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Osmotic Sensitivity and Cross-Resistance of Iprodione-Resistant Mutants of *Cochliobolus heterostrophus*

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Four resistant mutants (MIC > 1600 µg/ml), i.e. IRE002, IRE008, IRE009 and IRE012, were isolated in a study of genetics of iprodione resistance in *Cochliobolus heterostrophus* after ethyl methanesulphonate mutagenesis. Osmotic sensitivity and cross-resistance of the resistant mutants were investigated. The results showed that the iprodione-resistant mutant strains were osmotically sensitive and displayed cross-resistance to some fungicides, indicating a possibility that iprodione resistance in the fungus may be controlled by pleiotropic drug resistance genes.

INTRODUCTION

Pesticide resistance has been one of the most important and challenging topics in plant disease-related research nowadays. Because there have been indications that a number of pleiotropic drug resistance (PDR) genes are associated with sensitivity to osmotic pressure and cross-resistance, osmotic sensitivity and cross-resistance of fungicide-resistant mutant strains in a number of fungal species have for some extent been subjects of considerable interest (Shimanuki *et al*. 1992). Genes controlling phenotypes specific of PDR have been characterized in *Saccharomyces cerevisiae* (Balzi *et al*. 1987) and *Candida albicans* (Prasad *et al*. 1995). In all these cases, the genes confer resistance to some drugs with collateral hypersensitivity to others (Meyers *et al*. 1992, Prasad *et al*. 1995). PDR has also been a major concern in a number of other aspects of agricultural developments. The regulation of resistance of plant pathogens toward natural plant defense compounds such as preformed compounds and phytoalexins, as well as the development of parasite-toxin resistant crops, are of major economic importance in agriculture.

No investigation, however, has so far been reported on the relationship in *Cochliobolus heterostrophus* (Drechsler) Drechsler [anamorph: *Bipolaris maydis* (Nisikado & Miyake) Shoemaker], the causal agent of the southern corn leaf blight disease, although there has been a speculation that iprodione resistance in the fungus is governed by PDR genes. This contradicts the fact that genetic and molecular aspects of fungicide resistance in the fungus have to some extent been investigated (Gafur 1999, Gafur *et al.* 1998a, Gafur *et al*. 1998b, Gafur *et al*. 2000). Investigations leading to the confirmation whether or not iprodione resistance in *C. heterostrophus* is controlled by PDR genes would therefore be worth-pursuing. The current investigation was initiated in the framework of possible role of the PDR genes in conferring iprodione resistance in *C. heterostrophus*.

Non-agricultural scientific communities may also benefit from the present research by using *C. heterostrophus* in exploring molecular mechanisms of living processes because in many respects *C. heterostrophus* could be a valid model for higher eukaryotic cells. For example, PDR mechanism operating in the fungus would provide new information relevant to the corresponding function of multiple drug resistance (MDR) in mammalian cells. It is thus important to determine whether the well known process of MDR is also operating in *C. heterostrophus*. There is no report to date of PDR loci for multiple drug resistance in *C. heterostrophus*. Knowledge on the drug resistance mechanisms could assist the development of not only new plant disease control agents but also therapeutic chemicals. It was also for this reason that the present study was initiated.

MATERIALS AND METHODS

Cultures, Fungicides and Media. Cultures used in this study are listed in Table 1. HITO7711 and MASHIKI2-2 are wild type strains (Tanaka *et al*. 1991), IRE002, IRE008, IRE009 and IRE012 are resistant mutants with minimum inhibitory concentration (MIC) > 1600 µg/ml that were previously isolated, and the other strains are progenies of the crosses between each of the mutants and the wild type strain (Gafur *et al*. 2000). Fungicides employed included bialaphos, cycloheximide, 5-fluorouracil, hygromycin, imazalil, iprodione, methotrexate, polyoxin, and terbinafin. The compounds were either dissolved in sterile distilled water and filter-sterilized or dissolved in 70% (v/v) ethanol before addition to the respective basal medium. Complete medium (CM) was used to maintain the cultures and as basal medium in assessing sensitivity to cycloheximide, hygromycin, imazalil, iprodione, and terbinafin. The medium was prepared with distilled water to contain the following components (g/l): $Ca(NO_3)_2 \cdot 4H_2O$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; KH_2PO_4, 0.4; K_2HPO_4, 0.03; glucose, 10.0; tryptone, 1.0; yeast extract, 1.0 and agar, 15.0. The medium was autoclaved at 121^0C for 15 min. Minimal medium (MM) containing the components of (g/l) $Ca(NO_3)_2 \cdot 4H_2O$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; KH_2PO_4,

Table 1. Strains and some of their characteristics of *Cochliobolus heterostrophus* used in this study

Strain	Phenotype	Mating type	Source
HITO7711	Iprodione sensitive	*MATI-2*	Tanaka *et al.* 1991
MASHIKI2-2	Iprodione sensitive	*MATI-1*	Tanaka *et al.* 1991
IRE002	Iprodione resistant	*MATI 2*	Gafur *et al.* 2000
IRE008	Iprodione resistant	*MATI 2*	Gafur *et al.* 2000
IRE009	Iprodione resistant	*MATI 2*	Gafur *et al.* 2000
IRE012	Iprodione resistant	*MATI 2*	Gafur *et al.* 2000
IRE002-501	Iprodione sensitive	*MATI-1*	Gafur *et al.* 2000
IRE002-502	Iprodione sensitive	*MATI-2*	Gafur *et al.* 2000
IRE002-503	Iprodione resistant	*MATI-2*	Gafur *et al.* 2000
IRE002-504	Iprodione resistant	*MATI-1*	Gafur *et al.* 2000
IRE002-505	Iprodione resistant	*MATI-1*	Gafur *et al.* 2000
IRE002-506	Iprodione sensitive	*MATI-1*	Gafur *et al.* 2000
IRE002-507	Iprodione sensitive	*MATI-2*	Gafur *et al.* 2000
IRE002-508	Iprodione resistant	*MATI-2*	Gafur *et al.* 2000
IRE008-101	Iprodione resistant	*MATI-1*	Gafur *et al.* 2000
IRE008-103	Iprodione sensitive	*MATI-2*	Gafur *et al.* 2000
IRE009-305	Iprodione sensitive	*MATI-2*	Gafur *et al.* 2000
IRE009-307	Iprodione resistant	*MATI-1*	Gafur *et al.* 2000
IRE012-303	Iprodione resistant	*MATI-1*	Gafur *et al.* 2000
IRE012-307	Iprodione sensitive	*MATI-2*	Gafur *et al.* 2000

0.4; K_2HPO_4, 0.03; glucose, 10.0 and agar, 15.0 was used as basal medium in assessing sensitivity to bialaphos, 5-fluorouracil, methotrexate, and polyoxin. The media were cooled to 50^{0}C before respective fungicides were added.

Osmotic Sensitivity. Considering that iprodione resistance can be characterized by the level of osmotic sensitivity pleiotrophically conditioned as shown in *Talaromyces flavus* (Katan *et al*. 1984), those of iprodione resistance of *C. heterostrophus* was also osmotically investigated. Wild type, as well as resistant and sensitive progenies of crosses between each of the four resistant mutant and the wild type strains, were used in this experiment. Sodium chloride (NaCl) to the final concentration of 1M was added to CM before the medium was sterilized. The mycelial growth of resistant and sensitive strains were observed after five days of inoculation on the medium.

Sensitivity to Fungicides and Cross-resistance. Wild type, as well as resistant and sensitive progenies of crosses between each of the four resistant mutant and the wild type strains, were also checked for their resistance to some other fungicides. The concentration of the fungicides was determined based on MIC of the fungicides against the wild type strain as explained by Gafur *et al*. (1998b). MIC was defined as the lowest concentration of the fungicide that produced no visible fungal growth. It was estimated after three days of incubation at 27^{0}C. Sensitivity test was conducted according to the method described previously (Gafur *et al*. 1998b).

RESULTS

Osmotic Sensitivity. Osmotic sensitivity of the mutant and wild type strains was determined on the basis of mycelial growth on CM containing 1M NaCl. As shown in Table 2 and Figure 1, all strains resistant to iprodione were sensitive to osmotic pressure, and all iprodione-sensitive strains were resistant to osmotic pressure.

Sensitivity to Fungicides. Unrelated fungicides were selected and used in the present study. The reaction phenotype of *C. heterostrophus* against these fungicides is summarized in Table 3. As it is clearly shown in the table, the fungus exhibited a range of sensitivity to the fungicides. The MIC of the fungicides for mycelial growth ranged from 3.1 µg/ml (of imazalil, iprodione and terbinafin) to 400.0 µg/ml (of methotrexate).

Cross-resistance. The results of the cross-resistance experiment (Table 4) indicated that iprodione-resistant mutants of *C. heterostrophus* was positively cross-resistant to hygromycin and imazalil, but negatively cross-resistant against cycloheximide, 5-fluorouracil and methotrexate. No cross relationship was observed to bialaphos, polyoxin and terbinafin (data not shown).

Table 2. Osmotic sensitivity of mutant and wild type strains of *Cochliobolus heterostrophus* to complete medium supplemented with 1M NaCl

Strain	Number of isolates observed	Osmotic sensitivity
Mutant (iprodione-resistant)		
IRE002-503	4	sensitive
IRE008-101	4	sensitive
IRE009-307	4	sensitive
IRE012-303	4	sensitive
Mutant (iprodione-sensitive)		
IRE002-501	4	resistant
IRE008-103	4	resistant
IRE009-305	4	resistant
IRE012-307	4	resistant
Wild type (iprodione-sensitive)		
HITO7711	1	resistant
MASHIKI2-2	1	resistant

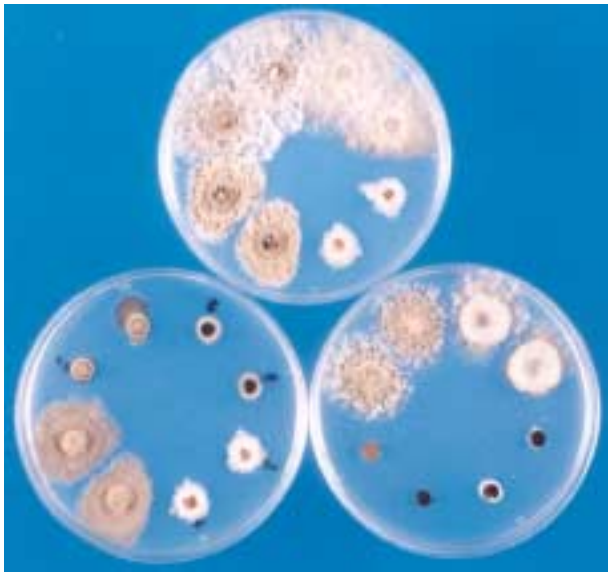

Figure 1. Osmotic sensitivity of iprodione-resistant segregants of *Cochliobolus heterostrophus*. All segregants grew well on complete medium, CM (top), but only iprodione-sensitive segregants (bottom right) grew on CM containing 1M of NaCl (bottom left). Photograph was taken after five days of inoculation on each medium.

Table 3. Sensitivity of *Cochliobolus heterostrophus* to some fungicides*

Fungicide	MIC (μg/ml)
Bialaphos	100.0
Cycloheximide	200.0
5-Fluorouracil	200.0
Hygromycin	100.0
Imazalil	3.1
Iprodione	3.1
Methotrexate	400.0
Polyoxin	25.0
Terbinafin	3.1

* Based on minimum inhibitory concentration (MIC) determined three days after inoculation

Table 4. Cross-resistance of mutant and wild type strains of *Cochliobolus heterostrophus* to some fungicides

Strain	Fungicide				
	A	B	C	D	E
Mutant (iprodione-resistant)					
IRE002-503	S	S	R	R	S
IRE002-504	S	S	R	R	S
IRE002-505	S	S	R	R	S
IRE002-508	S	S	R	R	S
Mutant (iprodione-sensitive)					
IRE002-501	R	R	S	S	R
IRE002-502	R	R	S	S	R
IRE002-506	R	R	S	S	R
IRE002-507	R	R	S	S	R
Wild type (iprodione-sensitive)					
HITO7711	R	R	S	S	R
MASHIKI2-2	R	R	S	S	R

A. Cycloheximide (50.0 μg/ml), B. 5-Fluorouracil (50.0 μg/ml), C. Hygromycin (100.0 μg/ml), D. Imazalil (1.6 μg/ml), E. Methotrexate (100.0 μg/ml), R. resistant, S. sensitive

DISCUSSION

Osmotic Sensitivity. Osmotic sensitivity of the resistant mutants was explored to elucidate possible relationship in *C. heterostrophus* between iprodione resistance and PDR genes which have previously been stated by Shimanuki *et al*. (1992) that sensitivity mightcorelate with osmotic pressure. In contrast to osmotic resistance of the iprodione-sensitive strains, all iprodione-resistant strains of *C. heterostrophus* investigated in the present study were found to be osmotically sensitive.

The same correlation have also been noted in some other fungi. Iprodione-resistant strains of *Botrytis cinerea* and *Fusarium nivale* showed increased sensitivity to high osmotic pressure (sodium chloride, glucose) (Leroux *et al*. 1992). All of iprodione-resistant strains of *Aspergillus nidulans, B. cinerea,* and *Penicillium expansum* were osmotically sensitive (Beever 1983). Grindle (1984), Grindle and Dolderson (1986) and Grindle and Temple (1985) noted that most of the dicarboximide-resistant mutants of *Neourospora crassa* were sensitive to high osmolarity. Similarly, osmotic-sensitive mutants which were initially isolated by Mays (1969) are resistant to fungicides of the dicarboximide group.

Osmotic sensitivity has been suggested to be one of the reasons for the relative lack of fitness of the resistant strains in the field. However, the *in vitro* selection in some fungal species of isolates of low osmotic sensitivity with high dicarboximide resistance (Beever 1983) cautions that such strains might become a problem in chemical control of diseases in the field in the future.

Sensitivity to Fungicides. Mycelial growth was used as the primary parameter of fungicide sensitivity of *C. heterostrophus* in this study. In a number of pathogen-fungicide systems, swelling and bursting of cells leading to alterations in hyphal morphology and mycelial growth is the most remarkable response of pathogens to fungicides (Hwang & Yun 1986). For example, whereas iprodione inhibits mycelial growth much more than spore germination in *B. cinerea* (Pappas & Fisher 1979), mycelial growth inhibition was the only vital process shown to be due to the fungicide in *Sclerotinia sclerotiorum* (Reilley & Lamoureux 1981).

Cross-Resistance. PDR genes have also been reported to confer resistance to some drugs and sensitivity to others (Meyers *et al*. 1992, Prasad *et al*. 1995). The same results were obtained in the present study. Iprodione-resistant strains of *C. heterostrophus* showed negatively cross-resistance to some fungicides and positively to some others. Similar phenomena have also been noted in a number of fungal species. Most of benomyl-resistant strains of pathogenic fungi display increased sensitivity to the *N*-phenylcarbamate fungicides. This negatively correlated cross-resistance between benomyl and *N*-phenylcarbamates was observed in some fungal species including *Rhynchosporium secalis* (Kendall *et al*. 1994), *Venturia inaequalis* (Jones *et al*. 1987, Shabi *et al*. 1987), *V. nashicola* (Ishii & van Raak 1988) and *V. pirina* (Shabi *et al*. 1986, Shabi *et al*. 1987). Elad *et al*. (1992), nevertheless, isolated strains of *B. cinerea* resistant to carbendazim, iprodione and diethofencarb.

Inheritance of MDR in most of the *S. cerevisiae* strains analyzed can be readily explained by assuming independent mutations in the same gene (Saunders & Rank 1982). Isolation, sequencing and complementation test of PDR1, responsible for pleiotropic drug resistance in the yeast, also ascertain the single gene inheritance (Balzi *et al*. 1987). Similarly, iprodione resistance in *C. heterostrophus* was earlier also found to be controlled by monogenic genes (Gafur *et al*. 2000). This, together with osmotic sensitivity and cross-resistance to some fungicides shown by iprodione-resistant mutants of *C. heterostrophus* in the present study, leads to a conclusion that the iprodione resistance trait in the fungus is most probably controlled by PDR genes, although further investigations are needed for confirmation. Isolation and cloning of the genes is for sure one of the next topics of interest.

The present study was also carried out in the awareness of steady change of the distribution of certain genes in nature due to interaction between pathogens and hosts or environmental factors. The use of chemicals to which the resistant strains show a negatively correlated cross-resistance is another option in reducing the use of agricultural chemicals (Ishii & van Raak 1988, Staskawicz *et al*. 1995). The basic knowledge obtained from this research should therefore also lead to reduced use of environmentally problematical pesticides.

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