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PHENOTYPIC CHARACTERISATION AND GENETIC ANALYSIS OF MUTANTS OF *ASPERGILLUS NIDULANS* RESISTANT TO THE FUNGICIDE TOLCLOFOS-METHYL

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Abstract

Spontaneous mutants of *Aspergillus nidulans* were recovered from $0{,}55.10^{+7}$ conidia incubated on synthetic medium supplemented with 100 µg tolclofos-methyl/ml. They differed considerably in morphology, growth rate, and level of resistance to two other fungicides. All mutants tested were cross-resistant to quintozene and vinclozolin; they produced fewer conidia than their wild-type parent. Some mutants required fungicides for maximum growth. Genetic analysis revealed that the mutants carried mutations in one gene located on linkage group III.

Keys words: *Aspergillus nidulans, tolclofos methyl, fungicide resistance, dicarboximide.*

Résumé

Des souches résistantes au fongicide tolclofos-methyl ont été sélectionnées d'une manière spontanée à partir de $0{,}55.10^{+7}$ spores d'*Aspergillus nidulans* incubés sur un milieu synthétique contenant 100 µg/ml tolclofos-methyl. Les colonies résistantes diffèrent considérablement dans leur morphologie, taux de croissance et niveau de résistance. Toutes les souches mutantes testées sont en outre tolérantes aux deux autres fongicides, quintozene et vinclozolin. Ils produisent moins de spores que leur parent sensible. Quelques mutants exigent la présence des fongicides dans le milieu pour une croissance maximum. L'analyse génétique a révélé que les souches résistantes portent des mutations dans un gène localisé sur le chromosome III.

Mots clés: *Aspergillus nidulans, tolclofos-methyl, resistance fongique, dicarboximide.*

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ملخص

تم اصطفاء عدد من الطفرات التلقائية لفطر *Aspergillus nidulans* من بين $0{,}55.10^{+7}$ بوغة خضرية حُضِنت على وسط مغذي يحتوي على تركيز 100 ميكروغرام tolclofos-methyl لكل ملل. وجدنا أن الطفرات المحصل عليها تختلف في شكل المستعمرة ونسبة النمو ودرجة المقاومة مقارنة بالسلالة الأم. كذلك كانت السلالات الطافرة المدروسة مقاومة للمبيدين vinclozolin وquintozene. كانت بعض الطفرات تعتمد على وجود المبيد في الوسط لتصل إلى أعلى درجات النمو. أظهر التحليل الوراثي أن الطفرات تحمل مورثة واحدة طافرة تقع على المجموعة الإرتباطية الثالثة.

الكلمات المفتاحية: *الفتر Aspergillus nidulans ، المبيد الفتري tolclofos-methyl ، مقاومة المبيدات.*

Tolclofos-methyl is a fungicide used to control various soil-borne fungal diseases of vegetables and ornamentals, especially those caused by *Rhisoctonia solani*. Its mode of action is unknown, but is probably similar to that of the aromatic hydrocarbon and dicarboximide fungicides [1]. Fungi can develop resistance to these fungicides, and few of these resistant mutants have been studied genetically [2]. Mutations in the same gene can give rise to different alleles of that gene, and allelic mutants can have various level of resistance to the same fungicide [3, 4]. However there are examples that resistance to fungicides could be controlled by a single major gene [5]. The mechanism of resistance to fungicides include a number of biochemical changes inside the fungal cell [6-10].

When resistance to a certain fungicide is detected, it is very important to know whether the effectiveness of other fungicides has been affected. Cross-resistance tests with inhibitors of known mechanism of action may provide an indication of the cellular processes affected by the fungicide [11]. Most pathogenic fungi such as *Botrytis cinerea* are not amenable to genetic analysis because there are no reliable methods for manipulating the genome to obtain recombinant progeny. The saprophytic fungus *Aspergillus nidulans* is of no importance in plant pathology, but nevertheless is an excellent model fungus for detailed genetic studies of fungicide resistance under laboratory conditions [12]. The aim of this work was to select mutants resistant to the fungicide tolclofos-methyl and characterise them phenotypically and genetically.

MATERIALS AND METHODS

Isolates

The stains used for mutants isolation, assigning genes to linkage groups and mapping the resistance genes were from the Glasgow collection [13].

Media and chemicals

Minimal medium (MM) comprised Czapek-Dox salts with 10 g glucose per litre as carbon source. Complete medium (CM) comprised MM supplemented with yeast extract, hydrolysed casein, hydrolysed nucleic acid and vitamins [14]. All media were solidified with agar (20 g/l) and sterilised for 15 min at 120° C. The sterilised media were amended with the three fungicides (added as suspensions in distilled water) after it had been cooled to 55° C. The formulations used were:

- Tolclofos - methyl (O,O - dimethyl- (2,6 - dichloro – 4 -methylphenyl) phosphorothioate (Risolex 50%),
- Quintozene (pentachloronitrobenzen, PCNB) (Brassicol 20 %),
- Vinclozolin (3 - (3,5 - dichlorophenyl) - 5- ethenyl – 5 – methyl - 2,4 - exazolinedione) (Ronilan 50 %).

Isolation of mutants

All mutants were obtained without mutagenic treatment. Conidia from 7-day-old colonies of the wild-type strain were suspended in saline (10 g NaCl/l distilled water), aliquots ($0{,}55.10^{+7}$ conidia) were added to 200 ml cool (55°C) CM supplemented with 20 mg tolclofos-methyl to give a final concentration of 100 µg/ml, then the medium was poured into Petri dishes. Colonies that developed after 7 days at 37°C were subcultured to tubes of CM for further analysis.

Phenotypic characterisation of mutants

Monoconidial isolates obtained by incubating conidia on CM for 16 hours were transferred to the centres of dishes of CM and CM amended with 5, 10, 20, 50, 100 µg fungicide. There were three replicates of each treatment. In the same manner the mutants and the wild type parent were tested on CM supplemented with 0.4 or 0.8 M of NaCl.

Growth rate was determined from colony diameters after 48 hours and 72 hours incubation. These were used to calculate the ED_{50} values (amount of fungicide that reduced the growth by 50%) from the regression of growth rate on log fungicide concentration.

Genetic analysis

Tolclofos-methyl-resistance genes were assigned to one of the eight linkage groups by methods of McCully and Forbes [15]. The resistance genes were mapped as described by Cluterbuck [12]. Also, pairs of mutants with complementary nutritional requirements were intercrossed, and more than 3500 ascospores from hybrid cleistothecia were grown on CM with 100 µg /ml tolclofos-methyl to distinguish fungicide-resistant and sensitive progeny. The mutants involved in the cross were considered to be allelic if the proportion of fungicide sensitive recombinants was less than 1%.

RESULTS

28 mutants were obtained from $0{,}55.10^{+7}$ conidia embedded in medium containing 100 µg tolclofos-methyl/ ml. This gives a spontaneous mutation frequency of $5{,}1.10^{-6}$.

Phenotype of mutants

The resistant mutants varied considerably in their morphology, growth rate and level of resistance to fungicides. The mutants obtained were classified into three main groups on the basis of differences in growth in the presence or absence of fungicides. Group 1 (19 mutants)

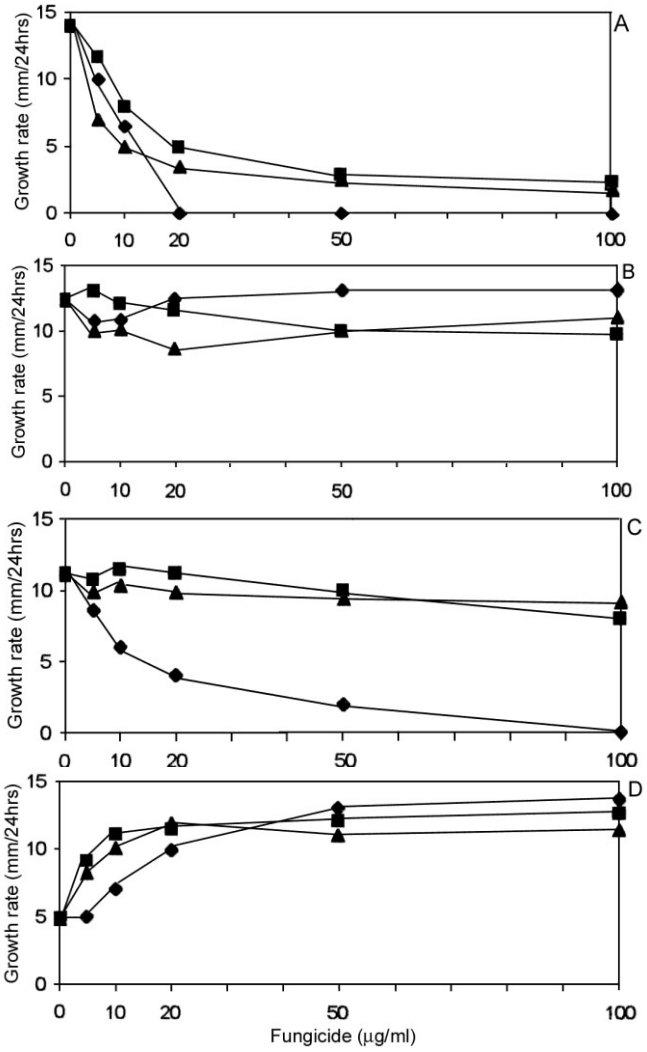

Figure 1: Effect of fungicides on growth rate of representative tolclofos-methyl-resistant mutants of *Aspergillus nidulans*. Group 1 (TMR2, B), group 2 (TMR 24, C) and group 3 (TMR 28, D) mutants were compared with the wild-type strain (A) on fungicide-free medium and medium supplemented with tolclofos-methyl (■), vinclozolin (♦) and quintozene (▲).

showed high level of resistance to tolclofos-methyl, quintozene and vinclozolin (ED_{50} > 100 µg/ml (for the wild-type strain the ED_{50} values were: 17,2, 11,3 and 7,3 µg/ml for tolclofos-methyl, quintozene and vinclozolin respectively) . Group 2 (7 mutants) were high resistant to tolclofos-methyl and quintozene but low resistant to vinclozolin (ED_{50} < 25 µg / ml). The last group was composed of 2 mutants that grew very poorly on CM (4,5 mm/24 hours), but when transferred on CM with fungicide they attain normal rate of growth (14 mm / 24 hours). Compared with the wild type the resistant mutants grew worse on media containing NaCl as osmoticum.

Representative mutant from group 1 (TMR 2), group 2 (TMR 24) and group 3 (TMR 28) were analysed in more details for their differences in growth rate and fungicide resistance (Fig. 1).

Linkage group	Genetic markers	TMR 2// MSE		TMR 24// MSE		TMR 28// MSE	
		Paren	Rec	Paren	Rec	Paren	Rec
I	*pro* A1 *bi* A1 + +	65	50	15	25	17	58
II	*w* A3 +	68	47	14	26	40	35
III	*gal* A1 +	115	00	39	01**	74	01**
IV	*Pyro* A4 +	52	63	14	26	30	45
V	*Fac* A303 +	59	56	13	27	32	43
VI	*s* B3 +	53	62	21	19	36	39
VII	*nic* B8 +	44	71	17	23	36	39
VIII	*Ribo* B2 +	47	68	15	25	41	34

* MSE genotype: **su A1 ad E20** (suppressor of ad E20) **y A2** (yellow conidia) **ad E20** (require adenine); **w A3** (white conidia); **gal A1** (galactose non-utilising); **pyro A4** (require pyridoxine); **fac A303** (acetate non-utilising); **s B3** (require thiosulphate); **nic B8** (require nocotinic acid); **ribo B2** (require riboflavin).

** Presumably due to mitotic crossing-over before haploidization.

Table 1: Number of parental and recombinant haploid mitotic segregants isolated from diploids: TMR 2// MSE*, TMR 24// MSE and TMR 28// MSE grown on CM and CM with 0.25 µg/ml benomyl as haploidizing agent.

Genotype of mutants

Each of the mutant isolates carried a single mutant gene causing resistance to the fungicides. Analysis of haploid mitotic progeny (sectors) formed on heterozygous (r/s) diploid colonies by the process of mitotic haploidization showed that the resistance gene segregated freely with all MES markers except with *gal* on linkage group III (Table 1). This means that the resistance gene is located on linkage group III. In order to map the resistance gene the three representative mutants were crossed with strain 34, which carry two markers on the left arm of linkage group III. Analysis of haploid meiotic progeny (ascospores) from the crosses revealed that the resistance gene is situated about 3.3 map units from the *meth* gene (Table 2).

Cross	Genotype of ascospore progeny				Distance between genes**		
	TMR + + + meth arg	+ + + TMR meth arg	TMR + arg + meth +	TMR meth + + + arg	TMR meth	meth arg	TMR arg
TMR2 X 34	105	03	04	0	2.7	3.6	6.25
TMR24X 34	321	11	20	0	3.1	5.7	8.8
TMR28X 34	150	6	11	01	3.6	6.6	10.2
Total	576	20	35	01	3.3	5.6	8.8

* Both genes are on the left arm of linkage group III - ** 1 Map unit = 1% recombination frequency.

Table 2: Analysis of meiotic progeny from crosses of strain 34 (methH arg*) with three resistant mutants (TMR2, TMR24 and TMR28).

The three mutants were intercrossed (TMR2 X TMR24; TMR2 X TMR28; TMR24 X TMR28) and ascospores from hybrid cleistothecia were analysed to detect recombinant progeny (tolclofos-methyl-sensitive). 3512 progeny were analysed and no recombinant was recovered confirming that the three resistant mutants were allelic.

DISCUSSION

This study has shown that spontaneous mutations of *A. nidulans* conferring resistance to tolclofos-methyl occur readily under laboratory conditions. The mutants were cross-resistant to quintozene and vinclozolin and abnormally sensitive to media of high osmolarity containing sodium chloride. Most mutants grew slower than the parent stain on CM, and some of them showed a marked improvement of growth on media containing fungicides.

Mutants of *A. nidulans* have been selected for resistance to tolclofos-methyl [16], quintozene [17], chloroneb [4], iprodione [18], and dichlobenil [19]. Many mutants were phenotypically similar to the mutants described here; for example, they were cross-resistant to other fungicides, grew better on media supplemented with fungicides.

Cross-resistance of mutants to dicarboximides and aromatic hydrocarbons has been reported in many species of saprophytic and pathogenic fungi [1, 20]. There is usually a good correlation between fungicide resistance and osmotic sensitivity [1, 21]. The biochemical basis of osmotic sensitivity has not been established. However, Ellis *et al*. [22] indicated that dicarboximide resistant/ osmotically sensitive mutant of *Neurospora crassa* was

abnormal in its retention of solute such as glycerol, may be due to a defective cell membrane.

The genetic basis of resistance to dicarboximides and aromatic hydrocarbons had been studied in various fungi [2]. Only one gene for resistance has been identified in most fungi, but at least six genes are distributed over the genome of *Neurospora crassa* [2]. Mutations giving resistance to dicarboximides and aromatic hydrocarbons in *A. nidulans* are probably at the same locus. Genetic studies of Caddick and Arst [23] have shown that the *daf* mutants of Beever [18] are allelic with the *pcnb* A mutants of Threlfell [17] and the *chl* mutants of Martinez and Azevedo [24]. The *daf* A locus is 2.6 map units distal to *meth* D on linkage group III [23], and *dic* locus of Chabani and Grindle [19] is 3.5 map units distal to *meth* H, whereas the gene responsible for resistance to tolclofos-methyl (*tol*) is 3.3 map units distal to *meth* H (table 2). However, *meth* H and *meth* D are probably identical loci [25], in which case *daf* A, *dic* and *tol* have been mapped to the same site on the *A. nidulans* genome. Chabani and Grindle [19] suggested that the same gene symbol *daf* (dicarboximides / aromatic hydrocarbons resistance) should be used to designate gene giving resistance to these fungicides.

Resistance to antifungal chemicals with applications in agriculture can cause problems with disease control. Phenotypic and genetic characterization of fungicide-resistant mutants can be crucial in providing data on their biological fitness. The most comprehensive information on the genetics of fungicide resistance comes from studies of non-pathogenic fungi such as *A. nidulans* that are easy to manipulate in the laboratory but have no direct relevance to pathology. However, results of these studies are applicable to pathogens with similar biological properties.

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