Mycoscience 39: 155–159, 1998

Genetic analysis of *Cochliobolus heterostrophus* polyoxin-resistant mutants

Abdul Gafur[1], Chihiro Tanaka[2], Kiminori Shimizu[2], Seiji Ouchi[1] and Mitsuya Tsuda[2]

[1] Laboratory of Plant Pathology, Faculty of Agriculture, Kinki University, 3327–204, Nakamachi, Nara 631–8505, Japan
[2] Laboratory of Environmental Mycoscience, Faculty of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606–8502, Japan

Accepted for publication 25 March 1998

Nine polyoxin-resistant mutants of *Cochliobolus heterostrophus* were isolated after ethyl methanesulphonate mutagenesis. All were highly resistant to polyoxin (MIC ≥ 1,600 ppm). Crosses between the mutants and a wild-type strain revealed that the resistance trait was inherited to the offsprings in different fashions. Four of the mutant strains inherited polyoxin resistance in a 1:1 segregation ratio, indicating that the phenotypes in these strains were due to alteration at a single locus. Allelism tests revealed four new loci, *Pol1*, *Pol2*, *Pol3* and *Pol4*, for polyoxin resistance in these mutant strains. The genes responsible for the phenotypes of the other five mutant strains were not determined, because of extremely slow growth of progenies in one cross, sterility in another cross, and inexplicable responses to polyoxin of the progenies in the other crosses. No linkage was detected between the genes for polyoxin resistance and mating type.

Key Words——*Bipolaris maydis*; *Cochliobolus heterostrophus*; drug resistance; mutant; polyoxin.

Polyoxin is an antifungal antibiotic isolated from *Streptomyces cacaoi* var. *asoensis* Isono et al. (Isono, 1969). It inhibits chitin synthetase from fungi and insects but is not toxic to mammalian cells (Becker et al., 1983). Though initially developed for the control of black spot of pears and apples caused by *Alternaria alternata* (Fr.: Fr.) Keissl. and rice leaf spot caused by *Cochliobolus miyabeanus* (Ito et Kurib.) Drechs. ex Dastur in Japan, polyoxin has been widely used as a component of control agents for different pathosystems, such as soil-borne diseases of turf grasses (Tanpo and Tani, 1992), powdery mildew of tobacco (Chen, 1988), *Botrytis cinerea* Pers.: Fr. on rose (Elad et al., 1993), and *A. carthami* Chowdhury on zinnia (Wu and Chou, 1995).

As polyoxin became more extensively used, the development of resistance to the fungicide in some plant pathogens posed a serious practical problem. Polyoxin-resistant strains have been reported in isolates of *A. alternata* in Japan and Korea (Hwang and Yun, 1986; Iida, 1975; Kawasaki et al., 1990; Kohmoto et al., 1974; Sakurai and Shimada, 1974; Tanaka and Takanashi, 1975; Tanaka et al., 1989) and *B. cinerea* in Japan (Iida, 1975). Polyoxin resistance in *A. alternata* apple pathotype (Eguchi et al., 1974), *A. solani* Sorauer, and *Sclerotium rolfsii* Sacc. (Maria and Sullia, 1986) was reported to persist on continued subculturing in fungicide-free media. However, since little work has been done on the genetic mechanisms of polyoxin resistance (Maria and Sullia, 1986), it is worthwhile to explore the genetic basis of polyoxin resistance. The present paper provides basic information on genetic regulation of the polyoxin resistance in *Cochliobolus heterostrophus* (Drechsler) Drechsler (anamorph: *Bipolaris maydis* (Nisikado et Miyake) Shoemaker), the causal agent of southern corn leaf blight and leaf spot diseases of various gramineous plants.

Materials and Methods

Cultures and media All the cultures used in this study are listed in Table 1. HITO7711 (*MAT1-2*) and MASHIKI2-2 (*MAT1-1*) (Tanaka et al., 1991) were used as wild-type strains. The former was used for mutagenesis, and the latter for crossing experiments. Mating types of resistant mutants and progenies were determined by polymerase chain reaction (PCR) amplification as described previously (Gafur et al., 1997). 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 cooled to 50°C before polyoxin AL WP (10% active ingredient, Nippon Noyaku, Tokyo) suspended in 70% (v/v) ethanol was added. Complete medium (CM), containing the components of MM with the addition of tryptone (1.0 g/L) and yeast extract (1.0 g/L), was used to maintain the cultures.

Mutagenesis and isolation of polyoxin resistant mutants Ethyl methanesulphonate (EMS) was used as mutagen. Mutagenesis was carried out as described previously (Tanaka et al., 1988). Following mutagenesis, a single

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

Strain	Phenotype[a]	Mating type	MIC (ppm)[b, c]	Origin	Assigned genotype[c]
HITO7711	S	*MAT1-2*	25	Wild type stock culture	Wild type
MASHIKI2-2	S	*MAT1-1*	25	Wild type stock culture	Wild type
PRE031	R	*MAT1-2*	>1,600	Mutant of HITO7711	ND
PRE051	R	*MAT1-2*	1,600	Mutant of HITO7711	*pol1*
PRE058	R	*MAT1-2*	1,600	Mutant of HITO7711	*pol2*
PRE059	R	*MAT1-2*	>1,600	Mutant of HITO7711	*pol3*
PRE062	R	*MAT1-2*	>1,600	Mutant of HITO7711	ND
PRE063	R	*MAT1-2*	>1,600	Mutant of HITO7711	ND
PRE068	R	*MAT1-2*	>1,600	Mutant of HITO7711	*pol4*
PRE069	R	*MAT1-2*	>1,600	Mutant of HITO7711	ND
PRE070	R	*MAT1-2*	>1,600	Mutant of HITO7711	ND
PRE051-003	R	*MAT1-2*	ND	Progeny of PRE051 × Mashiki2-2	*Pol1*
PRE051-008	R	*MAT1-1*	ND	Progeny of PRE051 × Mashiki2-2	*Pol1*
PRE051-205	R	*MAT1-1*	ND	Progeny of PRE051 × Mashiki2-2	*Pol1*
PRE058-301	R	*MAT1-2*	ND	Progeny of PRE058 × Mashiki2-2	*Pol2*
PRE059-009	R	*MAT1-2*	ND	Progeny of PRE059 × Mashiki2-2	*Pol3*
PRE059-010	R	*MAT1-1*	ND	Progeny of PRE059 × Mashiki2-2	*Pol3*
PRE068-007	R	*MAT1-1*	ND	Progeny of PRE068 × Mashiki2-2	*Pol4*
PRE068-105	R	*MAT1-2*	ND	Progeny of PRE068 × Mashiki2-2	*Pol4*

a) R, polyoxin resistant; S, polyxin sensitive.
b) MIC, minimum inhibitory concentration of polyxin.
c) ND, not determined.

conidium borne on the growing colony on MM amended with 100 ppm (w/v) of polyoxin was isolated, transferred to CM, and incubated at 27°C. Sensitivity of the mutants to polyoxin was evaluated mainly by measuring the minimum inhibitory concentration (MIC) using the plate dilution method. MIC was determined as the lowest concentration of the fungicide that produced no visible growth of the fungus. Mycelial disks, 6 mm in diam, were cut with a sterilized cork borer from the margin of 1-wk-old colonies, and each disk was placed upside down at the center of a 9-cm-diam Petri dish containing MM amended with 12.5, 25.0, 50.0, 100, 200, 400, 800 and 1,600 ppm (w/v) of polyoxin. The MIC was estimated after incubation for 3 d at 27°C by observing mycelial growth on the medium. Each experiment was carried out in triplicate.

Crossing and ascospore analysis Crossing was performed according to Ueyama and Tsuda (1975). Small pieces of mycelia of compatible strains to be crossed were inoculated on the opposite sides of a piece of sterilized rice straw placed on Sachs' agar medium, which 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; $FeCl_3$, trace, and agar, 12.0. The inoculated dishes were incubated at 24°C under intermittent fluorescent light. Mature pseudothecia were harvested after 3 wk of incubation. Ascospores were analyzed according to Taga et al. (1978) and isolated aseptically with a micromanipulator from several pseudothecia which had been crushed in water. Each ascospore was allowed to germinate on a small agar block on sorbose-amended CM or MM and incubated at 27°C. After mycelial growth was confirmed, ascospore isolates were cultured on CM slants.

Polyoxin-resistance test of ascospore-isolates Freshly growing mycelia were transferred from slant tubes to Petri dishes containing CM. Polyoxin resistance or sensitivity of ascopore-isolates was tested by cutting mycelial disks 6 mm in diam from the leading edge of an actively growing colony and placing them upside down on MM containing 100 ppm (w/v) of polyoxin. Resistance or sensitivity of isolates to the fungicide was determined on the basis of mycelial growth after incubation for 5 d at 27°C.

Results

Characterization of polyoxin resistant mutants After EMS mutagenesis, 11 putative polyoxin-resistant mutant strains were isolated. The mutants grew as fast as or faster than the wild-type strain on CM and MM. Nine of them were employed in genetic study of polyoxin resistance. The MIC of polyoxin for the mutants exceeded 1,600 ppm, while the wild-type strains were unable to grow at concentrations higher than 25 ppm (Table 1).

Identification of polyoxin-resistance genes To identify the genes for polyoxin resistance, mutants were crossed with the wild-type strain. The polyoxin resistance was genetically transmitted in all crosses, but the number of genes responsible for the resistance trait was apparently

different, as demonstrated by the differences in segregation ratio. Four mutant isolates, PRE051, PRE058, PRE059 and PRE068, inherited polyoxin resistance in a 1:1 segregation ratio in random ascospore analysis (Table 2) and in tetrad analysis (Fig. 1), suggesting that the polyoxin resistance was due to alteration at a single locus. To investigate the allelic nature of the mutation, resistant progenies of four mutant isolates presented in Table 2 were crossed to each other. When wild-type ascospores are produced in any cross, it should be concluded that resistance genes of the two parental strains are not allelic. The result of ascospore analysis in Table 3 shows that the genes controlling polyoxin resistance in these four mutant strains are not allelic. Segregation of

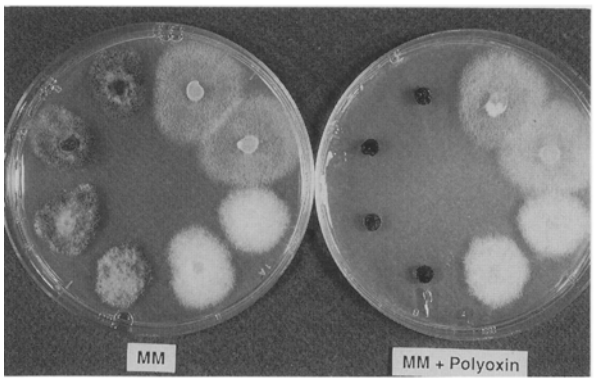

Fig. 1. The 1:1 segregation of ascospore isolates derived from a single ascus from the cross between a wild type (MASHIKI2-2) of *Cochliobolus heterostrophus* and a resistant mutant strain PRE068.
Photograph was taken 5 d after inoculation on minimal medium (MM, left) and MM amended with 100 ppm (w/v) of polyoxin (right).

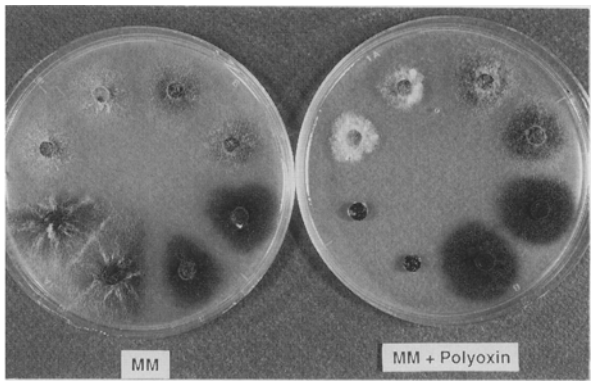

Fig. 2. Tetratype segregation of eight ascospores isolated from a single ascus in the cross between two mutants (PRE051 and PRE068) of *Cochliobolus heterostrophus* carrying a single polyoxin resistance gene.
Photograph was taken 5 d after inoculation on minimal medium (MM, left) and MM amended with 100 ppm (w/v) of polyoxin (right).

Table 2. Segregation of polyoxin resistance in random ascospore progenies from crosses between a wild-type strain and four polyoxin-resistant mutants of *Cochliobolus heterostrophus*.

Cross (Pol^R × Wild type)[a]	Number of progeny			$\chi^{2\,b)}$
	Total	Resistant	Sensitive	
PRE051 × MASHIKI2-2	59	28	31	0.07
PRE058 × MASHIKI2-2	71	33	38	0.23
PRE059 × MASHIKI2-2	72	32	40	0.68
PRE068 × MASHIKI2-2	79	38	41	0.05

a) Pol^R, polyoxin resistance.
b) A 1:1 ratio at $P=0.05$ is 3.84.

Table 3. Segregation of polyoxin resistance in allelism tests among four polyoxin-resistant mutants of *Cochliobolus heterostrophus* bearing mutation at a single locus.

Cross[a]	Genotype	Number of progeny			$\chi^{2\,b)}$
		Total	Resistant	Sensitive	
PRE051-003 × PRE059-009	*Pol1* × *Pol3*	57	39	18	0.99
PRE051-205 × PRE068-007	*Pol1* × *Pol4*	85	62	23	0.10
PRE059-010 × PRE068-105	*Pol3* × *Pol4*	56	32	24	1.93
PRE051-008 × PRE058-301	*Pol1* × *Pol2*	61	40	21	2.41
PRE051-010 × PRE058-301	*Pol3* × *Pol2*	54	37	17	0.89
PRE068-007 × PRE058-301	*Pol4* × *Pol2*	70	53	17	0.00

a) See Table 1 for polyoxin-resistant strains crossed.
b) A 3:1 ratio at $P=0.05$ is 3.84.

resistant and sensitive progenies fits the 3:1 ratio (Table 3, Fig. 2), indicating that the resistance loci of these four strains segregate independently. We tentatively assigned the resistance loci of PRE051, PRE058, PRE059 and PRE068 as *Pol1*, *Pol2*, *Pol3* and *Pol4*, respectively.

In the cross between PRE031 and the wild-type strain, 11 of the 58 ascospore offsprings obtained were resistant. The other 47 progenies seemed to be sensitive, 20 of which exhibited extremely slow growth even on MM without polyoxin. This characteristic complicated the classification of resistance or susceptibility of the progenies. Similarly, in the cross between PRE063 and the wild-type strain, 13 progenies showed resistance to polyoxin, whereas the other 51 were sensitive. Unfortunately, crosses involving the progenies of the strain including a backcross to the wild-type parent failed to produce ascospores. Thus, the genes responsible for polyoxin resistance in these two mutants were not fully characterized.

In other crosses between the wild-type strain and PRE062, PRE069 and PRE070, an odd manner of segregation of resistant and sensitive progenies was observed (data not shown). The segregation ratios seemed to be unstable and to change depending on the age of culture. A polygenic system was supposed to be involved in this phenomenon.

Linkage between loci for polyoxin resistance and mating type To investigate possible linkage between the loci for polyoxin resistance and the mating type, each of the polyoxin-resistant mutants carrying a mutation at a single locus were crossed with a wild-type strain of opposite mating type. The progenies in each cross segregated in a 1:1 ratio of parental type to recombinant type (Table 4), indicating that there was no linkage between mating-type loci and any of the locus for polyoxin resistance.

Discussion

The 1:1 segregation ratio of resistant and sensitive progenies in crosses of four resistant mutant strains, PRE051, PRE058, PRE059 and PRE068, with the wild-type strain indicated that polyoxin resistance in these four mutant strains was controlled by a single Mendelian gene. Progenies of allelism tests among resistant mutants segregated in a 3:1 ratio of resistant to sensitive phenotypes, implying that polyoxin resistance in these four strains was controlled by a single gene at different, unlinked loci, designated tentatively as *Pol1*, *Pol2*, *Pol3* and *Pol4*, respectively. The identification of four loci in four of the resistant mutant strains isolated in the present study is not surprising, because the control of resistance to certain fungicides by more than one gene is common in most plant pathogenic fungi (Georgopoulos, 1977).

Analysis of progenies of initial crosses between the wild type and two mutant strains, PRE031 and PRE063, led to the speculation that polyoxin resistance in each of these mutants might be governed by two genes because a 1:3 ratio of resistant and sensistive was observed. However, due to the extremely slow growth of some of sensitive progenies of the PRE031 strain and the failure of the cross between PRE063 and the wild type to yield progenies, the nature of polyoxin resistance in these two mutants remains unclear. Attempts to prove the two-gene hypothesis are now in progress. The slow-growing characteristic in some sensitive offsprings from the cross between PRE031 and the wild type may be attributed to mutation at gene(s) involved in mycelial growth. This could have confused the determination of the resistant and sensitive progenies and therefore complicated the genetic analysis of polyoxin resistance. The failure to obtain fertile pairings in the backcrosses of resistant progenies of PRE063 to the wild-type parent appears to be due to physiological disorders in these progenies because the wild-type strains crossed with them were suitable partners in many other matings.

Results of crosses involving PRE062, PRE069 and PRE070 indicated that their polyoxin resistance is also genetically controlled, but the mode of inheritance could not be clarified in this study. Provided that polyoxin resistance in these strains is under polygenic control, quantitative analyses on the sensitivity to polyoxin should provide more comprehensive results.

In the present study, the polyoxin resistance and mating type were independently segregated. There has been no report indicating linkage between mating-type gene and other genetic markers in the genus of *Cochliobolus*. The absence of linkage between the mating-type gene and colony or conidial color genes has been noted in *C. miyabeanus* (C. Tanaka et al., unpubl.), *C. sativus* (Ito

Table 4. Segregation of polyoxin resistance and mating type in crosses between four polyoxin-resistant mutants bearing mutation at a single locus and the wild-type strain of *Cochliobolus heterostrophus*.

Cross	Loci analyzed	Number of progeny[a]			χ^{2} [b]
		Total	Parental	Recombinant	
PRE051 × MASHIKI2-2	*Pol1-MAT1*	57	26	31	0.28
PRE058 × MASHIKI2-2	*Pol2-MAT1*	58	27	31	0.16
PRE059 × MASHIKI2-2	*Pol3-MAT1*	49	21	28	0.73
PRE068 × MASHIKI2-2	*Pol4-MAT1*	74	34	40	0.34

a) Parental: polyoxin sensitive-*MAT1-1* and polyoxin resistant-*MAT1-2*; Recombinant: polyoxin sensitive-*MAT-2* and polyoxin resistant-*MAT1-1*.
b) A 1:1 ratio at $P=0.05$ is 3.84.

et Kurib.) Drechs. ex Dastur (Tinline, 1988; Tinline and Harding, 1988), *C. carbonum* R. R. Nelson (Leonard, 1972), and *C. heterostrophus* (Tanaka et al., 1991, 1994). At present, no linkage between mating-type gene and genes for fungicide resistance is known. Taga et al. (1978, 1979) indicated that none of the three loci for kasugamycin resistance in *P. oryzae* Cavara, *kas-1*, *kas-2*, and *kas-3*, appeared to be linked to the mating type gene. Kiebacher and Hoffman (1981), in an attempt to correlate mating type with benzimidazole resistance in *Venturia inaequalis* (Cooke) G. Wint., also found no evidence of linkage between the two genetic markers. These phenomena are quite puzzling, and the exploration of mating type-linked genetic markers will be an interesting topic.

Literature cited

Becker, J. M., Covert, N. L., Shenbagamurthi, P., Steinfeld, A. S. and Naider, F. 1983. Polyoxin D inhibits growth of zoopathogenic fungi. Antimicrob. Agents Chemoter. **23**: 926–929.

Chen, H. L. 1988. Fungicidal control of tobacco powdery mildew in Taiwan. II. Bull. Tob. Res. Inst. Taiwan Tob. Wine Monop. Bur. **28**: 89–96.

Eguchi, J., Mogami, M. and Ota, S. 1974. Studies on characters of polyoxin-insensitive isolates of apple leaf spot fungi, *Alternaria mali*. Ann. Phytopathol. Soc. Japan **40**: 220.

Elad, Y., Kirshner, B. and Gotlib, Y. 1993. Attempts to control *Botrytis cinerea* on roses by pre- and postharvest treatments with biological and chemical agents. Crop Protection **12**: 69–73.

Gafur, A., Tanaka, C., Ouchi, S. and Tsuda, M. 1997. A PCR-based method for mating type determination in *Cochliobolus heterostrophus*. Mycoscience **38**: 455–458.

Georgopoulos, S. G. 1977. Development of fungal resistance to fungicides. In: Antifungal compounds, vol. 2, (ed. by Sisler, H. D. and Siegel, M. R.), pp. 439–495. Marcel Dekker, New York.

Hwang, B. K. and Yun, J. H. 1986. Variability in sensitivity to polyoxin B of isolates of *Alternaria mali* and decreased fitness of polyoxin-resistant isolates. J. Phytopathol. **115**: 305–312.

Iida, W. 1975. On the tolerance of plant pathogenic fungi and bacteria to fungicides in Japan. Japan Pesticide Information **23**: 13–16.

Isono, K., Asahi, K. and Suzuki, S. 1969. Studies on polyoxins, antifungal antibiotics. XXIII. The structure of polyoxins. J. Am. Chem. Soc. **91**: 7490–7505.

Kawasaki, N., Mametsuka, H. and Tashiro, N. 1990. Major diseases and insect pests of apple in the northern highlands of Saga Prefecture and occurrence of polyoxin-tolerance isolates of *Alternaria alternata* apple pathotype. Proc. Assoc. Plant Prot. Kyushu **36**: 72–75. (In Japanese.)

Kiebacher, J. and Hoffman, G. M. 1981. Zur Genetik der Benzimidazol-Resistenz bei *Venturia inaequalis*. Z. Pflanzenkrankh. Pflanzenschutz **88**: 189–205.

Kohmoto, K., Miyake, H., Nishimura, S. and Udagawa, H. 1974. Distribution and chronological population shift of polyoxin resistant strains of black spot fungi of Japanese pear, *Alternaria kikuchiana*, in field. Ann. Phytopathol. Soc. Japan **40**: 220.

Leonard, K. J. 1972. Color mutants in *Cochliobolus carbonum*. Can. J. Bot. **50**: 1283–1285.

Maria, R. and Sullia, S. B. 1986. Acquired resistance of *Alternaria solani* and *Sclerotium rolfsii* to polyoxin-D. J. Phytopathol. **116**: 60–66.

Sakurai, H. and Shimada, T. 1974. Studies on polyoxin-resistant strains of *Alternaria kikuchiana* Tanaka. III. Characters on media and pathogenicity. Bull. Agric. Chem. Inspect. St. **14**: 54–65.

Taga, M., Nagakubo, H., Tsuda, M. and Ueyama, A. 1978. Ascospore analysis of kasugamycin resistance in the perfect stage of *Pyricularia oryzae*. Phytopathology **68**: 815–817.

Taga, M., Nakagawa, H., Tsuda, M. and Ueyama, A. 1979. Identification of three different loci controlling kasugamycin resistance in *Pyricularia oryzae*. Phytopathology **69**: 463–466.

Tanaka, C., Kubo, Y. and Tsuda, M. 1988. Comparison of mutagens in *Cochliobolus heterostrophus* mutagenesis. Ann. Phytopathol. Soc. Japan **54**: 503–509.

Tanaka, C., Kubo, Y. and Tsuda, M. 1991. Genetic analysis and characterization of *Cochliobolus heterostrophus* color mutants. Mycol. Res. **92**: 49–56.

Tanaka, C., Taniguchi, T. and Tsuda, M. 1994. Genetic analysis of *Cochliobolus heterostrophus* beige mutants. Mycol. Res. **99**: 621–622.

Tanaka, S. and Takanashi, S. 1975. Studies on the tolerance of pear black spot fungus (*Alternaria kikuchiana* Tanaka) against an antibiotic, polyoxin. Bull. Fac. Agr. Tamagawa Univ. **15**: 101–107.

Tanaka, S., Shimomura, S., Takashima, E., Katsumoto, K. and Nishi, Y. 1989. Occurrence of the fungicide-resistance strains of *Alternaria mali* in Tokusa, Ato-cho, Yamaguchi Prefecture, Japan. Bull. Fac. Agric. Yamaguchi Univ. **37**: 49–60. (In Japanese.)

Tanpo, H. and Tani, T. 1992. Efficient chemical control of soil-borne diseases of turf grasses. I. *Rhizoctonia* spring dead spot and fairy ring on zoysia turf. Kagawa Daigaku Nogakubu Gakujutsu Hokoku **44**: 89–95. (In Japanese.)

Tinline, R. D. 1988. *Cochliobolus sativus*, a pathogen of wide host range. In: Genetics of plant pathogenic fungi (Advances in plant pathology, vol. 6), (ed. by Ingram, D. D. and Williams, P. H.), pp. 113–122. Academic Press, London.

Tinline, R. D. and Harding, H. 1988. Inheritance of some genetic markers in *Cochliobolus sativus*. Mycologia **80**: 863–865.

Ueyama, A. and Tsuda, M. 1975. Formation in culture of *Cochliobolus miyabeanus*, the perfect state of *Helminthosporium oryzae*. Ann. Phytopathol. Soc. Japan **41**: 434–440.

Wu, W. S. and Chou, J. K. 1995. Chemical and biological control of *Alternaria carthami* on zinnia. Seed Sci. Technol. **23**: 193–200.