

Efficacy of orysastrobin against blast and sheath blight in transplanted rice

Gerd STAMMLER,* Manabu ITOH,[†] Isao HINO,[†] Akihide WATANABE,^{††} Kenichi KOJIMA,^{††}
Masatoshi MOTOYOSHI,^{††} Andreas KOCH and Egon HADEN

BASF Aktiengesellschaft, 67114 Limburgerhof, Germany

[†] BASF Agro Ltd., Roppongi, Minato-ku, Tokyo, 106–0032, Japan

^{††} BASF Agro Ltd., Kamigaya, Mutsure, Tahara, Aichi, 441–3413, Japan

(Received July 10, 2006; Accepted October 19, 2006)

Oryastrobin is a new QoI fungicide with excellent fungicidal efficacy against leaf and panicle blast and against sheath blight in rice. Formulations developed for seedling box treatments provide long lasting residual control combined with excellent plant selectivity under different environmental conditions. The sensitivity was monitored based on genetic assays. No samples with reduced sensitivity could be detected in our extensive monitoring studies in 2004 and 2005. © Pesticide Science Society of Japan

Keywords: orysastrobin, blast, sheath blight, pyrosequencing, *Magnaporthe grisea*, *Thanatephorus cucumeris*.

Introduction

Oryastrobin, (2*E*)-2-(methoxyimino)-2- $\{2-[(3*E*,5*E*,6*E*)-5-(methoxyimino)-4,6-dimethyl-2,8-dioxo-7-diazinona-3,6-dien-1-yl]phenyl\}$ -*N*-methylacetamide, is the common name of a new QoI fungicide discovered and developed by BASF for the control of leaf and panicle blast (*Magnaporthe grisea*) and sheath blight (*Thanatephorus cucumeris*) in rice. Its biochemical mode of action is inhibition of the mitochondrial respiration chain by binding to the cytochrome *bc*₁ complex at the Qo site.¹⁾

Rice blast and sheath blight control in transplanted rice in Japan is mainly based on granule application in the seedling box. For this application type it is essential that the fungicide is safe for rice seedlings and shows long-lasting activity in the upper parts of the plant. Special granular formulations with orysastrobin have been developed for controlled release in seedling box application with or without an insecticide partner. The resulting outstanding long-lasting efficacy of orysastrobin in the field against leaf and panicle blast, and sheath blight under different conditions is here described.

Two mutations have been detected in the cytochrome *b* gene in several plant pathogens, which govern resistance to

QoI fungicides. One mutation leads to a substitution of glycine by alanine at codon 143 (found *e.g.* in *Blumeria graminis*, *Mycosphaerella graminicola*, *Plasmopara viticola*) and is the main mechanism of resistance to QoIs.^{2–4)} Another mutation at codon 129 (found *e.g.* in *Pyrenophora teres*, *Alternaria solani*), which leads to the substitution of phenylalanine by leucine results in lower resistance factors.^{5,6)} A monitoring program based on genetic analysis of the cytochrome *b* gene has been established, and the methods and data are reported.

Material and Methods

1. Chemicals

The chemical structure of orysastrobin is shown in Fig. 1. Oryastrobin was used in granular formulations containing 7% orysastrobin with or without insecticide partners. Efficacy

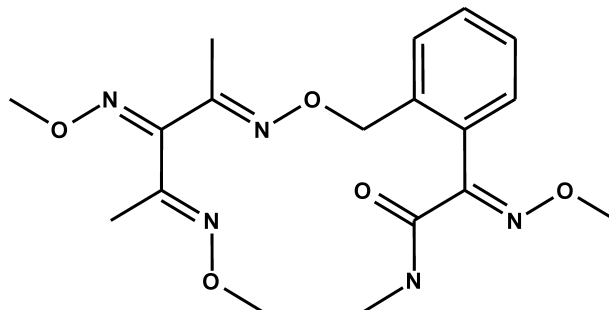


Fig. 1. Chemical structure of orysastrobin.

* To whom correspondence should be addressed.

E-mail: gerd.stammler@basf.com

Published online November 30, 2006

© Pesticide Science Society of Japan

against rice blast was compared with commercial available granular products for seedling box application containing 3% diclocymet, 24% probenazole or 12% pyroquilon. Controlling activity against rice sheath blight was compared with seedling box granular products containing 4% furametpyr or 3% thifluzamide.

2. Field trials

2.1. Application and transplanting

Fungicides were applied as granular formulations to seedling boxes. Each fungicide (50 g) was scattered in each seedling box (30×60 cm), corresponding to 10 kg product/ha. Applications were made on the day of transplanting. For trials investigating the influence of application timing on selectivity and efficacy, applications were done at the seeding stage (after seeding, before soil covering), at the greening stage (2 days after emergence) and on the day of transplanting.

2.2. Trial layout

Seedlings were transplanted into paddy fields using a commercial mechanical transplanter (Kubota, two rows). Trials were performed in 2002 and 2003 at different trial sites in Japan. In each trial, three randomised replicates per treatment were used with a minimum plot size of 15 m². The cultivation of the crop was according to normal practical standards. The rice variety used was Koshihikari.

2.3. Evaluation of disease

Leaf blast was evaluated at growth stage 24–34, which corresponds to 48–64 days after transplanting. Panicle blast was evaluated at growth stage 77–85, which corresponds to 85–103 days after transplanting. Leaf blast was assessed by estimating the infected leaf area in %, panicle blast by using the damage index calculation method⁷⁾ (damage index = $A + 0.66B + 0.26C$, A = % panicles attacked at panicle neck, B = % of panicles whose rachis area is damaged by >1/3, C = % of panicles whose rachis area is damaged by < or = 1/3). Sheath blight was assessed at growth stage 77–87 (86–106 days after transplanting) using the Hashiba method⁸⁾ (Total damage index = $A \times \% \text{ infected hills} / 100$; A, index of attacked hill = $1.62 \times B - 32.4$; B = lesion height / plant height × 100%).

2.4. Statistical analysis

Statistical analyses were performed with SAS. The Student–Newman Keuls test, ($P < 0.05$) was used for comparisons of disease severity. Percentage disease values were arc-sin-square-root transformed.

3. Sensitivity monitoring

3.1. Sampling preparation

Leaves or panicles diseased with *Magnaporthe grisea* were collected from different sites of commercial fields or trial sites in 2004 and 2005. At trial sites, samples were taken from plots treated with oryastrobin, reference products or from untreated plots. In total, 216 samples were analysed in 2004 and 135 samples in 2005. In the samples, lesions were cut out

from the leaves or diseased panicles were selected. These parts were dried and then homogenised by milling after freezing in liquid nitrogen with a MixerMill MM200 (Retsch, Hahn, Germany).

3.2. DNA isolation and PCR amplification

Total DNA was extracted using the DNA NucleoSpin Plant Extraction Kit (Macherey and Nagel, Düren, Germany) and suspended in 50 μ l H₂O. PCR was carried out with 5 μ l DNA solution, 1 μ l dNTP's (10 mM), 2.5 μ l of forward primer and reverse primer (10 pmol/ μ l), 5 μ l 10×ThermoStart buffer (Abgene, Hamburg, Germany) and 0.5 μ l ThermoStart DNA Polymerase (Abgene). The sequences used were for the forward primer: 5'-TGGAAGCTTTTAACTCAATAGAG-3' and the reverse primer: 5' Biotin-TGCCCTATTCAAGGTATAGCA-3'. PCR was performed on a Mastercycler Gradient (Eppendorf, Hamburg, Germany). PCR amplifications were carried out using the following parameters: Initial preheating for 15 min at 95°C, followed by 50 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s and a final amplification step at 72°C for 5 min. The length of the PCR product was 301 bp. For control of the PCR amplification, an aliquot of the PCR product was separated on a 2% agarose gel, stained with ethidium bromide and visualized with UV light.

3.3. Pyrosequencing

PCR products were used for further sequence analysis. Single nucleotide polymorphisms in the codons for amino acid 129 and amino acid 143 were analysed using the pyrosequencing method, as previously described^{9,10)} and according to the instructions by the manufacturer using PyroGold reagents and a PSQ 96 MA machine (Biotage, Uppsala, Sweden). The sequence primers used were for codon 129 5'-AATATTAATGATGGCTATCG-3' and for codon 143 5'-GGACAGATGCATTATGAG-3'. The denaturation step before sequencing was done at 75°C for 3 min and the whole sequencing procedure was carried out at 28°C. Sequence data were analysed quantitatively using software provided by the manufacturer (Biotage).

Results

1. Efficacy against leaf and panicle blast

In all field trials oryastrobin provided excellent control of leaf blast (Table 1) and panicle blast (Table 2) with outstanding long-lasting activity. The efficacy was equal or superior to the standard products under different disease pressure. Trials with different application timing (seeding, greening, transplanting) also showed excellent efficacy of oryastrobin against leaf and panicle blast (Table 3) independent of the application timing.

2. Efficacy against sheath blight

Excellent control of sheath blight was achieved by oryastrobin and this efficacy was comparable to standard sheath blight products such as thifluzamide or furametpyr under different disease pressure (Tables 4 and 5). As for blast control,

Table 1. Efficacy of orysastrobin-containing products on leaf blast in comparison to commercialised standard products

Product	Rate fungicide [g a.i./ha]	% Control	
		5.0% in untreated*	14.3% in untreated*
Orysastrobin GR	700	99 ^c	98 ^a
Orysastrobin+Fipronil GR	700	99 ^c	98 ^a
Orysastrobin+Clothianidin GR	700	99 ^c	99 ^a
Diclocymet combination GR	300	94 ^b	95 ^a
Probenazole combination GR	2400	99 ^c	95 ^a
Pyroquilon combination GR	1200	91 ^a	98 ^a
Trial location		Tahara, Aichi	Toyohashi, Aichi
Year		2002	2002

* Evaluation method: Diseased leaf area. Means followed by the same letter do not significantly differ (Student–Newman Keuls test, $P<0.05$).

Table 2. Efficacy of orysastrobin-containing products on panicle blast in comparison to commercialised standard products

Product	Rate fungicide [g a.i./ha]	% Control	
		3.0% in untreated*	12.5% in untreated*
Orysastrobin GR	700	92 ^a	97 ^c
Orysastrobin+Fipronil GR	700	94 ^a	97 ^c
Orysastrobin+Clothianidin GR	700	91 ^a	98 ^c
Diclocymet combination GR	300	80 ^a	90 ^b
Probenazole combination GR	2400	67 ^a	79 ^a
Pyroquilon combination GR	1200	67 ^a	81 ^a
Trial location		Tahara, Aichi	Toyohashi, Aichi
Year		2002	2002

* Evaluation method: Diseased leaf area. Means followed by the same letter do not significantly differ (Student–Newman Keuls test, $P<0.05$).

Table 3. Influence of the application timing of orysastrobin on the control of leaf and panicle blast in comparison to commercialised standard product

Product	Application timing	% Control	
		Leaf blast 3.0% in untreated*	Panicle blast 4.2% in untreated**
Orysastrobin+Fipronil GR (700 g a.i./ha)	seeding	97 ^b	97 ^a
	greening	96 ^b	94 ^a
	transplanting	96 ^b	96 ^a
Diclocymet combination GR (300 g a.i./ha)	seeding	83 ^a	82 ^a
	greening	87 ^a	79 ^a
	transplanting	84 ^a	77 ^a
Trial location		Tahara, Aichi	Tahara, Aichi
Year		2003	2003

* Evaluation method: Diseased leaf area. ** Evaluation method: Panicle damage index. Means followed by the same letter do not significantly differ (Student–Newman Keuls test, $P<0.05$).

Table 4. Efficacy of oryastrobin-containing products on sheath blight in comparison to commercialised standard product

Product	Rate fungicide [g a.i./ha]	% Control	
		7.0% in untreated*	40.2% in untreated*
Oryastrobin GR	700	95 ^b	89 ^a
Oryastrobin+Fipronil GR	700	99 ^b	90 ^a
Oryastrobin+Clothianidin GR	700	97 ^b	84 ^a
Thiﬂuzamide combination GR	300	52 ^a	85 ^a
Trial location		Toyohashi, Aichi	Tahara, Aichi
Year		2002	2002

* Evaluation method: Hashiba damage index. Means followed by the same letter do not significantly differ (Student–Newman Keuls test, $P<0.05$).

this efficacy was also independent of the application time point in the seedling box (seeding, greening, transplanting, Table 5).

3. Sensitivity monitoring

From most samples, sufficient DNA from *M. grisea* could be isolated for amplification of sufficient PCR product in one step for sequence analysis by pyrosequencing. Only from three samples with very weak blast symptoms in 2005 was no specific amplification of the cytochrome *b* sequence of *M. grisea* possible. All samples from plots treated with oryastrobin-containing products or products with other modes of action or untreated plots gave a full sensitivity picture in 2004 ($n=216$) and in 2005 ($n=135$). No F129L or G143A mutation in the cytochrome *b* gene could be detected in any sample described in Table 6. In all samples, the sequence of the

wild-type codon (for codon 129 TTC, coding for phenylalanine and for codon 143 GGT, coding for glycine) was identified.

Discussion

The development of the fungicide oryastrobin started in the late 1990s. For the seedling box application type a granular formulation with a time-released control was formulated. The combination of high intrinsic and systemic fungicidal activity with controlled release in a granular formulation is fundamental for the excellent and long-lasting control of leaf and panicle blast, and sheath blight. For seedling box products it is mandatory that the compounds are not phytotoxic to sensitive rice seedlings. Oryastrobin is a selective compound that caused no damage through the growth stages of all tested varieties of rice (Japonica and Indica varieties). Selectivity was tested on several Japonica and Indica varieties of rice, e.g. Koshihikari, Hinohikari, Hitomebore, Akitakomachi, Haenuki and others in Japan, Tainong 67, Taigeng 16 and others in Taiwan, IR50 and IR72 in the Philippines, IAC165, Metica-1 and others in Brazil, Balilla and Lemont, and others in Europe and US. The efficacy of oryastrobin against target pathogens has been tested for 10 years in numerous trials under different environmental conditions. Oryastrobin-containing products were competitive with the best standard products for leaf blast, panicle blast (e.g. SAR compounds, melanin biosynthesis inhibitors) and sheath blight (e.g. carboxanilides). The long-lasting and high efficacy against target diseases enables farmers to save additional field applications during the vegetation period and therefore results in a reduction of labor and costs.

New modes of action in the application strategy improved the efficiency of disease management since new modes of action are effective tools to manage the development of fungicide resistance. In this sense, oryastrobin can also be useful to reduce or slow down the distribution of resistance to melanin biosynthesis inhibitors (MBI-D-type) such as carpropamid or diclocymet.

Table 5. Influence of the application timing of oryastrobin on the control of sheath blight in comparison to commercialised standard product

Product	Application timing	Sheath blight
		% Control 6.5% in untreated*
Oryastrobin+Fipronil GR (700 g a.i./ha)	seeding	100 ^a
	greening	100 ^a
	transplanting	98 ^a
Furametpyr combination GR (400 g a.i./ha)	transplanting	98 ^a
Trial location		Tahara, Aichi
Year		2003

* Evaluation method: Hashiba damage index. Means followed by the same letter do not significantly differ (Student–Newman Keuls test, $P<0.05$).

Table 6. Origin of the samples collected for sensitivity monitoring of *Magnaporthe grisea* in 2004 and 2005

Number of samples	2004	2005
Aichi	9	6
Akita	9	2
Aomori	2	2
Ehime	0	8
Fukui	6	0
Fukuoka	12	0
Fukushima	4	9
Gifu	0	1
Gunma	3	1
Hiroshima	8	3
Hyogo	22	1
Ibaraki	8	8
Iwate	7	2
Kagoshima	3	4
Kochi	6	8
Kumamoto	5	4
Kyoto	0	1
Mie	3	0
Miyagi	3	4
Nagano	7	17
Niigata	11	2
Oita	11	1
Okayama	3	3
Saga	6	4
Shimane	0	1
Shizuoka	8	1
Tochigi	5	5
Tokushima	11	0
Tottori	34	8
Yamagata	7	29
Yamaguchi	3	0
Leaf blast	195	73
Panicle blast	21	62
Commercial sites	81	48
Trial sites (treated with oryzastrubin)	135 (62)	87 (42)
Total	216	135

To maintain the excellent activity of oryzastrubin, resistance management strategies have been developed. Non-chemical control methods *e.g.* crop culture according to good agricultural practice (*e.g.* cultivation of rice varieties with less susceptibility to blast, usage of healthy seeds) are efficient

tools for a significant reduction of infection pressure and are therefore appropriate to reduce selection pressure in subsequent fungicidal treatments. Additionally, the number of QoI applications per season should be restricted and other modes of action should be implemented in a season-long disease control strategy. For sustainable and responsible resistance management, monitoring of fungicide sensitivity is essential. The sensitivity status of *M. grisea* was monitored yearly during the season (leaf blast symptoms) and at the end of the season (leaf and panicle blast symptoms). Different methods were established to monitor sensitivity. *In vivo* assays (*e.g.* greenhouse trials with rice seedlings) and *in vitro* methods (*e.g.* microtiter assays) are appropriate to determine the sensitivity of isolates and ED values can be calculated.¹¹⁾ Intensive studies by BASF showed a stable and narrow distribution of sensitivity (determined as ED₅₀ values) in *M. grisea* towards oryzastrubin in microtiter assays over the years (data not shown). A disadvantage of both methods is the necessity of isolating viable strains from blast lesions, which is often not possible, since in some samples no viable spores or mycelia can be found. Furthermore, a high number of strains must be isolated and tested to detect less sensitive isolates in populations with a low frequency of resistance. Since the genetic background of QoI resistance is clearly understood, a genetic approach for resistance monitoring is possible. Several methods based on DNA analysis are possible and for all these methods no living material is necessary. Codons of the cytochrome *b* gene, which must be included in genetic analysis, are 129 and 143. The relevant mutations F129L and G143A are caused by single nucleotide exchanges. For F129L, three different codons are possible for leucine (CTC, TTA, TTG) with one nucleotide exchange from the wild-type phenylalanine codon (TTC), and all three mutated codons have been found in *Pyrenophora teres* in Europe (own unpublished data). For G143A, only one codon for alanine (GCT) is possible with one nucleotide exchange from the wild-type glycine codon (GGT). Qualitative PCR assays as described by Araki *et al.*¹²⁾ (amplification of the cytochrome *b* gene followed by subsequent digestion with a specific restriction enzyme) are appropriate for determination of the G143A mutation if the restriction enzyme *Sat*I is used. Detection of the F129L mutation by qualitative PCR using *Sty*I as a restriction enzyme can lead to false negative data, since only the leucine codons TTG and TTA, but not the leucine codon CTC show a different restriction pattern to the wild-type phenylalanine codon (TTC).

Quantitative real-time PCR based on the amplification refractory mutation system (ARMS)¹³⁾ provides a sensitive method for the detection of single mutations. For G143A it is an appropriate method, but for F129L it would be more labor and cost intensive because of the different codons possible for this exchange. In this case, detailed information on the whole sequence of codon 129 is needed and the pyrosequencing method represents an efficient quantitative one-step method to achieve this. Because of synergistic effects in the analysis

procedure, this method was used for the quantitative monitoring of both mutations, F129L and G143A.

No QoI-resistant isolate of *M. grisea* from rice could be detected in our extensive monitoring studies in 2004 and 2005. The data show the full QoI sensitive situation throughout Japan.

Acknowledgements

The authors wish to thank Sumika-Takeda, Nissan and Kyoyu Agri for providing samples for monitoring studies.

References

- 1) H. Sauter, W. Steglich and T. Anke: *Angew. Chem. Int. Ed.* **38**, 1328–1349 (1999).
- 2) S. P. Heaney, A. A. Hall, S. A. Davies and G. Olaya: “The BCPC Conference Pests and Diseases, 2000,” The British Crop Protection Council, Alton, Hampshire, UK, pp. 755–762, 2000.
- 3) I. Yamaguchi and M. Fujimura: *J. Pestic. Sci.* **30**, 67–74 (2005).
- 4) I. Ishii, B. Fraaije, T. Sugiyama, K. Noguchi, K. Nishimura, T. Takeda, T. Amano and D.W. Hollomon: *Phytopathology* **91**, 1166–1171 (2001).
- 5) Y. Kim, E. W. Dixon, P. Vincelli and M. L. Farman: *Phytopathology* **93**, 891–900 (2003).
- 6) J. S. Pasche, C. M. Wharam and N. C. Gudmestad: “The BCPC Conference Pests and Diseases, 2002,” The British Crop Protection Council, Alton, Hampshire, UK, pp. 841–846, 2002.
- 7) H. Suzuki: *JPPA (Japan Plant Protection Association)*, **52**, (1990).
- 8) T. Hashiba: *Bull. Hokuriku Natl. Agric. Exp. Stn.* **26**, 115–164 (1984).
- 9) M. Ronaghi: *Genome Res.* **11**, 3–11 (2001).
- 10) A. Alderborn, A. Kristofferson and U. Hammerling: *Genome Res.* **10**, 1249–1258 (2000).
- 11) G. Stammer, D. Strobel, M. Semar and K. Klappach: *Asp. Appl. Biol.* **78**, 29–36 (2006).
- 12) Y. Araki, M. Sugihara, H. Sawada, H. Fujimoto and M. Masuko: *J. Pestic. Sci.* **30**, 203–208 (2005).
- 13) C. R. Newton, A. Graham, L. E. Heptinstall, S. J. Powell, C. Summers, N. Kalsheker, J. C. Smith and A. F. Markham: *Nucl. Acids Res.* **17**, 2503–2516 (1989).