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REVIEW

Impact of Fungicide Resistance in Plant Pathogens on Crop Disease Control and Agricultural Environment

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Abstract

Fungicides have greatly contributed to sustaining quality food production through protecting a variety of crops from fungal diseases. However, such production is often threatened by the occurrence of pathogen strains resistant to fungicides. In this paper, the resistance to QoI and MBI-D fungicides in the pathogens of horticultural crops and rice blast fungus, respectively, are introduced as representative cases of resistance which have been experienced recently. Implications for integrated disease control that reduces the risk of fungicide resistance are discussed, then strategies to cope with resistance are overviewed. Application of PCR-based molecular techniques has become a powerful tool in the diagnosis of pathogens and/or fungicide resistance. Rapid and quantitative detection of resistant strains in fungal populations will make it easier to precisely predict the resistance risk without control failure by fungicides. The approach will also match the direction demanded by the society to reduce pesticide inputs to the agricultural environment.

Discipline: Plant protection
Additional key words: integrated disease control, MBI-Ds, QoIs, resistance management, strobilurin fungicides

Introduction

Use of modern fungicides greatly contributed to reducing damage caused by a variety of diseases and to increasing not only yields but also quality of crops. Since the early 1970's, however, emergence of fungicide-resistant strains in target pathogens has been continuing worldwide (Table 1). Concerns about toxicological and environmental problems which some but not all classical pesticides possessed undoubtedly prompted the development of selective fungicides, single-site (site-specific) inhibitors in particular. It was closely related with the continuous occurrence of fungicide resistance in practice.

It is well known that the use of agrochemicals per acreage of cultivated land was highest in Japan in 2002 according to the report made by OECD. Furthermore, Japan was the biggest national fungicide market in the world worth $818 million in the same year. This background might be explained by the warm and wet climate conditions that are suitable for the reproduction of many pathogens, as well as the high demand by the marketing industry and consumers for quality agricultural products. On the other hand, it is also true that public concerns about pesticide residues in the environment and agricultural products are extremely high these days although some of these concerns occasionally lack enough scientific evidence.

To change this situation, various trials such as the development of biocontrol agents are under investigation but it will probably be difficult to see most of the chemically synthesized pesticides replaced quickly by those novel agents. Biological control products currently account for only 0.5% of the ¥350,000 million ($3,300 million) Japanese crop protection market, although this share is expected to reach 5% in the future. Moreover, development of new classes of pesticides will also be hard due to the increasing cost and the lack of promising novel chemical structures, so dependence on pesticides currently available will further increase. Therefore, it will be important to combat pathogens resistant to fungicides as long as ordinary types of 'fungicides' are used for disease control. In this paper, the author will review recent outbreaks of fungicide resistance in Japan and propose some ideas, which seem to be useful for avoiding resistance development in fungal pathogens.

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Table 1. Occurrence of fungicide resistant strains in the field in Japan (representatives only)

Fungicide	Pathogen
Polyoxin	*Alternaria alternata*
Kasugamycin	*Magnaporthe grisea*, *Pseudomonas avenae***
Benzimidazoles	*Botrytis cinerea*, *Venturia nashicola*, *Monilinia fructicola*, *Colletotrichum theae-sinensis*, *Gibberella fujikuroi*, *Tapesia yallundae*, *Fusarium graminearum*, *Cercospora kikuchii*, *B. allii*, *C. gloeosporioides*, *Elsinoe fawcetti*, *E. ampelina*
Organophosphorous fungicides	*M. grisea*
Dicarboximides	*B. cinerea*, *A. alternata*
Streptomycin*	*Xanthomonas campestris* pv. *pruni***, *P. syringae* pv. *lachrymans***
Phenylamides	*Pseudoperonospora cubensis*, *Phytophthora infestans*
Sterol demethylation inhibitors	*Podosphaera* (*Sphaerotheca*) *fusca*, *Erysiphe* (*Blumeria*) *graminis* f.sp. *tritici*, *S. aphanis* var. *aphanis*, *Mycovellosiella nattrassii*
Fluazinam	*B. cinerea*
Oxolinic acid*	*P. glumae***, *P. avenae***
QoIs (Strobilurins)	*P. fusca*, *P. cubensis*, *M. nattrassii*, *Corynespora cassiicola*, *C. gloeosporioides*
MBI-Ds	*M. grisea*

*: Bactericide. **: Bacterial pathogen.

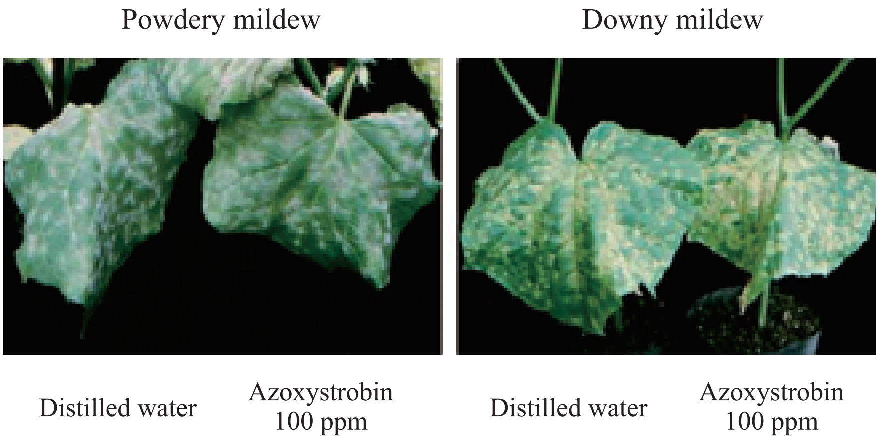

Fig. 1. Resistance of cucumber powdery mildew and downy mildew to strobilurin fungicide azoxystrobin

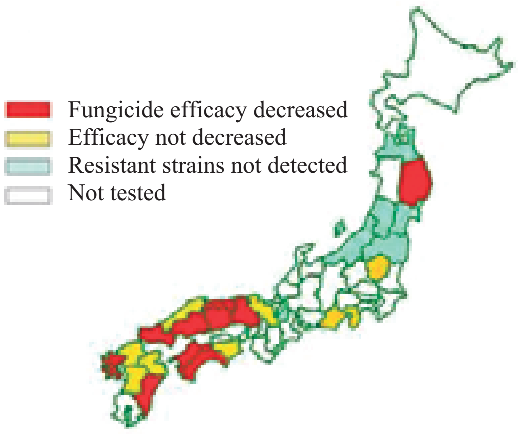

Fig. 2. Distribution of MBI-D-resistant strains in rice blast fungus in Japan

Resistance to QoI fungicides

QoI fungicides (strobilurin fungicides), which inhibit mitochondrial respiration at the Qo site of cytochrome *bc1* enzyme complex, are the most important class of fungicides at present and azoxystrobin is the world's biggest-selling fungicide[1]. They have a broad spectrum of control activity against a large number of pathogens on various crops. As of September 2005, five QoI fungicides (kresoxim-methyl, azoxystrobin, metominostrobin, famoxadone, and trifloxystrobin) were registered in Japan, but some others (orysastrobin, pyraclostrobin, and fenamidone) will also get fungicide registration in the near future.

As experienced with benzimidazole fungicides earlier, site-specific inhibitors such as QoI fungicides generally carry a high risk of resistance development in pathogens. Between 1998 and 1999 in fact, shortly (only one year in some cases) after the introduction, control failure of powdery mildew (*Podosphaera fusca* = *Sphaerotheca fusca*) by QoI fungicides was frequently reported in cucumber and melon growing areas in Japan. Subsequently, reduced efficacy of these fungicides was also claimed in cucumber downy mildew (*Pseudoperonospora cubensis*). Bioassays carried out using intact plants and leaf disks clearly showed the occurrence of resistant pathogen isolates (Fig. 1)[10].

Most growers followed the manufacturers' usage recommendation and applied their products only a couple of times per crop in alternation with other fungicides, which possess different modes of action. Unfortunately, however, they couldn't escape from incredibly rapid development of QoI resistance. When control efficacy of the fungicides was lost due to resistance, the growers stopped using them. Results from monitoring tests showed that resistant strains were still predominant in a greenhouse after withdrawal of fungicides in question for 3 years[11]. In addition, monitoring tests performed in 2004 revealed only resistant strains were detected in cucumber powdery mildew irrespective of the usage history of QoI fungicides indicating that resistant strains are widely distributed in this air-borne pathogen. It is less likely that resistant fungal populations rapidly shift back to wild-type sensitive populations due to a fitness penalty for the time being. This seems to be the typical example which shows how fungicide applications have strong potentials for disturbing microbial populations in the agricultural environment.

So far, QoI resistance has been reported in over 20 pathogens in the world (Table 2)[9]. The most recent

Table 2. Occurrence of strobilurin resistant strains in the field in the world

Disease	Pathogen
Wheat powdery mildew*	*Erysiphe* (*Blumeria*) *graminis* f.sp. *tritici*
Wheat speckled leaf blotch	*Mycosphaerella graminicola*
Barley powdery mildew	*E. graminis* f.sp. *hordei*
Potato early blight	*Alternaria solani*
Cucurbit powdery mildew*	*Podosphaera* (*Sphaerotheca*) *fusca*
Cucumber downy mildew*	*Pseudoperonospora cubensis*
Cucumber Corynespora leaf spot*	*Corynespora cassiicola*
Cucurbit gummy stem blight	*Didymella bryoniae*
Eggplant leaf mold*	*Mycovellosiella nattrassii*
Banana black Sigatoka	*Mycosphaerella fijiensis*
Grapevine downy mildew	*Plasmopara viticola*
Grapevine powdery mildew	*Uncinula necator*
Apple scab	*Venturia inaequalis*
Apple Alternaria leaf blotch*	*A. alternata*
Pistachio Alternaria late blight	*A. alternata* etc.
Citrus gray mold*	*Botrytis cinerea*
Chrysanthemum white rust	*Puccinia horiana*
Turf grass anthracnose*†	*Colletotrichum graminicola*
Turf grass leaf spot	*Pyricularia grisea*
Turf grass blight	*Pythium aphanidermatum*

*: Detected in Japan. †: Strobilurins not registered in Japan.

examples in Japan are resistance in gray mold (*Botrytis cinerea*) on citrus, and Alternaria leaf blotch (*Alternaria alternata* apple pathotype) on apple. But it is uncertain yet in these two cases whether resistant strains relate with the loss of field performance of fungicides or not.

Resistance to MBI-D fungicides in rice blast fungus

Magnaporthe grisea (*Pyricularia oryzae*), the rice blast fungus, produces melanized appressoria, essential for penetration into cuticle layers of rice plants. Melanin biosynthesis inhibitors (MBIs), widely used for rice blast control, are divided into two classes: inhibitors of polyhydroxynaphthalene reductase (MBI-R fungicides) and those of scytalone dehydratase (MBI-D fungicides). The former group containing tricyclazole, pyroquilon, and phthalide have been used for over 30 years with no sign of resistance development in the target pathogen. Only very recently, laboratory resistant mutants were first obtained in China but no resistant strains have been isolated from the field yet[17].

Currently in Japan, seedling box treatment with MBI-D fungicides, e.g. carpropamid, is a common cultural practice in many rice growing areas as carpropamid exhibits long-lasting control efficacy against blast disease. The treatment is labor-cost effective, and greatly contributed to diminishing fungicide applications in paddy fields, in other words lowering the pesticide input to the environment.

However, in summer 2001, the efficacy of carpropamid against leaf blast was lost suddenly in Saga Prefecture, Kyushu. Results from extensive studies indicated that resistant strains appeared and played a significant role in the decrease of the fungicide efficacy[14,16]. It is very likely that the long-lasting efficacy based on the persistent properties of this fungicide has acted as a strong selection pressure and resistant strains rapidly increased in fungal populations. Distribution of resistant strains has spread from Kyushu to Shikoku, Chugoku, and Kinki District gradually, then eventually those strains have been detected in a couple of prefectures in Tohoku District, the north of Japan in 2004 (Fig. 2). Based on theoretical considerations and circumstantial evidence accumulated from field data as described below, the author of the present paper proposes to use MBI-D fungicides, if they are still effective, in alternation with other fungicides such as MBI-R fungicides or resistance inducers e.g. probenazole every 2 to 3 years for the treatment of rice seedling boxes.

Integrated disease control and fungicide resistance

Fungicide resistance is a critical factor limiting not only quality food production but also achievement of integrated disease control as farmers may often use fungicides in a higher dose or more frequently when they come across resistance problems. In addition, if application of fungicides has to be reduced according to the strong demands from the public, environmentalists and others, after all farmers would rely more on fungicides carrying higher efficacy, broader spectrum and properties to persist longer which might result in an increased risk for resistance development in pathogens.

QoI fungicides were originally developed from naturally occurring products (e.g., strobilurin A in mushrooms), not persistent in the environment ('low-risk pesticides' by the category of the Environmental Protection Agency, USA), and are regarded to be highly suitable for inclusion in integrated pest management (IPM) programs. Actually, in Aomori Prefecture, where about half of Japanese domestic apples are produced, concerned people succeeded in reducing fungicide applications from 16 times per year in 1981 to 11 times in 2003. However, dependence on both QoI fungicides and DMIs (sterol 14α-demethylation inhibitors) has rather increased to more than was previously used.

The summer diseases such as flyspeck (*Schizothyrium pomi*) and sooty blotch (*Gloeodes pomigena*) often reduce the commercial value of harvested apple fruit as 'cosmetic diseases', and thereby growers must spray fungicides. In Aomori, only QoI fungicides have been available for summer disease control until very recently as they are allowed to apply until one day before harvest under the official pesticide-usage guideline. Furthermore, apple fruits, dropped on the ground by strong typhoon winds, will be assessed for the residue of pesticides when they are processed for juice production. Due to these reasons, it has not been uncommon for apple growers to successively spray QoI fungicides as a solo product 2 to 4 times in the summer.

DMI fungicides are also widely used for controlling plenty of diseases on fruit trees, vegetables, cereals, and other crops. In pear growing, DMIs have been used as a major class of fungicides to control scab disease caused by *Venturia nashicola*. Development of DMI resistance was carefully monitored in this pathogen as well as in *V. inaequalis*, the apple scab fungus. The loss of fungicide efficacy has never been reported in commercial orchards although fungal isolates with reduced DMI sensitivity were often detected[6,15]. In spring 2005, however, the early sign of resistance development has been found in *V.*

nashicola isolates collected from Fukuoka Prefecture. Spores of this fungus used for inoculation tests produced lesions on the leaves pretreated with a DMI fungicide (Ishii and Kikuhara, unpublished). DMI fungicides have been used for scab control in pear since 1983 and resistance has been successfully managed. Sparse spray applications of DMIs in alternation with other classes of fungicides or their tank-mixed applications were strongly recommended by experts. Nevertheless, some pear growers have sprayed DMI fungicides 3 to 6 times successively starting at the pre-blossom period of pear.

How to manage fungicide resistance

To combat fungicide resistance, use of the at-risk fungicides in a mixture or a rotation with a fungicide of a different type has been recommended for a long time. Table 3 shows a simulation model indicating the effects of combined or alternating use of fungicides on build-up of resistant fungal populations[2]. Similarly the resistance risk of fungicide spray programs is shown in Table 4[5]. More recently, the effectiveness of strategies aiming to retard the development of QoI resistance was determined in the field populations of barley powdery mildew fungus[3]. Mixtures of fungicides with different modes of action appeared to slow down the increase in the frequency of the mutation in the gene encoding the fungicide-targeted protein.

However, such a general strategy might be successful only for delaying resistance development but not always effective for stopping the development of resistance itself. If so, can we predict the risk for resistance development precisely at a previous stage? The answer might be presumably ‘No, not enough yet’. Actually some fungicides are used under the situation that their modes of action, i.e., either they have a specific target site or multiple target sites, are still unknown. A methodological approach was conducted to assess the development risk for QoI resistance in wheat and barley pathogens[13]. Different concentrations of azoxystrobin were applied on cotyledons to ensure a selection pressure. The higher fungicide sensitivity of barley powdery mildew required more generations to manifest resistance than in the experiment with wheat powdery mildew. The significant differences in fungicide sensitivity between the two powdery mildews are in a good accordance with differences in the speed of resistance development observed in the field.

We know that the risk assessment for resistance development was a failure in some cases, e.g. benzimidazole resistance in cereal eyespot, dicarboximide resistance in gray mold, and QoI resistance in apple scab. Limitations in model experiments exist as the size of pathogen populations in those experiments is generally small, and so the fungicide pressure to select resistant strains in populations will be too low to cause the decrease of fungicide efficacy. Less genetic diversity of the pathogen will require a much longer time until resistant individuals appear and spread widely in the fungal populations. Taken together, it should be recommended

Table 3. Effects of combined or alternating use of two fungicides on build-up of a resistant pathogen population (Simulation model from Dekker (1982))

Fungicide application	Proportion of the resistant populations after various numbers of sprayings				
	5	10	20	30	40
S repeated	0.000	0.826	1.000	1.000	1.000
(S + C) repeated	0.000	0.000	0.996	1.000	1.000
S alternating with C	0.000	0.000	0.826	1.000	1.000
(S + C) alternating with C	0.000	0.000	0.000	0.261	0.996

S: Vulnerable fungicide, e.g. systemic fungicide. C: Conventional fungicide. Initial resistance frequency = 10^{-9}.

Table 4. Resistance risk of spray programs (Hewitt, 1998)

Program sequence	Program type	Risk
H-H-H-H	Repeat	High
H-L-H-L	Alternation	
(H + L)-(H + L)-(H + L)-(H + L)	Mixture	
(H + L)-H-(H + L)-L	Combination	
L-L-(H + L)-L	Combination	Low

to use fungicides at risk or at unknown risk as sparsely as possible. The routine monitoring conducted in commercial vineyards in Champagne, France, known to be a 'hotspot of resistance', showed the presence of gray mold resistant to benzimidazoles, dicarboximides, and anilinopyrimidines[12]. However, the limitation in the use of these 3 families of fungicides, once for each per year when used, prevented the build-up of resistant fungal populations.

Application of modern technology for resistance monitoring

Monitoring for detecting resistance development is important; however it is definitely more important to notice the early shifts of fungicide sensitivity to resistance in fungal populations than to notice a drop in control efficacy of fungicides. To enable rapid detection of resistance, which is often difficult with conventional bioassays including mycelial growth and spore germination tests *in vitro* or fungus inoculation tests by using plants pretreated with fungicides, some molecular methods have already been developed[7].

The method most commonly used is PCR (polymerase chain reaction), which can amplify the fragments of target genes, e.g. genes encoding fungicide-binding proteins. Molecular mechanisms of QoI resistance have been well characterized and a single point mutation of a nucleotide (e.g. GGT to GCT) at the amino acid position 143 in the fungicide-target cytochrome *b* protein governs the expression of high resistance in many fungal pathogens (Fig. 3)[4,9]. Using the mechanism, QoI resistance is diagnosed when PCR fragments of the cytochrome *b* gene are cut with the restriction enzyme *Ita* I, which recognizes the nucleotide sequence of GCNGC (Fig. 4).

Recently, the author of this paper successfully introduced a novel method, the PCR-Luminex system, for identifying MBI-D resistance in *M. grisea* isolates (Ishii et al., in press). This detection system is based on hybridization between a fluorescent PCR product and an oligonucleotide probe, which can specifically recognize the single point mutation of the gene conferring resistance. The PCR-Luminex system has been further employed for the rapid diagnosis of pathogen species, *Fusarium graminearum*, *F. culmorum*, and *Microdochium nivale*, which cause Fusarium head blight of cereals.

Resistant isolate R: *Ita* I
↓
・・・GGACAAATGAGTTTTTGGGCTGCAACTGTTATTACT・・・
143
Sensitive isolate S:
・・・GGACAAATGAGTTTTTGGGGTGCAACTGTTATTACT・・・

Fig. 3. Partial nucleotide sequences of cytochrome *b* gene in strobilurin-resistant and -sensitive strains of cucumber downy mildew and restriction site of the enzyme *Ita* I

What can we do to cope with fungicide resistance?

What have we learned from the history of fungicide resistance which started a long time ago? It may certainly be rare these days to see farmers applying the same group of fungicides continuously. Many of them may be making efforts to minimize fungicide applications due to an interest in environmental issues or in order to reduce costs of production. Although it is often argued, we should stop using the fungicide in question if we detect resistant strains over certain levels but not carry on using it as long as alternative control measures are available. We might be able to start using the fungicide again if resistant populations decline and the risk for control failure by the fungicide is judged to be low. Strict regulation of fungicide usage and education of dealers and farmers through transferring necessary information will make it possible to manage resistance more effectively.

Approaches including the use of disease tolerant or resistant cultivars of crops together with other cultural practices, e.g. sanitation, should be considered more seri-

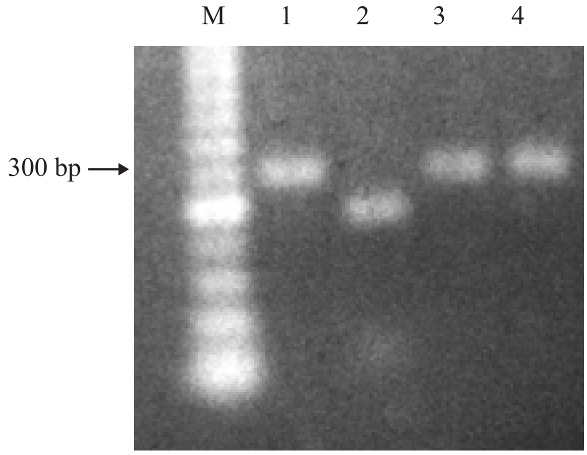

Fig. 4. *Ita* I digestion pattern of cytochrome *b* gene of cucumber downy mildew isolates
M, 50 bp ladder;
1, Untreated (Resistant isolate R); 2, *Ita* I-treated (Resistant isolate R); 3, Untreated (Sensitive isolate S); 4, *Ita* I-treated (Sensitive isolate S).

ously so that the population density of fungicide-resistant populations and/or pathogens themselves would decrease, therefore lowering the pressure to select resistant pathogens. Those trials will surely contribute to diminishing the input of fungicides to the agricultural environment. It is also required to develop novel types of disease-control agents such as systemic disease-resistance inducers, not antimicrobial but instead agents that activate host defense responses, which most plants have acquired inherently during their evolution. It is less likely that pathogens will develop resistance to those resistance inducers as induced systemic (systemic acquired) resistance is based on and orchestrated by a number of defense mechanisms[8]. In fact, no resistance issue has arisen in probenazole, which was commercialized around 30 years ago as the first disease-resistance inducer in the world. This product has been one of the biggest-selling disease control agents for many years in Japan.

Finally, international cooperation and collaboration, e.g. exchange of experimental materials and information, will be quite useful to combat resistance as pathogen isolates resistant to fungicides or bactericides can also occur independently in many parts of the world although they are also transmitted from one place to another.

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