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Note

A Method for Monitoring the Sensitivity of *Botrytis cinerea* to Mepanipyrim

Makiichi TAKAGAKI,* Ichirou MIURA and Kozo NAGAYAMA

Kumiai Chemical Industry Co., Ltd. Life Science Research Institute, 3360 Kamo, Kikugawa, Ogasagun, Shizuoka 439–0031, Japan

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Mepanipyrim is an anilinopyrimidine fungicide with a broad spectrum of activities. Mepanipyrim did not inhibit mycelial growth of *Botrytis cinerea* completely on complex media and therefore, this method is considered not to be useful for evaluation of the sensitivity of isolated *B. cinerea* to mepanipyrim. As a result of our studies, we have established new techniques to determine the fungal sensitivity to mepanipyrim *in vitro* by utilizing the inhibitory activity of mepanipyrim against protein secretion and germ-tube elongation. The FGA-paper disc method is considered to be more useful and more reliable for evaluation of the sensitivity of *B. cinerea* to mepanipyrim.

Keywords: anilinopyrimidine fungicides, mepanipyrim, *Botrytis cinerea*, monitoring method, baseline.

INTRODUCTION

Mepanipyrim [*N*-(4-methyl-6-prop-1-ynylpyrimidin-2-yl)aniline], cyprodinil and pyrimethanil are anilinopyrimidine fungicides with a broad spectrum of activity.[1–4] Anilinopyrimidine fungicides can control fungal diseases caused by *Botrytis cinerea* (gray mold), *Venturia* spp. (scab), *Alternaria mali* (alternaria leaf spot), *Podosphaera leucotricha* (powdery mildew) and others. Their mode of action, however, has not been fully clarified. In *Botrytis cinerea*, mepanipyrim and pyrimethanil are considered to inhibit the secretion of hydrolytic enzymes such as pectinases, cellulases and proteinases in a site-specific manner.[5–7] In contrast, it is reported that cyprodinil inhibits the biosynthesis of methionine and other amino acids in target species.[8] Anilinopyrimidines are considered to inhibit the pathogenesis because the above enzymes are involved in the pathogenic process.

The development of resistant *B. cinerea* to conventional fungicides has been widely recognized, posing increasingly serious problems. It is, therefore, important to set up resistance management programs to reduce potential risks of resistance to new classes of fungicides. A useful, reliable and practical test for evaluating fungal sensitivity is therefore needed for resistance monitoring programs.

The compound mepanipyrim little inhibits spore germination in *B. cinerea* and does not fully inhibit mycelial growth in complex media. The test method used to evaluate these inhibitory activities is considered inappropriate for examining sensitivity. Several methods have so far been proposed to evaluate sensitivity to anilinopyrimidine fungicides. The inhibitory activity of pyrimethanil against the growth of *B. cinerea* was reported to be affected by the composition of the medium.[7] Most of the methods proposed to date are considered too time-consuming to be put to practical use and inappropriate for monitoring resistance on a large scale. Consequently, we have attempted to establish a simple, quick and inexpensive method of evaluating the sensitivity of *B. cinerea* to mepanipyrim for use in resistance monitoring.

MATERIALS AND METHODS

1. Sample Collection and Spore Preparation of B. cinerea

Four hundred and twenty single spore isolates were obtained from a wide range of crops in different locations in Japan where the anirinopyrimidine group of fungicides had never been applied. These isolates were cultured on potato-dextrose agar (PDA Difco) slants at 20°C in the dark for 4 days in an incubator and then stored at 4°C. Spores of the fungi were produced by illuminating with near ultraviolet light for 2–3 days after the 4-day incubation on PDA plates at 20°C. The spores were used for setting a sensitivity baseline of wild types to mepanipyrim and a preliminary monitoring study.

2. Reference Isolates

Three isolates (CH12.92, CH15.93 and CH164.94) of *B. cinerea* provided by Novartis Agro, Basel, Switzerland were tested as resistant strains to anilinopyrimidine fungicides. These resistant strains were collected from grapes in Switzerland. The sensitive strains KU-01 and KU-59, maintained in the Life Science Research Institute of Kumiai, were used as standards. Spores of these reference isolates were prepared in the same manner as the wild types.

3. In Vitro Sensitivity Measurement on FGA Media

3.1. Preparation of basal medium

FGA medium, a synthetic medium containing fructose 10 g, gelatin 2 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $NaNO_3$ 2 g and agar 15 g per liter was prepared. After autoclaving at 121°C for 10 min, 20 ml each of the medium was put into plastic Petri dishes having a diameter of 9 cm.

3.2. Incorporation of fungicide into the medium

Frupica (40% mepanipyrim) SC was used to prepare a series of concentrations of mepanipyrim in the FGA medium. The final

* To whom correspondence should be addressed.
E-mail: m-takagaki@kumiai-chem.co.jp

concentrations in the FGA medium were set at 0, 0.1, 0.3, 1, 3, 10, 30 and 100 mg a.i. per liter.

3.3. Measurement of mycelial growth

The fungal sensitivities to mepanipyrim were determined as follows. Spores of *B. cinerea* were suspended in distilled water (10^5 spores/ml). Paper discs (Toyo Roshi Kaisya, Ltd, 6 mm thick) were dipped in the spore suspensions. The paper discs were then placed upside-down onto plates containing mepanipyrim at different concentrations. The plates were incubated at 20°C for 4 days. The colony diameter in each plate was measured and the average diameter relative to that of the untreated control (100%) was calculated.

4. In Vivo Assay, Strawberry Fruit Gray Mold

Ten surface-sterilized strawberry fruits were each sprayed with a series of concentrations at 0, 50, 100 and 200 mg a.i. per liter of mepanipyrim. After being dried in air (around 2–3 hr), the fruits were inoculated with the spore suspension (10^5 spores/ml). The number of infected fruits was counted after a 5-day incubation in a chamber at 20°C with high humidity.

RESULTS

1. Mycelial Growth Assay

With our new *in vitro* assay method (FGA-paper disc method), the key is to use a unique basal medium, FGA medium consisting of fructose, gelatin and minerals, and to place paper discs dipped in the spore suspension onto the said FGA medium containing mepanipyrim. Using this method, the diameters of colonies were measured after incubation for a certain period of time. Subsequently, EC_{50} values of mepanipyrim for the sensitive strains were placed within a range of 0.01 to 0.07 mg a.i. per liter with a MIC value of 0.3 mg a.i. per liter. In contrast, MIC values for the isolates provided by Novartis were greater than 100 mg a.i. per liter (Table 1).

Using the FGA-paper disc method, preliminary monitoring tests were conducted utilizing 420 isolates sampled from several fields in Japan where no anilinopyrimidine fungicides had ever been applied. MIC values distributed in a range from 0.1 to 3 mg a.i. per liter; *viz* 37.6% of the isolates for 0.1 mg, 51.1% for 0.3 mg, 10.6% for 1 mg and 0.7% for 3 mg, a.i. per liter (Fig. 1). Based on these results, it was concluded that the baseline of sensitivity to mepanipyrim should be set at 3 mg a.i. per liter in wild type isolates of *B. cinerea*.

2. Strawberry Fruit Assay

An *in vivo* assay using strawberry fruits was carried out to find out whether or not the sensitive strains and the three isolates from Novartis are resistant to mepanipyrim. Mepanipyrim showed 100% control of the sensitive strains at 50 mg a.i. per liter. On the other hand, mepanipyrim showed no efficacy against the three isolates from Novartis even at 200 mg a.i. per liter, the highest recommended dosage, revealing significant differences in the sensitivity to mepanipyrim between the sensitive strains and Novartis's resistant isolates (Table 2).

Using the strawberry fruit assay, preliminary monitoring tests were conducted utilizing 100 isolates randomly selected from the 420 isolates. When each selected isolate was inoculated, mepanipyrim at 200 mg a.i. per liter showed perfect control of all the isolates.

Table 1. Mycelial growth inhibition of *B. cinerea* by mepanipyrim on the FGA medium

Mepanipyrim (mg/l)	Inhibition of mycelial growth (%)				
	CH12.92	CH15.93	CH164.94	KU-01	KU-59
0.1	0	0	0	75.5	86.4
0.3	19.8	0	0	100	100
1	42.6	34.9	38.3	100	100
3	60.1	65.7	57.9	100	100
10	65.7	72.6	67.1	100	100
30	63.2	74.1	66.2	100	100
100	70.4	75.4	70.8	100	100

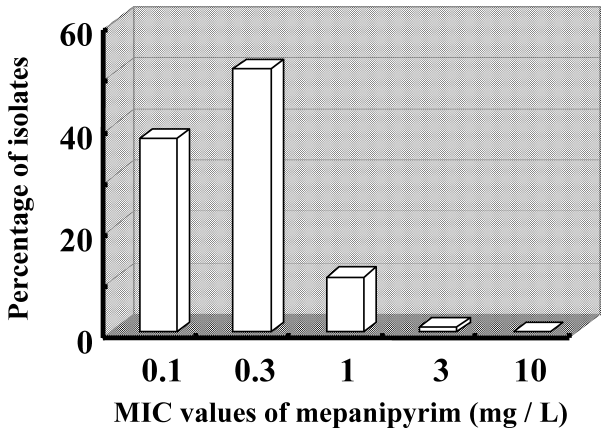

Fig. 1. Distribution of isolates of *B. cinerea* from Japan based on sensitivity to mepanipyrim in a mycelial growth test on the FGA medium. A total of 420 isolates were collected from several fields in Japan where anilinopyrimidine fungicides had never been applied and tested by the FGA-paper disc method. MIC values distributed in a range from 0.1 to 3 mg a.i. per liter; *viz* 37.6% of the isolates for 0.1 mg, 51.1% for 0.3 mg, 10.6% for 1 mg and 0.7% for 3 mg, a.i. per liter.

Table 2. Effect of mepanipyrim on strawberry gray mold

Mepanipyrim (mg/l)	Control (%)				
	CH12.92	CH15.93	CH164.94	KU-01	KU-59
50	0	0	0	100	100
100	0	0	0	100	100
200	0	0	0	100	100

DISCUSSION

Mepanipyrim did not inhibit the mycelial growth of *B. cinerea* completely even at 300 mg a.i. per liter on complex media and therefore, this method is considered not to be useful for evaluation of the sensitivity of isolated *B. cinerea* to mepanipyrim. However, in general, mycelial growth tests are regarded as the most simple, quick and inexpensive means of evaluating the sensitivity of fungicides and for monitoring. As a result of our studies, we have established new techniques to determine the fungal sensitivity to mepanipyrim *in vitro* by utilizing the inhibitory activity of mepanipyrim against protein secretion and germ-tube elongation.

As shown in Table 1, the test results showed that the FGA-paper disc method is well suited to determining the isolates of *B. cinerea* sensitive and resistant to mepanipyrim with good reproducibility. From the results of our preliminary monitoring studies, the sensitivity baseline for mepanipyrim was set at 3 mg a.i. per liter in wild type isolates of *B. cinerea*.

The FGA-paper disc method is thus considered useful and reliable for evaluation of the sensitivities of *B. cinerea* to mepanipyrim. However, it is also recommended that appropriate *in vivo* assay methods be used for confirmation of the resistant strains. This is because mepanipyrm has a unique mode of action to inhibit the secretion of enzymes such as cutinase, pectinase and cellulase which play important roles in pathogenic infection. Wild type isolates that do not show good sensitivity to mepanipyrim at the baseline of 3 mg a.i. per liter, should be re-evaluated using the strawberry fruit assay. Re-evaluation using a quantitative *in vivo* assay can give important indications about the pathogenicity and fitness of such strains, and can be useful in assessing their relevance to field conditions.

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