassembly of the Golgi complex and the accumulation of secretory proteins in the endoplasmic reticulum.\(^4,5\) Vacuolar-type ATPase inhibitors such as monensin and concanamycin also affected intracellular transport of secretory proteins,\(^6\) and are antibiotic. Few inhibitors of protein secretion have been chemically synthesized. Mepanipyrim is thought to be the first agrochemical to inhibit protein secretion.

Unlike many other fungicides, disease control in the field of mepanipyrim is not logically predicted by its relatively low fungicidal or fungistatic activity *in vitro*. Mepanipyrim hardly inhibited the spore germination of *B. cinerea* at 100 \(\mu g/ml\) and mycelial growth even at 300 \(\mu g/ml\) (its inhibition rates were 9.4% after 24 hr of incubation and 84.2% after 3 days of incubation, respectively), although it exhibited marked disease control activity against *B. cinerea* at 3–10 \(\mu g/ml\) in a pot test. However, mepanipyrim strongly affected the infection process of *B. cinerea* to cucumber at 1–10 \(\mu g/ml\), especially germ tube elongation, secondary appressorium formation and penetration.\(^1\) Triforine and buthiobate, 14-\(\alpha\)-demethylase inhibitor (DMI) fungicides, hardly inhibited the spore germination of various fungi, while they strongly inhibited germ tube elongation and mycelial growth.\(^7,8\) Mepanipyrim also did not affect spore germination and it did not fully inhibit mycelial growth despite its strong inhibition of germ tube elongation. In our previous study,\(^9\) mepanipyrim was assumed to generally retard the development and growth of *B. cinerea* rather than to kill or to fully inhibit it. For example, secondary appressorium formation of *B. cinerea* was delayed by 48–120 hr after incubation in the presence of 1–10 \(\mu g/ml\) mepanipyrim. Similar retardation occurs in spore germination. Mepanipyrim at 100 \(\mu g/ml\) moderately inhibited spore germination of *B. cinerea* in the early stages (67.2% at 4 hr).\(^9\) The factor of time might be an important key to clarify the biological activities.

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**Original Article**

**Effect of culture age on mepanipyrim-mediated inhibition of pectinase secretion in *Botrytis cinerea***

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The fungicide mepanipyrim tended to affect both mycelial growth and pectinase secretion of younger *B. cinerea* mycelia more strongly than older cultures, which is suggestive of its mode of action. Mepanipyrim was more effective in inhibiting pectinase secretion and in disease control activity against *B. cinerea* in the early stages of growth and infection. The uptake of mepanipyrim into the mycelia of *B. cinerea* was reduced as the culture aged and was inhibited by the mitochondrial uncoupling agent CCCP. This suggested that active energy metabolism results in greater mepanipyrim uptake, resulting in good inhibition of mycelial growth, pectinase secretion and disease control in younger *B. cinerea*. © Pesticide Science Society of Japan

Keywords: anilinopyrimidine, mepanipyrim, fungicide, *Botrytis cinerea*, mode 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 was prepared in methanol (2000–20,000 µg/ml) and diluted appropriately for each assay. A 40% suspension concentrate formulation (Frupica®) of mepanipyrim was also used for in vivo assays. [U-14C]mepanipyrim (14C-Be-mepanipyrim, 0.562 GBq/mmol) was synthesized from [U-14C]-aniline by Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan).

B. cinerea (Kumiai stock culture No. 50-01) was grown and maintained on a potato-sucrose agar (PSA) plate at 20°C, and was illuminated with near-ultraviolet light (FL15B, Toshiba Lighting & Technology Co., Tokyo, Japan) for 2–3 days to induce spore formation. Spores were harvested by autoclaved paintbrush with sterilized distilled water and obtained by filtration with sterilized double gauze.

2. Mycelial growth assay in liquid culture
Spores of B. cinerea were suspended in a liquid medium containing 0.3% yeast extract and 2% glucose (YG-medium), and incubated with reciprocal shaking in the absence of mepanipyrim for 4 or 24 hr at 20°C. Mycelia were harvested by filtration through a filter paper (No. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) using an aspirator, and washed thoroughly with distilled water. Washed mycelia (17 mg wet weight) were resuspended in YG-medium with or without mepanipyrim. After 48 hr incubation with reciprocal shaking at 20°C, mycelia were harvested by filtration through the filter paper and washed with distilled water. Harvested mycelia were dried at 60°C overnight, and the dry weight was measured. Three replications were made for each treatment.

3. Plants and disease assays
Cucumber plants (Cucumis sativa cv. Sagami-hanjiro) at the second or third leaf stage were employed for disease assays. Freshly harvested leaves were placed on wet filter papers in a plastic box. Paper discs (6 mm dia., thick type; Toyo Roshi Kaisha, Ltd.) dipped in a spore suspension of B. cinerea (1×10⁶ spores/ml, containing 0.3% yeast extract and 2% glucose) were placed on the leaves and incubated at 20°C. The paper discs were removed after 0, 6, 12, or 18 hr and new paper discs containing mepanipyrim in a solution with 0.3% yeast extract and 2% glucose were placed on the site of inoculation. Two days after inoculation, the lesion diameter was measured. The efficacy of disease control by mepanipyrim was assessed as a percentage of reduced lesion size in comparison with untreated plants. Three replications were made for each treatment.

Twenty-four hours after inoculation, part of the inoculated and treated leaves was excised, fixed and decolored in formaldehyde/ethanol/acetic acid (1/1/1, v/v; FAA) to observe the mycelia of B. cinerea on the leaves by light microscopy. Degrees of mycelial growth were investigated with four indexes (A: Spores did not germinate; B: Spores germinated, but mycelia were very short; C: Mycelia developed moderately, but secondary appressoria were not observed; D: Mycelia developed long and secondary appressoria were observed).

4. Pectinase secretion assay
Two slightly different spore preparation methods were used. Spores of B. cinerea were suspended in Czapek liquid medium (0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl and 0.001% FeSO₄·7H₂O, w/v) supplemented with 0.5% sodium polypectate as the sole carbon source (Czapek P medium) and incubated with shaking in the absence of mepanipyrim for 6–48 hr at 20°C. Mycelia were washed three times with distilled water by centrifugation, resuspended in Czapek P medium and incubated by shaking with and without mepanipyrim for 48 hr at 20°C.

For another experiment, spores of B. cinerea were suspended in Czapek P liquid medium supplemented with 0.1% yeast extract. The culture was incubated with shaking in the absence as well as the presence of mepanipyrim for 31 hr at 20°C. Mycelia were washed three times with distilled water by centrifugation and resuspended in Czapek P medium supplemented with 10 µg/ml of blasticidin S (Wako Pure Chemical Industries, Ltd., Osaka, Japan), an inhibitor of protein biosynthesis, to prevent newly biosynthesized pectinase and incubated by shaking in the absence as well as the presence of mepanipyrim for 6 hr at 20°C.

After incubation, mycelia were harvested 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 for pectinase secretion were described previously.³ Data presented are the means of two replications.

5. Uptake of mepanipyrm
Spores of B. cinerea were inoculated into Czapek liquid medium supplemented with 0.3% yeast extract and 2% glucose (Czapek YG medium), and incubated by shaking in the absence of mepanipyrim for 6–48 hr at 20°C. Mycelia were washed three times with distilled water by centrifugation. Washed mycelia (10 mg or 25 mg wet weight) were resuspended in Czapek liquid medium supplemented with 0.5% glucose. In assays using 10 µM of the mitochondrial uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP, Wako Pure Chemical Industries, Ltd.), mycelia were incubated in the medium for 30 min prior to labeling. Radioisotopically labeled mepanipyrim was added to give a final concentration of
3.7 KBq/ml (1.47 μg/ml). After 60 min, labeling was stopped by adding cold distilled water with a cold chase of non-labeled mepanipyrim (NLM-solution) at 10 μg/ml. Labeled mycelia were then washed three times with NLM solution by centrifugation, suspended in cold 5% trichloroacetic acid (TCA) and kept on ice overnight. Major radioisotopic activities extracted by TCA were assumed to be proportional to the mepanipyrim 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, Boston, MA, USA). Two or three replications were made for each treatment.

Results

1. Effects of mepanipyrim on mycelial growth of B. cinerea in liquid culture

Although mepanipyrim strongly inhibited germ tube elongation of B. cinerea, it did not fully inhibit mycelial growth. When the difference between mycelium and germ tube could be characterized as the difference in the growth period after spore germination, it remained a possibility that younger mycelia are more affected by mepanipyrim than older ones. To address this possibility, the effect of mepanipyrim against mycelial growth under different cultural ages was examined.

Mepanipyrim (1 and 10 μg/ml) more strongly inhibited mycelial growth when mycelia were pre-incubated for 4 hr before treatment than for 24 hr (Fig. 1). A similar inhibition trend was observed with pre-incubation for 8 hr (70.0% inhibition) and 48 hr (54.3% inhibition) at 1 μg/ml (data not shown). These results suggested that the activity of mepanipyrim on mycelial growth was influenced by the age of the culture.

2. Effects of mepanipyrim on control of cucumber gray mold at different application timings

Next, we assessed the connection between disease controlling efficacy and treatment timing by mepanipyrim. When mepanipyrim was applied within 6 hr after inoculation, there was excellent disease control efficacy against cucumber gray mold.

![Fig. 1. Effect of mepanipyrim on mycelial growth of Botrytis cinerea. Spores of B. cinerea were incubated with shaking in YG-medium for 4 or 24 hr at 20°C. Harvested mycelia (17 mg wet weight) were resuspended in YG-medium and incubated with or without mepanipyrim for 48 hr at 20°C. Harvested mycelia were dried at 60°C overnight, and dry weight was measured. Horizontal bars represent standard deviations of the mean (n=3).](image)

<table>
<thead>
<tr>
<th>Application timing (hr after inoculation)</th>
<th>Lesion diameter (cm)a)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>0</td>
<td>0 (100.0)</td>
</tr>
<tr>
<td>B)b)</td>
<td>B</td>
</tr>
<tr>
<td>6</td>
<td>0 (100.0)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>12</td>
<td>0.43±0.06 (63.6)</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>18</td>
<td>0.60±0.00 (51.2)</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

a) Cucumber plants (Cucumis sativa cv. Sagami-hanjiro) at the second or third leaf stage were employed for disease assays. Freshly harvested leaves inoculated with the spores of Botrytis cinerea were placed on wet filter papers in a plastic box. Lesion diameter was measured two days after inoculation. b) Means±SD (n=3). Values in parentheses indicate % of the control. c) Concentration of mepanipyrim. d) Inoculated and treated leaves were detached 24 hr after inoculation, fixed and decolored in FAA, and observed under light microscopy. Degrees of mycelial growth were estimated with four indexes. A: Spores did not germinate; B: Spores germinated, but mycelia were very short; C: Mycelia developed moderately, but secondary appressoria were not observed; D: Mycelia developed long and secondary appressoria were observed.
Table 2. Effect of mepanipyrim on *Botrytis cinerea* pectinase secretion at different ages of culture

<table>
<thead>
<tr>
<th>Mepanipyrim (µg/ml)</th>
<th>Secreted pectinase activity (unit/mg protein)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hr pre-incubated mycelia</td>
</tr>
<tr>
<td>1</td>
<td>1.34±0.11 (19.4)</td>
</tr>
<tr>
<td>0</td>
<td>6.89±0.14 (100)</td>
</tr>
</tbody>
</table>

¹ Means±SD (n=2). Spores of *B. cinerea* were incubated in Czapek P medium with shaking for 6–48 hr at 20°C. Harvested mycelia were resuspended in Czapek P medium and incubated with or without mepanipyrim for 48 hr at 20°C. Pectinase activity was measured following Miura et al.³ Values in parentheses indicate % of the control.

(Table 1); however, efficacy was significantly reduced when it was applied after 12 hr. The degree of mycelial growth on cucumber leaves was observed under a light microscope 24 hr after inoculation. Spores started to germinate 3 hr after inoculation and secondary appressoria started to form at 24 hr, and a lesion was recognized 36 hr after inoculation. Light microscopy confirmed the absence of secondary appressoria and moderate mycelial growth 24 hr after inoculation when mepanipyrim was applied within 6 hr of inoculation, leading to full control. When mepanipyrim was treated 0 hr after inoculation, spores germinated as well as in the mock control, but mycelia elongated very shortly and secondary appressoria were not observed. Treatment with mepanipyrim 12 hr after inoculation resulted in long developed mycelial and secondary appressorium formation like the untreated control. These results suggest that mepanipyrim application is only effective to provide good disease control in the very early stages of fungal infection.

3. Effects of mepanipyrim on *B. cinerea* pectinase secretion

Since the activity of mepanipyrim on mycelial growth was influenced by the age of the culture, an attempt was made to demonstrate whether the inhibition of pectinase secretion by mepanipyrim is related with the age of the culture. Mepanipyrim inhibited mycelial pectinase secretions in an age-dependent manner (Table 2). Pectinase secretion was most affected in the youngest cultures, and the oldest cultures were nearly the same as untreated controls, reflecting the normal decrease in enzyme secretion as the culture ages.

The next attempt examined which treatment timing of mepanipyrim was more effective to inhibit pectinase secretion. As shown in Fig. 2, mepanipyrim strongly inhibited pectinase secretion both pre- and post-treatment, only pre-treatment, and only post-treatment in order. Pectinase activity 6 hr after incubation is due to enzymes that were synthesized and accumulated during the 31 hr pre-incubation period. Contamination from *de novo* pectinase activity was eliminated by the washing and resuspension in blasticidin S. After 31 hr pre-incubation, levels of secreted pectinases from cells pre-treated with mepanipyrim (second bar in Fig. 2) were much lower than cells which had not received pre-incubation treatment (third bar in Fig. 2).

Since mepanipyrim inhibited the secretion of pectinase and accumulated it in the cells,³ the pectinase accumulated during pre-incubation with mepanipyrim must exceed those without mepanipyrim and pre-incubation-treated mycelia have a large potential for pectinase secretion; however, secreted pectinases from mycelia treated with mepanipyrim only during pre-incubation were significantly lower than those treated only during post-incubation. Mepanipyrim treatment is assumed to be more effective to inhibit pectinase secretion in the very early stages of fungal growth, like its disease control activity described above.

4. Uptake of mepanipyrim by mycelia of *B. cinerea*

Cumulatively, the results described above indicate that in-
The uptake of mepanipyrim into mycelia appears to have a greater effect on younger mycelia than older mycelia. In this study, we focused on the relationship between mepanipyrim uptake and the age of the mycelium.

Incorporation of 14C-mepanipyrim into 5% TCA soluble fraction was measured. These data suggest that mepanipyrim is mainly taken into mycelia through active transport.

### Discussion

In this study, we focused on the relationship between mepanipyrim activity and the age of *Botrytis cinerea* cultures. Mepanipyrim appears to have a greater effect on younger mycelia for both growth and pectinase secretion than older mycelia.

### Table 3. Uptake of 14C-mepanipyrim by mycelia of *Botrytis cinerea* at different ages of culture

<table>
<thead>
<tr>
<th>Mycelial culture age (hr of pre-incubation)</th>
<th>Radioactivity/mg mycelial wet weight (dpm)</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>1431±36</td>
</tr>
<tr>
<td>12</td>
<td>1207±12</td>
</tr>
<tr>
<td>24</td>
<td>1582±21</td>
</tr>
<tr>
<td>48</td>
<td>471±26</td>
</tr>
</tbody>
</table>

(a) Means±SD (n=2). Spores of *B. cinerea* were incubated in Czapek YG medium with shaking for 6–48 hr at 20°C. Harvested mycelia were washed three times with distilled water and the washed mycelia (10 mg wet weight) were labeled for 60 min. Incorporation of 14C-mepanipyrim into 5% TCA soluble fraction was measured.

### Table 4. Effect of carbonylcyanide m-chlorophenylhydrazone (CCCP) on uptake of 14C-mepanipyrim by mycelia of *Botrytis cinerea* a)

<table>
<thead>
<tr>
<th>CCCP (μM)</th>
<th>Radioactivity/mg mycelial wet weight (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>361 b (28.7)</td>
</tr>
<tr>
<td>0</td>
<td>1259 a (100.0)</td>
</tr>
</tbody>
</table>

(a) Spores of *B. cinerea* were inoculated in Czapek YG medium with shaking for 24 hr at 20°C. Harvested mycelia were washed three times with distilled water, and washed mycelia (25 mg wet weight) were labeled for 60 min after 30 min pre-incubation with CCCP. Incorporation of 14C-mepanipyrim into 5% TCA soluble fraction was measured. Figures [dpm] (n=3) followed by a common letter do not differ significantly according to Tukey’s multiple range test (p<0.05). a) Mitochondrial uncoupler. a) Values in parentheses indicate % of the control.

Mepanipyrim is usually applied as a protective fungicide because it is not effective enough to be practical for disease control when applied after infection. The present results indicate that mepanipyrim treatment needs to be in the early stage of fungal development before infection and that treatment within 6 hr after inoculation is most effective. Mepanipyrim retards the developmental processes and growth of *B. cinerea* rather than fully inhibiting them or completely inhibiting the secretion of macerating enzymes such as pectinase. Since the action of mepanipyrim is basically a retardant, its inhibitory activity *in vitro* and disease control are far more effective if applied in the early stage of infection. Early application before fungal germination or up to several hours after germination might bring mepanipyrim into a chance contact with younger mycelia and provide enough time for mepanipyrim to retard fungal development before infection.

Differences in the disease control efficacy of mepanipyrim by treatment timing were observed in several pot trials by mepanipyrim. Although mepanipyrim exhibits good translaminar activity on many fungi,1,16) it shows little curative effect on the diseases caused by the pathogens including *B. cinerea*. Weak inhibition against mycelial growth may be indicative of its poor performance on established pathogens. However, mepanipyrim exhibited good palliative activity against apple scab caused by *Venturia inaequalis*, whereas mycelial growth inhibition on *B. cinerea* and *V. inaequalis* is about the same.

Reduced as the cultures aged. Since the uptake of mepanipyrim is inhibited by CCCP, mepanipyrim uptake into mycelia must have an active component. This suggests that more active energy metabolism in young mycelia results in greater mepanipyrim uptake, resulting in stronger inhibition of mycelial growth, inhibition of pectinase secretion and disease control. Respiratory inhibitors and uncouplers (e.g., CCCP) enhanced the accumulation of DMI fungicides in *B. cinerea*, owing to reductions of synthesized ATP and the activity of energy-dependent ATP-binding cassette (ABC) transporters.10,11) As the amount of mepanipyrim in mycelia was reduced by CCCP, it was assumed not to be affected by energy-dependent efflux.

The biological and cytological properties of fungi change from the mycelial base to the top,12,13) and different genes are expressed in the germ tube than in the vegetatively growing mycelium.14,15) Therefore, these differences can affect the activity of mepanipyrim. However, our results suggested that the differences in mepanipyrim activities on germ tubes and mycelia correlate with the age of the culture. Since mepanipyrim strongly inhibited germ tube elongation against *B. cinerea* 24 hr after germination, it could inhibit not only the germ tube (before first septum formation) but also apical elongated hypha (after nuclear division in the germ tube and first septum formation). Younger mycelia such as germ tubes and apical elongated hyphae may thus actively take in more mepanipyrim, and thus are more significantly affected by it than older mycelia.
(inhibition rates at 100 µg/ml mepanipyrim are 73.1% and 67.7%, respectively). The rates of mycelial growth and disease development of V. inaequalis are significantly smaller than those of B. cinerea (mycelial growth rates of V. inaequalis and B. cinerea are 0.91 mm/day and 24.9 mm/day in PSA, respectively, and the lesions of V. inaequalis and B. cinerea first emerged 13 to 15 days and two days after inoculation, respectively). The slower growth of V. inaequalis may allow mepanipyrim to inhibit earlier disease development. Mepanipyrim (100–30 µg/ml) scarcely inhibited cucumber powdery mildew (Podosphaera xanithii) when applied five days after inoculation and the lesions started to emerge, while it showed almost the same activity as the standard fungicide (100–10 µg/ml quinomethionate) when applied within three days after inoculation (data not shown).

A factor of time such as ageing of pathogenic fungi and treatment timing must be an important key to biological activities of mepanipyrim. While brefeldin A sharply and perfectly inhibited intracellular transport of secretory proteins in mammalian cells, mepanipyrim partially affected these processes in the cells at only higher dosages. Retardant action for fungal growth seen in mepanipyrim can be apart from its mode of action. Retardant actions by mepanipyrim are questions for further studies.

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References