Research and Development of a Novel Fungicide 'Fenpyrazamine'

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Fenpyrazamine is a novel fungicide with an aminopyrazolinone structure developed by Sumitomo Chemical Co., Ltd. Fenpyrazamine has good fungicidal properties, such as high antifungal activity, preventive efficacy, translaminar ability, inhibition activity in lesion development, long lasting activity and short pre-harvest intervals (PHIs). Fenpyrazamine also shows safer profiles for human health and the environment. Formulated products, PROLECTUS® and PIXIO®DF, have been registered since 2012. PROLECTUS® was first launched in Italy in 2012, and PIXIO®DF was launched in Japan in 2014.

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Introduction

In the agricultural industry worldwide, gray mold (*Botrytis cinerea*) and stem rot (*Sclerotinia sclerotiorum*), which attack the fruits of fruit trees and vegetables, greatly reduce the yields and productivity of crops, so they are one of the most important diseases to control.

Many fungicides, such as benomyl, have been developed up to now to control gray mold. However, the life-cycle of gray mold in particular is short, and many spores are produced; therefore, it is widely known as a disease-causing fungus which easily acquires fungicide resistance.[1)] Therefore, novel fungicides are always expected in the market for gray mold controls.

Fenpyrazamine is a novel fungicide that was discovered and developed by Sumitomo Chemical Co., Ltd., and it has a particularly high efficacy against gray mold, stem rot and brown rot in fruits and vegetables. In terms of its chemical structure, it has an aminopyrazolinone structure (**Fig. 1**), for which there are no previous examples in existing agricultural chemicals. In addition, the only other fungicide having the same mode of action as fenpyrazamine is fenhexamid from Bayer CropScience.

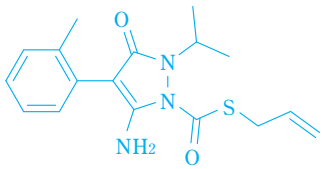

Fig. 1 Chemical structure of fenpyrazamine

In April 2012, Sumitomo Chemical Co., Ltd. received Korean registration for fenpyrazamine fungicide, and thereafter received EU registration for fenpyrazamine and then launched "PROLECTUS®" first in Italy in October 2012. Thereafter, Sumitomo Chemical Co., Ltd also received registration in Switzerland, Austria, Japan, Chile and the United States in 2013 and launched the product in Korea and Chile in the same year. In Japan, Sumitomo Chemical Co., Ltd. launched "Pixio® DF" in January 2014.

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Furthermore, Sumitomo Chemical Co., Ltd. will also receive registration and launch fenpyrazamine in the various EU countries and Australia. In this article, we will report on the history of the discovery of fenpyrazamine, its efficacy, manufacturing method, physical and chemical properties, formulation and safety for mammals and the environment.

Discovery

1. Discovery of a Lead Compound

As a method for discovering new lead compounds with biological activity, we not only made improvements to our original compounds in our chemical library, but also in parallel, evaluated biological activities of compounds which had unique chemical structures and which were introduced from external libraries.

In such evaluation work, a compound introduced from an external library showed a disease controlling efficacy on powdery mildew in wheat. As the compound had a unique chemical structure, we chose it as a lead compound and initiated screening processes such as synthesis and biological evaluations of varieties of compounds. When we investigated carefully the effect of the substituents of the benzene, it was interestingly found that we were able to give the compound a disease controlling efficacy for gray mold by introducing a chlorine atom at the *ortho* position of the benzene ring (compound A). The discovery was an extremely important one for us because we were seeking the next generation of gray mold controlling agent at the time. Then we decided upon the compound A as a lead compound for discovering a gray mold fungicidal compound, and as a result of careful investigation, the following outline of structure activity-relationships for gray mold controlling efficacy became clear (**Fig. 2**).

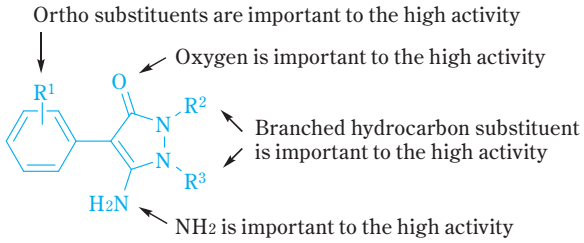
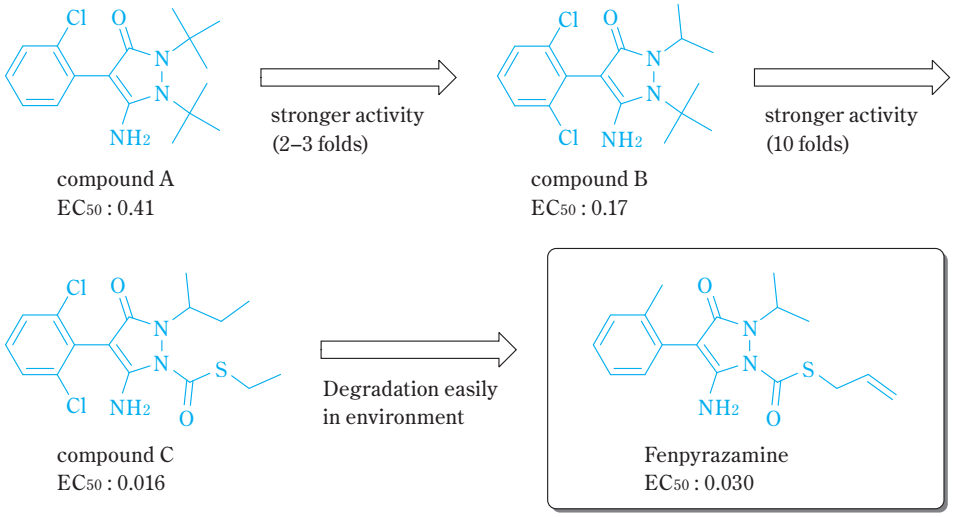


Fig. 2 Structure-activity relationship of aminopyrazolinone derivatives

— The introduction of a substituent at the *ortho* position of the benzene ring is important to high activity in disease control.
— The ketone structure of the pyrazolinone ring is important to high activity in disease control.
— Branched hydrocarbon substituents bonded to the nitrogen atoms at the first and second positions in the pyrazolinone ring is important to high activity in disease control.
— The bonding of an amino group (NH_2) at the fifth position in the pyrazolinone ring is important to high activity in disease control.

As a result of intensive research into the synthesis and biological evaluation of several hundred compounds, we discovered compound B (**Fig. 3**), which showed high activity in disease control.[2)]



EC_{50} : Antifungal activity (mg ai/L) against *Botrytis cinerea*

Fig. 3 Discovery of fenpyrazamine

2. Discovery of Compound C

Through further improvements in optimization of the structures of these compounds, we found that conversion of the branched hydrocarbon group R^3 bound to the nitrogen atom at the first position in the pyrazolinone ring to a $-C(=O)OCH_3$ group with an ester structure, especially to a $-C(=O)SCH_3$ group with a thiol ester structure, drastically improved the efficacy against gray mold.[3] After we re-evaluated a variety of combinations of the substituents (R^2, R^3) bonded to the nitrogen atoms in the first and second positions of the pyrazolinone ring and the substituent R^1 in the benzene, we selected compound C (**Fig. 3**), which had excellent activity in gray mold disease control. Since the activity of compound C appeared to be the same or greater than that of benchmarked products, it was determined to proceed with development of compound C from the standpoint of biological activity.

However, because of the slow degradation of compound C in the soil, further improvements were required.

3. Discovery of Fenpyrazamine

Along with making further modifications to a series of compounds in order to improve on the persistence in the soil, we carried out simple soil residue evaluations in parallel. As a result, we were successful in discovering fenpyrazamine. Fenpyrazamine has a thiol ester group terminally substituted by an allyl group as an unsaturated hydrocarbon, at the nitrogen atom at the first position in the pyrazolinone ring and a methyl group at the *ortho* position in the benzene ring.

While fenpyrazamine has excellent activity in gray mold disease control substantially the same as compound C, it is rapidly degraded in the soil.

Furthermore, as a result of giving comprehensive consideration to various development criteria, we finally selected fenpyrazamine as the compound for development (**Fig. 3**).

Manufacturing Methods

Fenpyrazamine has an allyl thiocarbonyl group and an isopropyl group on the two nitrogen atoms in the pyrazole ring. The practicable manufacturing routes are shown in **Fig. 4**. One route is where an isopropyl group is introduced into the pyrazole ring first, and the other is where an allyl thiocarbonyl group is introduced into the pyrazole ring first. After energetically carrying out investigations, we established an industrial manufacturing method-for fenpyrazamine that regioselectively subsutitutes onto the two nitrogen atoms in the pyrazole ring.

Biological Effects

1. Antifungal Spectrum

Fenpyrazamine shows a high antifungal activity for the Sclerotiniaceae family such as *Botrytis* spp.

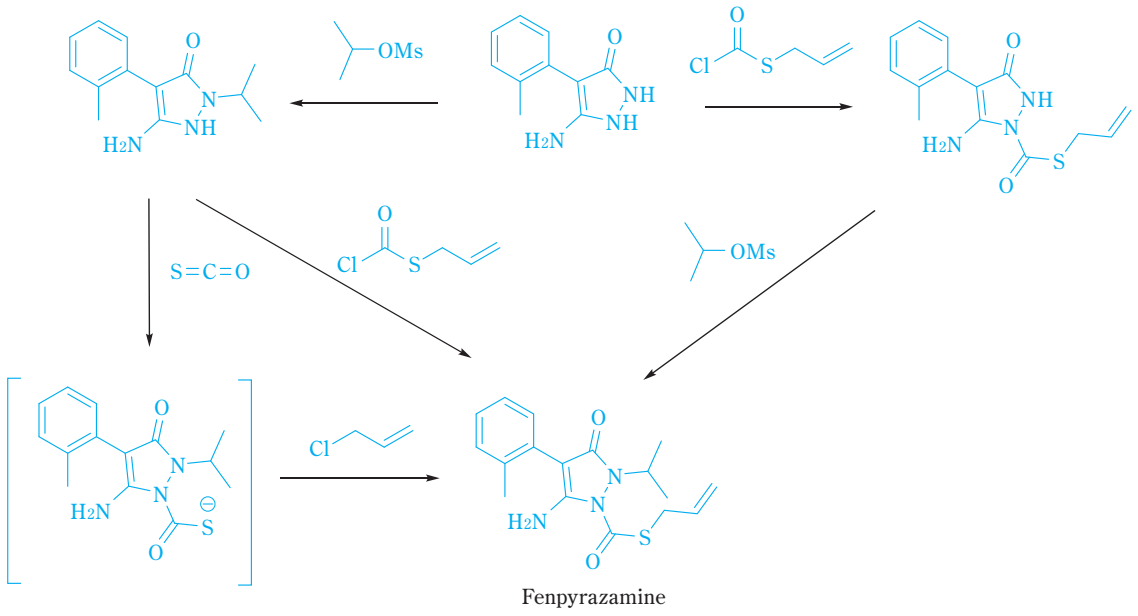


Fig. 4 Synthesis route of fenpyrazamine

(gray mold, etc.) and *Sclerotinia* spp. (Sclerotinia rot, etc.), which are significant diseases in agriculture. (**Table 1**).[4)]

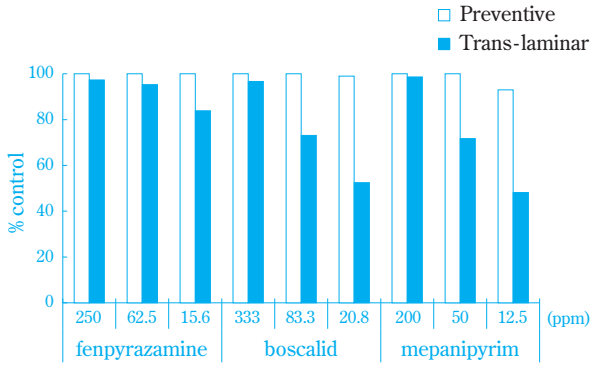
Table 1 Antifungal activity of fenpyrazamine

Fungal species	EC_{50} (mg ai/L)	EC_{90} (mg ai/L)
Botrytis cinerea	0.030	0.14
Botrytis allii	0.030	0.67
Botrytis tulipae	0.030	0.67
Sclerotinia sclerotiorum	0.11	0.47
Sclerotinia minor	0.049	0.25
Sclerotinia triformis	0.012	0.041
Monilinia laxa	0.020	0.15
Monilinia fructigena	0.048	0.31
Monilinia fructicola	0.079	0.58
Magnaporthe grisea	> 5	> 5
Septoria tritici	> 5	> 5
Pythium aphanidermatum	> 5	> 5
Rhizoctonia solani	> 5	> 5
Rhizopus oryzae	> 5	> 5

2. Characteristics of Action

(1) Preventative effect

In potted tests, fenpyrazamine had a high preventive efficacy (% control was 100) against gray mold at a low concentration of 1/16 of the concentration (250 ppm) registered in Japan for gray mold on cucumbers (**Fig. 5**).



Preventive: *Botrytis cinerea* was inoculated one day after fungicide application
Trans-laminar: *Botrytis cinerea* was inoculated on adaxial side of leaves one day after fungicide application on abaxial side of leaves

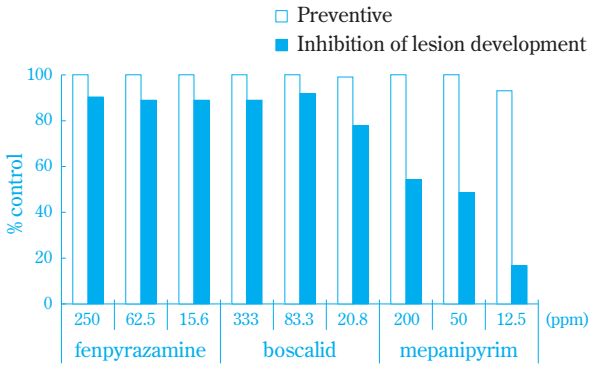
Fig. 5 Trans-laminar ability of fenpyrazamine

(2) Trans-laminar ability

In trans-laminar ability tests (fungicides were applied on the abaxial side of leaves and *Botrytis cinerea* was inoculated on the adaxial side of leaves one day after application.), fenpyrazamine had a high efficacy (% control was more than 80) against gray mold even a low concentrations of 1/16 of the concentration (250 ppm) registered in Japan (**Fig. 5**). This result indicated that fenpyrazamine was rapidly absorbed into the plant after treatment and was transferred to untreated areas. With the trans-laminar ability, fenpyrazamne would be expected to show a good efficacy in controlling gray mold in cases of a little uneven application in the field.

(3) Inhibition activity of lesion development

In inhibition of lesion development tests (*Botrytis cinerea* was inoculated one day before application.), fenpyrazamine had a high efficacy (% control was more than 80) against gray mold even a low concentrations of 1/16 of the concentration (250 ppm) registered in Japan (**Fig. 6**). This result indicated that fenpyrazamine can suppress the disease development at the initial stages of disease after infection. With the inhibition activity of lesion development, fenpyrazamine would be expected to show good efficacy in controlling gray mold on vegetables even with application after 1st symptoms.

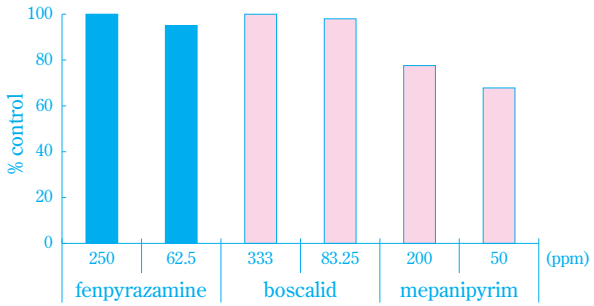


Preventive: *Botrytis cinerea* was inoculated one day after fungicide application
Inhibition of lesion development: *Botrytis cinerea* was inoculated one day before fungicide application

Fig. 6 Fenpyrazamine-mediated inhibition of lesion development

(4) Long lasting activity

In 14-day long lasting activity tests, fenpyrazamine had a high efficacy (% control was more than 90) against gray mold even at a low concentration of 1/4 of the concentration (250 ppm) registered in Japan for gray mold on cucumbers (**Fig. 7**).

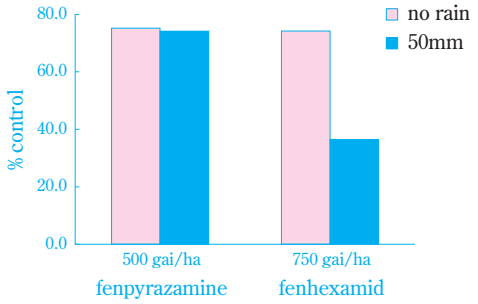


Long lasting: *Botrytis cinerea* was inoculated 14 days after fungicide application

Fig. 7 Long lasting activity of fenpyrazamine

(5) Rainfastness

In rainfastness tests for gray mold on grapes, fenpyrazamine showed a high efficacy against gray mold on grapes (% control was more than 75) under conditions in which artificial rain was dispersed to total of 50mm (25mm/hour) two hours after application (**Fig. 8**).



Rainfall: artificial rainfall was conducted 2 hours after fungicide application
Inoculation: *Botrytis cinerea* was inoculated one day after fungicide application

Fig. 8 Rainfastness of fenpyrazamine on grape

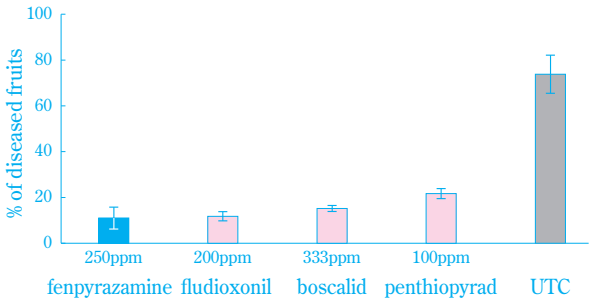
From the results of biological property tests, fenpyrazamine can be expected to have a high efficacy against diseases in practical fields[5]. The following shows evaluations of field tests.

3. Practical Evaluation

(1) Gray mold on eggplant (Japan)

Fenpyrazamine showed a high efficacy against gray mold on eggplant in field tests under curative conditions in which there were some diseased fruits before 1st application and conditions of severe incidence (% of diseased fruits at UTC was more than 70%) (**Fig. 9**).

Fenpyrazamine can be thought of as having high practical performance to control gray mold on eggplant.[5]

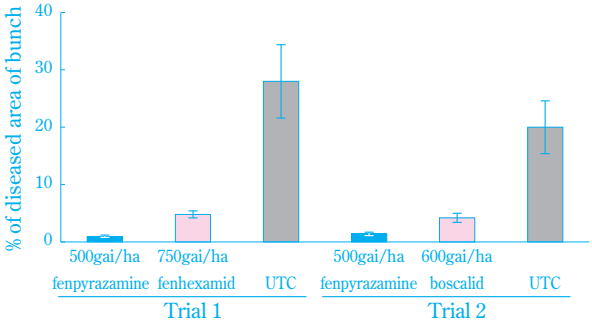


Location: Hyogo prefecture, Japan
Inoculation: One week before 1st application (Curative condition)
Application: 7 days interval application from December 16th to January 31th.
Final assessment: 7 days after final application, February 7th
Error bar: Standard deviation

Fig. 9 Efficacy of fenpyrazamine against gray mold on eggplant

(2) Gray mold on grapes (Italy)

Fenpyrazamine showed a high efficacy against gray mold on grapes in multiple field tests (**Fig. 10**). Fenpyrazamine can be thought of as having high practical performance in controlling gray mold on grapes.[6]



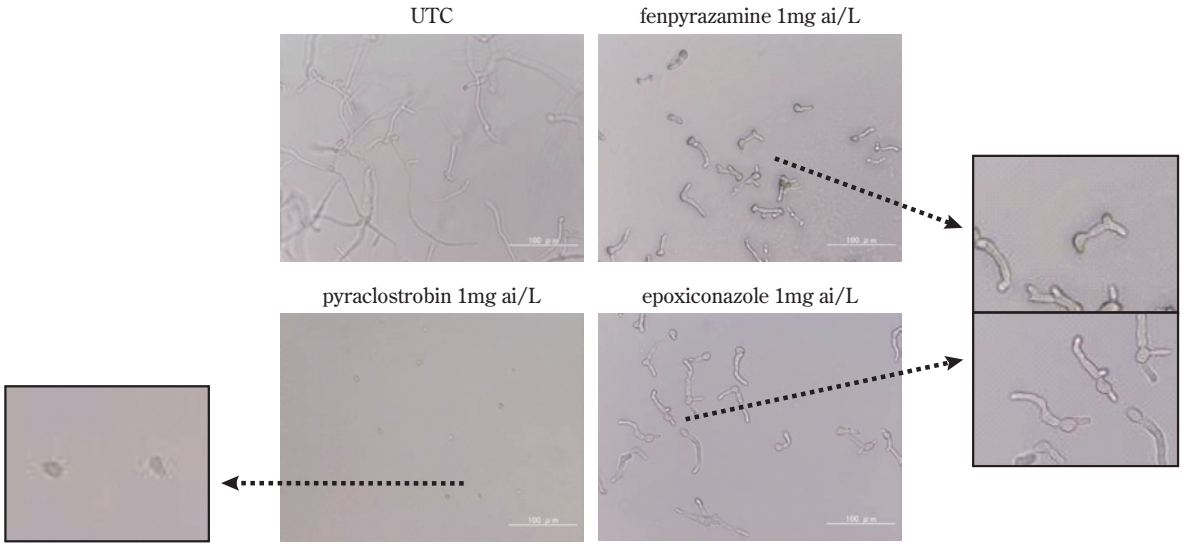
Location: Trani, Italy
Application No: Twice
Application timing:
Trial 1 Bunch touching completed and beginning of ripening
Trial 2 Flowering and beginning of ripening
Final assessment: Harvest, % diseased area of bunches (100 bunches/plot, 4 plots / treatment)
Error bar: Standard deviation

Fig. 10 Efficacy of fenpyrazamine against gray mold on grape

Mode of Action

1. Inhibition of Germ Tube Elongation and Morphological Change of Germ Tube of *Botrytis Cinerea* Induced by Fenpyrazamine

Conidia of *Botrytis cinerea* were inoculated onto a PDA medium containing 0.1–1 mg/L of fenpyrazamine,



Medium: PDA, Incubation temp.18°C, Incubation time: 18 hours

Fig. 11 Morphological change of germ tube of *Botrytis cinerea* induced by fenpyrazamine

Table 2 Fenpyrazamine-mediated inhibition of germ tube elongation of *Botrytis cinerea*

	fenpyrazamine		
Conc. (mg ai/L)	0.5	0.2	0.1
% inhibition	92.4*	86.3	75.5

UTC: 174.6 µm
*: % of inhibition of germ tube elongation
Medium: PDA, incubation temp. 18°C
Incubation time: 18 hours

and incubated for 18 hours at 18°C. After the incubation, growth of the germ tubes was observed under a microscope.

Fenpyrazamine did not inhibit spore germination, but remarkably inhibited germ tube elongation after germination (**Table 2**) and caused swelling of the germ tubes. Moreover, the swelling was a morphological change (**Fig. 11**) similar to that due to epoxiconazole, which is an ergosterol biosynthesis inhibitor (abbreviated as EBI agent in the following).

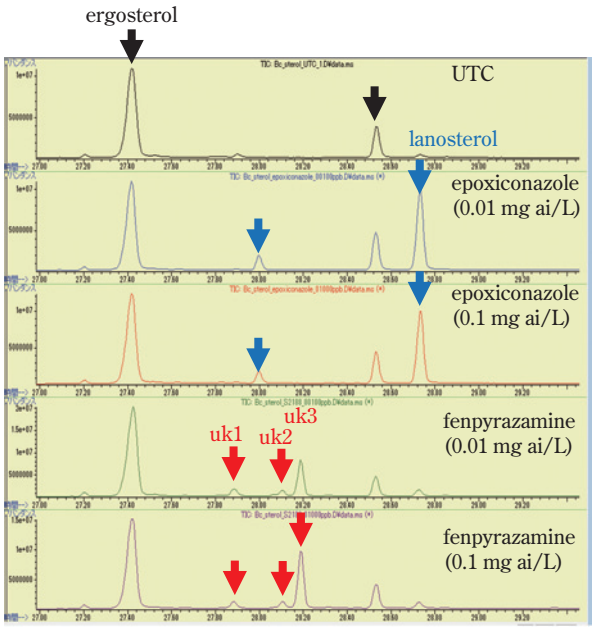
From these results, we deduced that fenpyrazamine has a mode of action that involves changes in biological membrane structures like EBI agents.[2)]

2. Metabolomics Analysis

The analysis of metabolomics was carried out in the metabolics of *Botrytis cinerea* untreated and treated with fenpyrazamine. The results suggest that fenpyrazamine involves the biosynthesis of ergosterol.[7)]

3. Analysis of Abnormal Accumulation of Intermediate Sterols

The morphological changes in the germ tubes and the metabolomics analysis suggest that fenpyrazamine involves the biosynthesis of ergosterol. Thus, we compared accumulation patterns for sterols in gray mold treated with fenpyrazamine and epoxiconazole (EBI).



Incubation temp: 18°C, Incubation time: 6 hours

Fig. 12 Analysis of sterols accumulation pattern in unsaponifiable lipids of *Botrytis cinerea* incubated in presence of fungicide with gas-chromatography

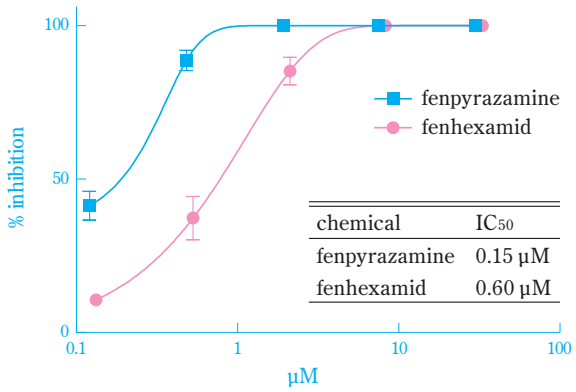
An abnormal accumulation of intermediate sterols other than ergosterol was detected in unsaponifiable lipids from *Botrytis cinerea* treated with fenpyrazamine. Besides, the pattern of abnormal accumulation for the intermediate sterols was different from that for epoxiconazole (**Fig. 12**). These results suggest that fenpyrazamine involves the biosynthesis of ergosterol, but the target site is different from that of epoxiconazole.

4. Identification of Accumulated Sterols

We examined identifications for abnormally accumulated sterols detected by gas chromatography (uk1, uk2 and uk3) with CG/MS. Uk1 couldn't be identified, but uk2 was identified as fecosterone and uk3 as 4-methylfecosterone (**Fig. 13**). The intermediates have a ketone at the third position. Therefore, the result suggests strongly that fenpyrazamine involves 3-keto reductase in the biosynthesis of ergosterol.

5. Inhibitory Activity of 3-keto Reductase on Gray Mold

In collaboration with Sumitomo Dainippon Pharma Co., Ltd., Sumitomo Chemical Co., Ltd. established a purification system for 3-keto reductase of *Botyrtis cinerea* from recombinant yeast and an *in vitro* evaluation system for inhibition of 3-keto reductase.[7] 3-keto reductase inhibitory activity of fenpyrazamine and fenhexamid, which is deduced to inhibit 3-keto reductase, was evaluated using the systems. Both compounds clearly inhibited 3-keto reductase directly (**Fig. 14**). In addition, the IC_{50} value for fenpyrazamine was 0.15μM, while the IC_{50} value for fenhexamid was 0.60μM. From the results, it has been proven that the target site of fenpyrazamine is the 3-keto reductase in the biosynthesis of ergosterol. (**Fig. 15**).[7]



Enzyme: 20 μg/ml 3-keto reductase
Substrate: 2 μM Zymosterone
Incubation time: 2 hours
Incubation temp: 18°C

Fig. 14 Inhibitory activity against 3-keto reductase from *Botrytis cinerea* with fenpyrazamine or fenhexamid

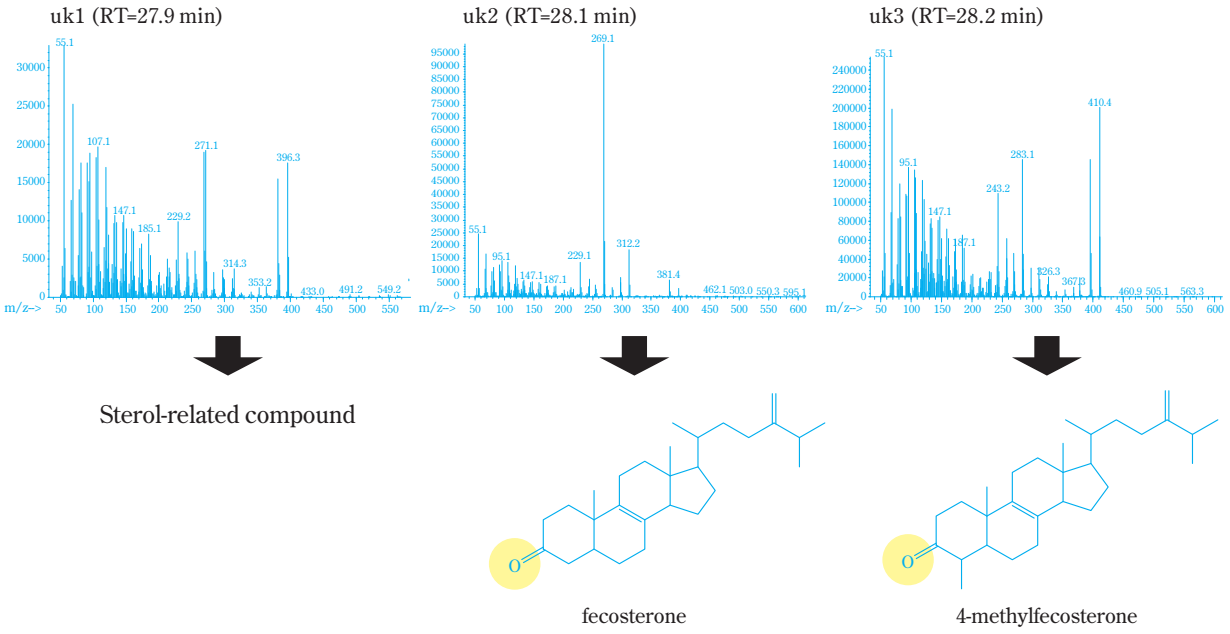


Fig. 13 Identification of accumulated unknown sterols (uk1, uk2 and uk3) of *Botrytis cinerea* incubated in presence of fenpyrazamine with GS / MS

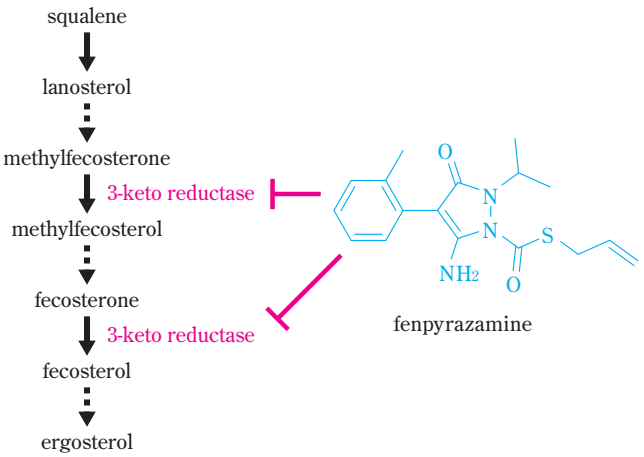


Fig. 15 Target enzyme of fenpyrazamine in the pathway of ergosterol biosynthesis

Physical Properties and Formulation

1. Physical Properties

Table 3 shows the physical and chemical properties of the fenpyrazamine technical grade. The fenpyrazamine technical grade is a white crystalline powder. The vapor pressure at 25°C is 2.89×10^{-8} Pa, and the partition coefficient log Pow is 3.52. Solubility in water is 20.4 mg/L, and it comparatively easily dissolves in organic solvents such as ethyl acetate and acetone.

Table 3 Physical and chemical properties of fenpyrazamine

Common Name (ISO)	fenpyrazamine
Chemical Name (IUPAC)	*S*-allyl 5-amino-2, 3-dihydro-2-isopropyl-3-oxo-4-(*o*-tolyl)pyrazole-1-carbothioate
CAS RN	473798-59-3
Molecular Formula	$C_{17}H_{21}N_3O_2S$
Molecular Weight	331.43
Physical Form	Solid (Ta)
Color	White (Ta)
Odor	Slight
Density	1.262 g/mL (20°C)
Melting Point	116.4°C
Vapor Pressure	2.89×10^{-8} Pa (25°C)
Solubility	Water : 20.4 mg/L (20°C) Hexane : 902 mg/L (20°C) Toluene : 113 g/L (20°C) Ethyl acetate : > 250 g/L (20°C) Acetone : > 250 g/L (20°C) Methanol : > 250 g/L (20°C)
Octanol-water partition coefficient (logPow)	3.52

2. Formulation

In Japan fenpyrazamine had been developed for stone fruit trees and vegetables, and Pixio® DF (50% fenpyrazamine), which is a water dispersible granule with excellent handling properties, was registered in July 2013 and launched in January 2014. The feature of this product is a high efficacy against gray mold which is resistant to various fungicides, which is becoming a problem in Japan at present. The product is expected to become a primary fungicide in application programs to control gray mold in the field. Overseas, it was launched first in Italy in 2012. In addition, a 40% suspension concentrated for Australia and other countries is being developed for launching in the near future.

The main points in the formulation design for Pixio® DF were to achieve both good biological efficacy and dilution properties in water. As a result of screening on adjuvants, it was found that the addition of a specific surfactant was effective in improving the biological efficacy.

On the other hand, there was a need to improve on foaming tendencies and dispersion properties because of the addition of the surfactant, but various compositions and manufacturing methods were investigated and a formulation recipe that satisfied the two aspects of performance described above was established.

In addition, in the development of the 40% suspension concentrated, a formulation recipe showing excellent dispersion properties and storage stability properties was established by selecting an optimal surfactant and thickening agent.

Table 4 and **Fig. 16** show representative physical and chemical properties for the Pixio® DF. This formulation has extremely good physical and chemical properties and storage stability.

Table 4 Physical and chemical properties of PIXIO®DF

Items	Typical value
Appearance	Brown micro granule (Visual observation)
Apparent density	0.64g/mL
pH	6.9
Suspensibility	96.9%
Stability	No decomposition of fenpyrazamine was observed after storage of 40°C 3 months

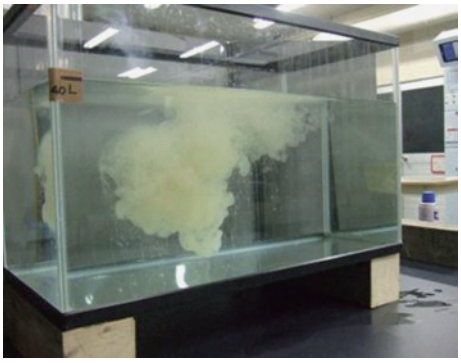


Fig. 16 Dispersibility of PIXIO®DF in water several tens of seconds after treatment

Toxicity, Metabolism and Persistence

1. Toxicity in Mammals

(1) Acute toxicity, irritation and skin sensitization

In tests of the acute oral, dermal and inhalation toxicity of the fenpyrazamine TG and fenpyrazamine 50%WG, there were no mortality or severe toxicity symptoms even at high doses, and the acute toxicity of both was low. Fenpyrazamine TG was classified as a minimal eye irritant and fenpyrazamine 50% WG was as a mild eye irritant, therefore the eye irritation potential of both was low. No skin irritation was observed for fenpyrazamine TG, and mild skin irritation was observed for fenpyrazamine 50% WG. Although the fenpyrazamine TG showed a weak potential for skin sensitization in the maximization test, fenpyrazamine 50%WG was negative in the Buehler test (**Table 5**).

Table 5 Acute toxicity summary of fenpyrazamine

Test type	fenpyrazamine	fenpyrazamine 50%WG
Rat acute oral (LD_{50})	>2000 mg/kg	>2000 mg/kg
Rat acute dermal (LD_{50})	>2000 mg/kg	>2000 mg/kg
Rat inhalation (LC_{50})	>4840 mg/m^3 of air (4-hour, nose only exposure)	>1974 mg/m^3 of air (4-hour, nose only exposure)
Eye irritation (Rabbit)	Minimally irritant	Mild irritant
Skin irritation (Rabbit)	Non-irritant	Mild irritant
Skin sensitization (Guinea pig)	Mild sensitizer	Non-sensitizer

(2) Subacute toxicity, chronic toxicity and carcinogenicity

In the results of subacute and chronic toxicity as well as carcinogenicity tests in rats, dogs and mice (**Table 6**), an inhibition of body weight gain and anemia were observed when repeated doses of fenpyrazamine TG were administered, and toxic effects were primarily found in the liver and thyroid gland.

In the liver, increases in organ weight and centrilobular hepatocellular hypertrophy were found, and accompanying follicular hypertrophy in the thyroid was observed. However, these changes are all adaptive changes related to drug metabolizing enzyme induction and can be concluded to be of no toxicological significance. No carcinogenicity was found in rats and mice.

(3) Reproductive and developmental toxicity

In developmental toxicity tests using rats and rabbits, no teratogenicity was found in fetuses. In two-generation reproductive toxicity test using rats, a low number of implantation sites, increases in post-implantation loss,

Table 6 Subacute and chronic toxicity summary of fenpyrazamine

Species	Administration route and duration	Dose	NOAEL (mg/kg/day)
Rat	Dermal, 28 days	100, 300, 1000 mg/kg/day	Male: 1000 Female: 1000
Rat	Oral (in diet), 13 weeks	300, 600, 1000, 3000 ppm	Male: 64.0 (1000ppm) Female: 68.6 (1000ppm)
Rat	Oral (in diet), 24 months	100, 300, 1200, 2400 ppm	Male: 12.7 (300ppm) Female: 15.6 (300ppm) No carcinogenicity
Dog	Oral (in capsule), 13 weeks	25, 50, 150 mg/kg/day	Male: 25 Female: 50
Dog	Oral (in capsule), 12 months	5, 25, 100 mg/kg/day	Male: 25 Female: 100
Mouse	Oral (in diet), 18 months	Male: 100, 1500, 3000 ppm Female: 100, 2000, 4000 ppm	Male: 176 (1500ppm) Female: 283 (2000ppm) No carcinogenicity

Table 7 Developmental and reproductive toxicity summary of fenpyrazamine

Study	Species	Administration route and duration	Dose	NOAEL (mg/kg/day)	
Developmental toxicity	Rat	Oral (gavage) Days 6-20 of gestation	30, 125, 500 mg/kg/day	Maternal Fetal	30 125
	Rabbit	Oral (gavage) Days 6-27 of gestation	30, 50, 90 mg/kg/day	Maternal Fetal	30 90
Two-generation reproductive toxicity	Rat	Oral (in diet)	400, 1000, 3000 ppm	Parental	Systemic Male; 27.4 (400ppm) Female; 32.0 (400ppm) Reproductive Male; 68.6 (1000ppm) Female; 79.9 (1000ppm)
				Offsprings	Systemic 32.0 (400ppm)

Table 8 Neurotoxicity and immunotoxicity summary of fenpyrazamine

Study	Species	Administration route and duration	Dose	NOAEL (mg/kg/day)
Neurotoxicity	Rat	Acute oral (gavage)	80, 400, 2000 mg/kg/day	Male & Female: >2000
	Rat	Oral (in diet), 13 weeks	500, 1200, 3000 ppm	Male: >223.6 (3000ppm) Female: >248.4 (3000ppm)
Immunotoxicity	Rat (female)	Oral (in diet), 4 weeks	500, 1500, 4000 ppm	>392 (4000ppm)

reduction in numbers of offspring born and increases in the mortality rate of offspring born alive were found, but all of these could be considered to be secondary effects related to maternal toxicity (**Table 7**).

(4) Neurotoxicity and immunotoxicity

In acute neurotoxicity and subacute neurotoxicity tests using rats, no specific neurotoxic effect was observed in either. In addition, in immunotoxicity test using rats, no effects on immune function were observed (**Table 8**).

(5) Genotoxicity

The results of carrying out reverse mutation test using *Salmonella typhimurium* and *Escherichia coli*, chromosomal aberration test using a CHL cell line (Chinese hamster lung cells), genetic mutation test using a Chinese hamster V79 cell line and micronucleus test in mice were all negative (**Table 9**).

Table 9 Mutagenicity summary of fenpyrazamine

Study	Study design	Results
Reverse mutation (Ames test)	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2uvrA –/+S9 mix: 156 – 5000 μg/plate	Negative
Gene mutation	Chinese hamster V79 –/+S9 mix: 10 – 100 μg/mL	Negative
In vitro chromosomal aberration	Chinese hamster CHL/IU –/+S9 mix: 22.5 – 160 μg/mL	Negative
Micronucleus	CD-1 mice 500, 1000, 2000 mg/kg	Negative

2. Metabolism in Animals and Plants

(1) Metabolism in animals

When ^{14}C labeled fenpyrazamine was dosed to rats, it was absorbed rapidly in the body and distributed systemically. Thereafter, it was rapidly metabolized and mainly excreted in the urine. The absorption rate with oral administration was estimated to be 90% or greater, and there was no persistence or accumulation in the tissue.

The principal metabolic reaction for fenpyrazamine was thiocarbamate cleavage to release a propenyl-sulfanyl-carbonyl group. Also, hydroxylation of the methyl group, release of the isopropyl group and 4-hydroxylation of the pyrazole ring occurred. In addition, glucuronic acid or sulfuric acid conjugation products of these metabolites were found.[8)]

(2) Metabolism in plants

Plant metabolism studies using ^{14}C labeled fenpyrazamine with three different types of crops (grape, lettuce and oilseed rape) demonstrated that the metabolism of fenpyrazamine proceeded *via* cleavage of the thiocarbamate linkage and subsequent hydroxylation at the 4-position of the pyrazolyl ring in all of them.

3. Behavior and Residue in the Environment

(1) Degradation in water

In the hydrolysis study, ^{14}C labeled fenpyrazamine was stable in buffer solutions at pH 4 and 7, while it degraded *via* cleavage of the thiocarbamate linkage and subsequent hydroxylation at the 4-position of the pyrazolyl ring at pH 9 with half-lives of 11–24 days (20–25°C). In addition, the degradation of the ^{14}C labeled fenpyrazamine in a buffer solution (pH 7) and natural water (pH 6.9–7.2) was significantly accelerated by exposure to light, and the photodegradation half-lives (natural sunlight at Tokyo in spring) was 5.2–5.5 days and 11.8–12.0 days, respectively, *via* cleavage of the thiocarbamate linkage and subsequent opening of the pyrazolyl ring.

(2) Metabolism in soil

Fenpyrazamine labeled with ^{14}C was degraded in aerobic soil with half-lives of 62–63 days (25°C), *via* cleavage of the thiocarbamate linkage and subsequent hydroxylation at the 4-position of the pyrazolyl ring, and finally mineralized to carbon dioxide or firmly bound by the soil residues. In addition, degradation of the fenpyrazamine on the surface of the soil was similar irrespective of light exposure, and the photodegradation half-lives were 74–80 days (20°C).

(3) Field dissipation

Terrestrial field dissipation studies were conducted by applying an 800-fold diluted solution of fenpyrazamine 50%WG three times with a seven-day interval at a rate of 300 L/10 a onto two upland fields in Ibaraki and Yamanashi. The dissipation half-lives were estimated to be 30–31 days with maximum residue concentrations of 5.03–8.78 mg/kg.

(4) Mobility in soil

The adsorption coefficient $K_{Foc(ads)}$ and desorption coefficient $K_{Foc(des)}$ of fenpyrazamine corrected with the soil organic carbon content were calculated using the Freundlich adsorption isotherm, to be 112–731 and 133–954, respectively.

(5) Residue in crops

Residue trails for cucumbers, eggplant, strawberries, tomatoes and cherry tomatoes were conducted with four applications of 2000-fold diluted formulation of fenpyrazamine 50%WG with a seven-day interval at a rate of 200–300 L/10 a. The maximum mean residues ranged from 0.28 to 3.04 ppm.

Residue trials were conducted for Unshu oranges, Japanese summer oranges, grapes, kabosu citrus and sudachi citrus. The same diluted formulation as that in the crop residue trails above was applied three times with a seven-day interval at a rate of 300–700 L/10 a to five crops. The maximum mean residues range from 0.02 to 6.52 ppm.

(6) Residue in rotational crops

A field rotational crop study was conducted by cultivating turnips and peppers as rotational crops in the field after the cultivation of tomatoes as a primary crop. Tomato plants were treated four times with 2000-fold diluted formulation of fenpyrazamine 50%WG at a rate of 300 L/10a with a seven-day interval. The residues in both rotational crops were less than the limit of quantification (0.01 ppm).

4. Effects on Non-target Species

Test results of ecotoxicological studies on aquatic organisms, honeybees, silkworms, natural enemy insects and birds are summarized in **Table 10**.

(1) Effects on aquatic organisms

The acute toxicity value (LC_{50}/EC_{50}) for the fenpyrazamine TG in carp, *Daphnia magna* and fresh water green algae was 6.0, 5.5 and >0.92 mg/L, respectively. In addition, the corresponding toxicity values of fenpyrazamine 50%WG in these aquatic organisms were 13, 6.0 and 1.5 mg/L, respectively. These values were significantly higher than the predicted environmental concentrations, which suggested no significant effects of fenpyrazamine on aquatic organisms in practical use.

(2) Effects on honeybees, silkworms, natural enemy insects

The acute oral and contact toxicity values (LD_{50}) of fenpyrazamine TG on honeybees (*Apis mellifera*) were both >100 μg/bee. In the oral toxicity study of silkworms with fenpyrazamine 50%WG, the mortality was 0% and

Table 10 Ecotoxicological summary of fenpyrazamine on non-target organisms

Test substance	Test organisms	Test species	Test type	Results
fenpyrazamine	Aquatic organisms	Carp	Acute (96 hrs)	LC_{50} = 6.0 mg/L
		Daphnia magna	Acute (48 hrs)	EC_{50} = 5.5 mg/L
		Pseudokirchneriella subcapitata	Acute (72 hrs)	ErC_{50} > 0.92 mg/L
	Honeybee	*Apis mellifera*	Acute oral (48 hrs)	LD_{50} > 100 μg/bee
		Apis mellifera	Acute contact (72 hrs)	LD_{50} > 100 μg/bee
	Bird	Bobwhite quail	Acute oral	LD_{50} > 2000 mg/kg
fenpyrazamine 50%WG	Aquatic organisms	Carp	Acute (96 hrs)	LC_{50} = 13 mg/L
		Daphnia magna	Acute (48 hrs)	EC_{50} = 6.0 mg/L
		Pseudokirchneriella subcapitata	Acute (72 hrs)	ErC_{50} = 1.5 mg/L
	Silkworm	*Bombyx mori*	Acute oral (7 days)	mortality 0% (at 175 g a.i./ha)
	Natural enemy	*Aphidius colemani* (adult)	Acute contact (7 days)	mortality 7.6% (at 100 mg a.i./L)
		Orius strigicollis (adult)	Acute contact (7 days)	mortality 6.8% (at 100 mg a.i./L)
		Amblyseius californicus (adult)	Acute contact (7 days)	mortality 6.0% (at 100 mg a.i./L)

no effect on development was observed. The mortality of natural enemy insects, *Aphidius colemani*, *Orius strigicollis* (Poppius) and *Neoseiulus californicus*, ranged from 6.0 to 7.6% in the contact toxicity studies. Based on these results, the effects of fenpyrazamine on honeybees, silkworms, natural enemy insects are considered to be low in practical use.

(3) Effects on birds

The acute oral toxicity of fenpyrazamine TG in bobwhite quail was negligible at an LD_{50} value of >2000 mg/kg. Accordingly, the effects of fenpyrazamine on birds are considered to be low in practical use.

Based on the above, it is suggested that fenpyrazamine has low acute toxicity toward mammals, and does not have any adverse effects on the next generation such as carcinogenicity and teratogenicity, nor adverse effects on fertility, even if it is taken for a long period of time. Furthermore, based on assessments of the environmental fate and the effects on non-target organisms, it is suggested that safe use is possible.

Domestic Registration of Fenpyrazamine (PIXIO®DF)

The domestic registration of fenpyrazamine is as shown in **Table 11**. Fenpyrazamine can be used up to one day before harvest on all crops. In the future we plan to expand target diseases for fenpyrazamine to stem rot on melon (proposed), stem rot on watermelon (proposed), stem rot on pepper, stem rot on strawberry and other like diseases.

Conclusion

Fenpyrazamine not only has a high preventative efficacy against gray mold and other diseases, but also superior trans-laminar ability, curative efficacy and long lasting activity; in practical fields it exhibits a high efficacy against gray mold. Therefore, we think that it can become a primary fungicide for controlling gray mold. However, as mentioned above, *Botrytis cinerea* is widely known as a fungus which acquire fungicide resistance easily; therefore, fenpyrazamine should be used within

Table 11 Domestic registration of fenpyrazamine (PIXIO®DF)

Target Crops	Target disease	Dilution rate	Spray volume	PHIs	Maximum number of applications	Application method
Citrus	Gray mold	2000	200-700 L/10a	One day	3	Spray
Grape						
Strawberry						
Cucumber	Gray mold Sclerotinia rot		100-300 L/10a		4	
Tomato						
Mini tomato						
Eggplant						

an application program which implements combined use with other fungicides having different modes of action.

With regard to the controlling of gray mold, there are scheduled applications such as those to fruit trees and grapes, particularly in the EU and other places, and applications such as those to vegetables in Japan, which is initiated after a few diseases occur. The role of the first fungicide in an application program is important for both cases. Therefore, it is desirable to use fenpyrazamin, as the first fungicide in an application program.

In the future, we think it is necessary to build up examples of the effects of fenpyrazamine in application programs and promote it.

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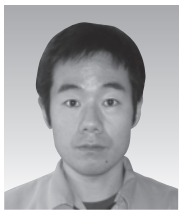

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