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GENETIC RELATIONSHIP BETWEEN COLONY COLOUR AND POLYOXIN RESISTANCE IN *COCHLIOBOLUS HETEROSTROPHUS*

HUBUNGAN GENETIK ANTARA WARNA KOLONI DAN KETAHANAN TERHADAP POLIOKSIN PADA COCHLIOBOLUS HETEROSTROPHUS

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INTISARI

Tiga mutan tahan polioksin (MIC > 1600 µg/ml) Cochliobolus heterostrophus *(Drechsler) Drechsler dengan warna koloni kemerahan (NA1, PRE058 dan TE2) dan dua berwarna keputihan (PRE062 dan PRE070) diisolasi setelah mutagenesis kimiawi menggunakan ethyl methanesulphonate (EMS). Mutan tahan tersebut disilangkan satu dengan lainnya atau dengan galur liar yang berwarna kehijauan dan sensitif pada polioksin dalam upaya untuk mengkaji hubungan genetik antara warna koloni keputihan dan ketahanan terhadap polioksin pada jamur tersebut. Analisis segregasi fenotip keturunan dari persilangan tersebut menunjukkan bahwa warna koloni dan ketahanan terhadap polioksin pada tiga galur mutan berwarna kemerahan tersebut dikendalikan oleh gen monogenik. Pada penelitian ini tidak ditemukan adanya keterpautan antara gen yang mengendalikan warna koloni keputihan dan ketahanan terhadap polioksin pada jamur tersebut.*

Kata kunci: Bipolaris maydis, Cochliobolus heterostrophus, *ketahanan terhadap polioksin, keterpautan, warna koloni*

ABSTRACT

Three reddish (NA1, PRE058 and TE2) and two whitish (PRE062 and PRE070) polyoxin-resistant mutant strains of *Cochliobolus heterostrophus* (Drechsler) Drechsler (MIC > 1600 µg/ml) were isolated after ethyl methanesulphonate (EMS) mutagenesis. The resistant mutants were crossed to each other or to the greeny, sensitive wild type strain to investigate genetic relationship between the whitish colony colour and polyoxin resistance traits in the fungus. Progeny analyses showed that colony colour and polyoxin resistance in the three reddish resistant mutants were conferred by monogenic genes. No linkage was detected in the present study between the genes controlling whitish colony colour and polyoxin resistance traits in the fungus.

Key words: *Bipolaris maydis*, *Cochliobolus heterostrophus*, colony colour, linkage, polyoxin resistance

INTRODUCTION

Polyoxin, an antifungal antibiotic originally isolated from *Streptomyces cacaoi* var. *asoensis* (Isono *et al.*, 1969), inhibits chitin synthetase from fungi and insects but is not toxic to mammalian cells (Becker *et al.*, 1983). It has in the past been widely used as a component of disease control agents for different pathosystems. As polyoxin became more extensively used, development of resistance to the fungicide in some plant pathogens posed a serious practical problem. Polyoxin-resistant strains have since been reported in isolates of different fungal species.

In a genetic investigation of polyoxin resistance in *Cochliobolus heterostrophus* (Drechsler) Drechsler [anamorph: *Bipolaris maydis* (Nisikado & Miyake) Shoemaker], the incitant of southern corn leaf blight, resistant mutants with different colony colours were isolated (Gafur, 1998). The colour of most of the recovered resistant mutant strains resembled that of the wild type strain, *i.e.* greeny, although reddish and albino mutants were also generated.

Genetic and biochemical aspects of the colony colour trait in the fungus have been extensively analyzed (Shimizu *et al.*, 1997; Tanaka *et al.*, 1991; Tanaka *et al.*, 1994). Melanins, whose production is widespread in the fungal kingdom, were shown to determine colony colours of *C. heterostrophus*. The pathway of the melanin biosynthesis was then established (Tanaka *et al.*, 1991; Tanaka *et al.*, 1994) and genes involved in the process were subsequently identified and characterized (Shimizu *et al.*, 1997).

Since the importance of melanins for the survival and longevity of fungal propagules has long been recognized (Shimizu *et al.*, 1997), investigations on the genetic relationship between colony colour and other important traits including fungicide resistance are of primary importance. Genetic and molecular aspects of fungicide resistance in the fungus have recently also been examined (Gafur, 1999; Gafur *et al.*, 1998a; Gafur *et al.*, 1998b; Gafur *et al.*, 2000). The present study was aimed at exploring genetic relationship between colony colour and polyoxin resistance. The importance of the study was not merely because of the fact that such investigation has not been conducted, but more importantly because it was also intended to contribute to the minimization of agricultural crop losses due to plant diseases as well as to the reduction of unexpected impacts of the use of agricultural fungicides which is surely crucial for a sustainable agricultural production.

MATERIALS AND METHODS

Strains and media. Strains of *C. heterostrophus* used in this study are listed in Table 1. The reddish (NA1, PRE058, and TE2) and whitish (PRE062 and PRE070) isolates were polyoxin-resistant mutant strains (MIC > 1600 µg/ml), whereas HITO7711 (*MAT 1-2*) and MASHIKI2-2 (*MAT 1-1*) were greeny, poyoxin-sensitive wild type strains. Minimal medium (MM) was used as basal medium in assessing sensitivity to polyoxin. It 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 and agar, 15.0. The medium was autoclaved at 121°C for 15 min and left cooled to 50°C before polyoxin AL WP (10% active ingredient, Nippon Noyaku, Tokyo) dissolved in 70% (v/v) ethanol was added to the final concentration of 100 µg/ml. Complete medium (CM), containing MM, tryptone (1.0 g/l) and yeast extract (1.0 g/l), was used to maintain the cultures. Sachs' agar medium (SAM), which was used for crossing experiments, was prepared as follows (g/l): KNO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; $Ca(NO_3)_2$, 0.5; $Ca_3(PO_4)_2$, 0.5; $FeCl_2$, trace, and agar, 12.0. SAM was also autoclaved at 121°C for 15 min.

Determination of ascospore phenotypes. Resistance or sensitivity of ascospore isolates to polyoxin was tested by cutting mycelial disks 6 mm in diameter from the leading edge of a freshly growing colony. The isolates were then placed upside down on MM containing 100 μg/ml of polyoxin. Mycelial growth was observed after incubation for five days at 27°C. Resistance or sensitivity of the isolates to the fungicide was determined on the basis of the mycelial growth.

Table 1. Characteristics of *C. heterostrophus* strains used in this study

Strain	Phenotype	Mating Type	Source
HITO7711	Greeny, polyoxin sensitive	*MAT1-2*	Tanaka *et al.*, 1991
MASHIKI2-2	Greeny, polyoxin sensitive	*MAT1-1*	Tanaka *et al.*, 1991
PRE058	Reddish, polyoxin resistant	*MAT1-2*	Gafur *et al.*, 1998b
NA1	Reddish, polyoxin resistant	*MAT1-2*	C. Tanaka, personal communication
TE2	Reddish, polyoxin resistant	*MAT1-2*	C. Tanaka, personal communication
PRE062	Whitish, polyoxin resistant	*MAT1-2*	Mutant of HITO7711
PRE070	Whitish, polyoxin resistant	*MAT1-2*	Mutant of HITO7711
PRE058-301	Reddish, polyoxin resistant	*MAT1-2*	Gafur *et al.*, 1998b
PRE058-302	Reddish, polyoxin resistant	*MAT1-1*	Gafur *et al.*, 1998b
NA1-AS13	Reddish, polyoxin resistant	*MAT1-1*	C. Tanaka, personal communication
TE2-AS53	Reddish, polyoxin resistant	*MAT1-1*	C. Tanaka, personal communication
TE2-AS57	Reddish, polyoxin resistant	*MAT1-2*	C. Tanaka, personal communication

Genetic constituent and linkage analyses. The three reddish, resistant mutant strains were crossed to each other to explore genetic nature of the reddish colony colour and polyoxin resistance traits in the mutant strains. To investigate possible linkage between the gene for whitish colony colour and the gene for polyoxin resistance, each of the two whitish, resistant mutant strains was crossed with the greeny, sensitive wild type strain of opposite mating type, MASHIKI2-2. Crossings were made on SAM with rice straw as described by Ueyama & Tsuda (1975). Polymerase chain reaction (PCR) amplification method (Gafur *et al.*, 1997) was employed to determine mating types of resistant mutants and progenies of all crosses. Ascospores were isolated using a micromanipulator according to the method of Taga *et al.* (1978).

RESULTS AND DISCUSSION

Phenotypic expression of polyoxin resistance. Hyphal growth of the *C. heterostrophus* resistant mutants was examined on MM and MM containing polyoxin. The growth rate of the resistant and wild type strains was indistinguishable from each other in culture on MM. However, testing of isolates on polyoxin-amended MM resulted in the growth of only the resistant mutant strains.

Although the growth rate of mutant strains each bearing single colony colour and polyoxin resistance genes was, as mentioned earlier, similar to that of the wild type strains on MM, the mutant strains were readily distinguishable from the wild type strains because of their reddish or whitish colour compared with the greeny colour of the wild type strains. The difference was also evident on MM amended with polyoxin because only the mutant strains grew well, whereas the wild type strains did not.

Genetic constituents of the reddish mutants. Mature pseudothecia were evident in all matings after three weeks of incubation. All progenies of the crosses among the reddish, resistant mutant strains were reddish, resistant (Figure 1), indicating the absence of recombination or segregation of genes controlling colony colour and polyoxin resistance in the mutants. Although the intensity of reddish colour in the mutant strains was slightly different, the results of ascospore analysis (Table 2) also confirmed that the reddish colour and polyoxin resistance in these mutant strains were conferred by alleles at the same locus. The absence of wild type ascospore progenies from all crosses is somehow intriguing because the colony colour and

resistance to certain fungicides in plant pathogenic fungi are usually regulated by more than one gene (Georgopoulos, 1977; Tanaka *et al.*, 1991; Tanaka *et al.*, 1994).

Linkage analysis. From crosses between each of the whitish, resistant mutant strains and the greeny, sensitive wild type strain, 137 ascospores were examined for four possible phenotypes, *i.e.* whitish, resistant; whitish, sensitive; greeny, resistant; and greeny, sensitive, to determine whether or not the gene for whitish colony colour and the gene for polyoxin resistance resided at the same locus. No indication of linkage between the genes was found in the mutant strains. Each of the crosses yielded the four phenotypes approximately in a ratio of 1:1:1:1 of parental (whitish, resistant and greeny, sensitive) and recombinant (whitish, sensitive and greeny, resistant) phenotypes (Table 3), an indication of an independent assortment of the genes.

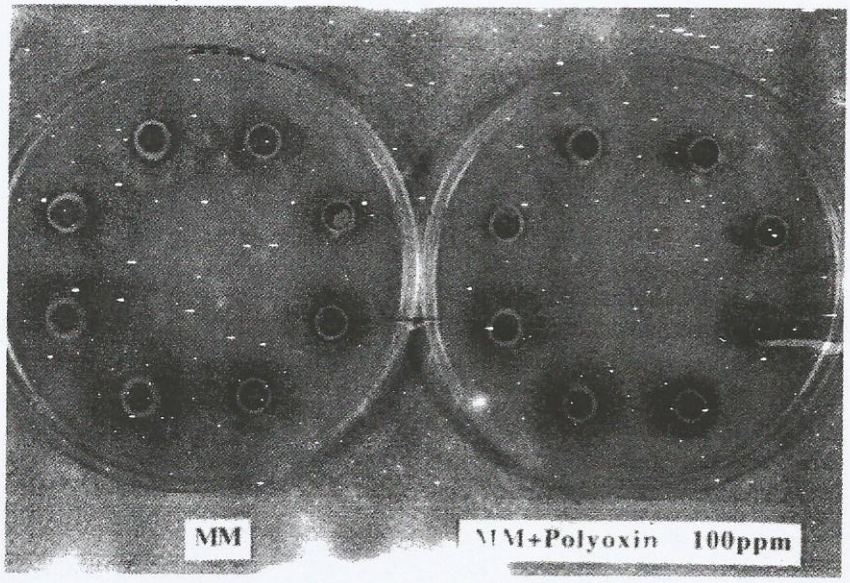

Figure 1. Phenotypes of progenies derived from a single ascus in a cross between two reddish, resistant mutants of *C. heterostrophus* (PRE058-301 X TE2-AS53), each bearing a single polyoxin resistance gene. Photograph was taken after five days of inoculation on minimal medium, MM (left), and MM amended with 100 μg/ml of polyoxin (right).

Table 2. Segregation of polyoxin resistance in allelism tests among reddish, polyoxin-resistant mutants of *C. heterostrophus* each bearing mutation at a single locus

Cross	Number of progeny					$\chi 2^{a)}$
	Total	Resistant	Sensitive	Reddish	Greeny	
PRE058-301 × NA1-AS13	42	42	0	42	0	NS
PRE058-301 × TE2-AS53	74	74	0	74	0	NS
PRE058-302 × TE2-AS57	29	29	0	29	0	NS
Total	145	145	0	145	0	NS

Note: [a)] NS, no segregation.

Table 3. Segregation for whitish colony colour and polyoxin resistance traits in crosses between each of the three reddish, resistant mutant strains bearing mutation at a single locus and the greeny, sensitive wild type strain of *C. heterostrophus*

Cross	Number of progeny			$\chi^{2a)}$
	Total	Parental	Recombinant	
PRE062 × MASHIKI2-2	70	37	33	0.32
PRE070 × MASHIKI2-2	67	32	35	0.17

Note: a) A 1:1 ratio at $P = 0.05$ is 3.84.

The results of the present study, in which independent segregation of whitish colony color and polyoxin resistance traits in the fungus was observed, found supports from similar reports of earlier investigations on benomyl-resistant mutants of *Venturia inaequalis* (Cke.) Wint. Kiebacher & Hoffman (1981) noted that mycelial coloration was not correlated with benzimidazole resistance in this fungus. Genetic analysis of the UV-induced white, benomyl resistant mutant obtained by Martin *et al.* (1981) showed that the traits of white colony and benomyl resistance segregated independently. Shabi *et al.* (1983) also found no evidence of linkage between a gene for green colony color and the gene for benomyl resistance in the same fungus. Similarly, analysis of progenies of crosses between resistant isolates with three colony color mutants by Stanis & Jones (1984) did not reveal linkage between any of the colony color genes with the gene for benomyl resistance.

For years, anamorphic states of *C. heterostrophus* have been considered as principal forms in nature (Tsuda & Ueyama, 1987). However, assuming that sexual reproduction of the fungus also occurs in the field, the absence of linkage between whitish colony colour and iprodione resistance traits discovered in the present study may have contributed to the genetic diversity of the fungus.

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