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Studies of the Inheritance of Virulence in the Entomopathogenic Fungus *Metarhizium anisopliae*

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A forced heterocaryon was established between two auxotrophic conidial color mutants of *Metarhizium anisopliae*. From the heterocaryon, a prototrophic somatic diploid was selected which, in turn, yielded somatic segregants. The virulence of the original mutants, the somatic diploid, and the somatic segregants was evaluated on three species of mosquitoes as well as on *Ostrinia nubilalis* larvae. The virulence of the somatic diploid was comparable to that of the wild-type parental strain while the auxotrophic somatic segregants exhibited virulence approximately equal to that of the auxotrophic components of the heterocaryon. Putative somatic diploids were obtained between morphological mutants of the two species varieties (*M. anisopliae* var. *minor* and var. *major*). The presumptive diploids were avirulent for the insect species to which the parental strains exhibited virulence.

KEY WORDS: *Metarhizium anisopliae; Anopheles stephensi; Aedes aegypti; Culex pipiens;* auxotrophic mutants; diploid hybrids; segregants; mortality by mycosis.

INTRODUCTION

Metarhizium anisopliae is an entomopathogenic hyphomycete with potential practical value for the control of agriculturally important insect pests (Guagliumi et al., 1974; Ferron, 1981) as well as for mosquito control (Roberts, 1970).

Heterocaryosis resulting from interhyphal and interconidial anastomoses has been described for *M. anisopliae* var. *minor* (see Tinline and Noviello, 1971). Somatic diploids and somatic segregants have been isolated in this species by Al-Aidroos (1980) and Messias and Azevedo (1980), thus completing the demonstration of the parasexual cycle. We have shown that putative somatic hybrids can be isolated between varieties of this species (*M. anisopliae* var. *minor* and *M. anisopliae* var. *major*), opening the way for the investigation of the genetic control of virulence within and between varieties of this insect pathogen (Riba et al., 1980). In this paper evidence is presented concerning the virulence of mutants and somatic segregants within a variety of this fungal species. In addition, experiments designed to test the virulence of putative somatic diploids between varieties of this fungus are described.

MATERIAL AND METHODS

Strains. The source and properties of the *M. anisopliae* strains used in these investigations are presented in Table 1.

Media. The complex medium (CM) consisted of 0.5% yeast extract (Difco) and 1.0% glucose solidified with 1.5% agar (Difco). The minimal medium (MM) contained 0.6% $NaNO_3$ as the nitrogen source and 1.0% glucose solidified with 1.5% agar (Difco). Cultures growing on agar were incubated at 28°C.

For cultures grown in liquid medium, Adamek's (1965) medium was used. The medium consists of 4% yeast extract, 3% corn steep liquor (Soc. Française des pro-

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TABLE 1
STRAINS OF *Metarhizium anisopliae* WITH THE ORIGINAL HOST AND GEOGRAPHIC ISOLATION

Strain	Species Variety	Insect host	Location	Mutations
Ma 140	Minor	*Ostrinia nubilalis*	Ablis (France)	Wild type
Ylo arg.		(UV-induced mutant of strain Ma 140)		Yellow conidia (ylo). arginine requirement (arg)
whi thi		(UV-induced mutant of strain Ma 140)		White conidia (whi). thiamine requirement (thi)
Ma 91	Minor	Spring beetle	Rennes (France)	Wild type
Ma 51	Major	*Oryctes rhinoceros*	La Minière (France)	Wild type

Note. On deposit in the Fungal Collection of the Biological Control Research Station INRA, La Minière, France.

duits du maïs), and 4% glucose. Conidia were inoculated into 150-ml Erlenmeyers flasks and aerated by shaking on a rotary shaker (100 rpm). The shaken cultures were incubated at 25°C for 6 days, at which time the cultures were harvested. The cultures were harvested by centrifugation at 3000*g* for 10 min at 4°C.

Production of mutants. Strain Ma 140 was irradiated by ultraviolet light (254 nm). Auxotrophic and color mutants were obtained by the same process described by Messias and Azevedo (1980).

Selection of putative diploids and somatic segregants. The auxotrophic conidial color mutants (*ylo arg* + *whi thi*) were crossed using the methods described by Messias and Azevedo (1980). Briefly, this method involves spreading the mixed conidial suspension of the two mutants on minimal medium followed by incubation. The presumptive heterocaryons which develop are then inspected for sectors exhibiting green conidia. The presumptive diploid is then grown on CM until sporulation has occurred. The conidia are diluted and plated on CM such that single, discrete, putative diploid colonies develop. Using sterile velveteen pads, the conidia from the diploid colonies are replicated to CM containing 1.5 ppm benomyl. Segregants are recognized as non-green outgrowths from the replica colony.

For the crosses between the two varieties of the fungus *minor* and *major* types, we used methods identical to those already described. Briefly, 0.1 ml of conidial suspension (1.0×10^7 condia/ml) from each of the strains was mixed and spread on the surface of CM plates. The plates were incubated until the resulting colonies had sporulated. Conidial suspensions were prepared from the plates, diluted appropriately such that single colonies developed. The putative hybrids (presumably somatic diploids) were recognized on the basis of conidial size as determined with a Coulter Counter.

Testing for virulence. Using a Bugerjon's (1956) tower, the virulence of a strain was evaluated by spraying 10 ml of a conidial or blastospore suspension of know concentration on diapausing larvae of *O. nubilalis* (see Riba et al., 1983) and 3rd-instar larvae of *O. rhinoceros*. In addition, we used 12th-instar larvae of *Aedes aegypti, Anopheles stephensi,* and *Culex pipiens,* which are susceptible to *M. anisopliae* (see Daoust and Roberts, 1982). For these tests, we prepared eight lots of 10 larvae in 50 ml of propagule suspension (10^6 propagules/ml). The percentage mortality was measured on the 5th day following treatment. Daily observations were made but only the mean mortality on the final day of observation is reported. The LT_{50} (lethal time) was calculated after transformation by probit mortality.

RESULTS

Virulence of somatic diploids and so-

matic segregants from a cross within a variety. When the two auxotrophic color mutants were crossed (*ylo arg* + *whi thi*), putative somatic diploids were isolated. Although these presumptive diploids did not produce conidia significantly larger than either of the component mutants, the fact that the putative diploids produce green conidia is consistent with the strains being diploid. Further support for the interpretation of these strains producing green conidia as being diploid comes from a consideration of the segregants recovered from these strains. Segregants 4 and 5 (Table 2) are clearly recombinant types while segregants 1, 2, 3, 6, and 7 represent parental types. Segregant 8 might be interpreted as either a revertant or, more likely, a somatic crossover.

When tested for virulence, the somatic diploid strains and segregant 8 appear comparable to the wild type (Table 2). Strains carrying auxotrophic markers, either the original mutants or segregants 1–7, are reduced in virulence. That auxotrophy is probably responsible for the reduced virulence rather than the conidial color mutation is attested to be segregant 8 (Table 2) which is a color mutant (*ylo*) while being phototrophic and exhibiting nearly wild type virulence.

Virulence of strains exhibiting conidial size intermediate between that of the two M. anisopliae varieties (major or minor). As shown here (Table 2) and as described by others (Al-Aidroos, 1980; Paris and Ferron, 1979), auxotrophy frequently leads to reduced virulence. Auxotrophic mutants are a convenient means to "force" heterocaryosis on MM and ultimately to select somatic diploids. Since the effect of auxotrophy on virulence is dramatic, we attempted to isolate putative somatic diploids by crossing wild strains carrying morphological differences.

The *major* variety is only pathogenic to *O. rhinoceros* larvae whereas the *minor* variety can attack the *O. nubilalis* larvae and mosquitoes (Table 3). Of course, Ma 91 is less aggressive against European corn borer larvae than Ma 140, a minor strain isolated from this insect pest.

The putative diploids selected on the basis of conidial size intermediate between that of the two parental varieties proved to be avirulent (Table 3).

DISCUSSION

From the wild strain, Ma 140, two auxotrophic and color mutant strains were used; one was arginine-requiring and had yellow color spores, and the other was a thiamine-requiring strain with white conidia. Crosses between such strains gave diploids. All the diploids obtained were green like the wild parental strain, whereas the segregants from them were yellow or white. There was no difference of spore size between the wild strain, the mutants, and the segregants. Almost all of the segregants have a parental phenotype; only three of them were clearly recombinant, two being auxotrophic and one being prototrophic (Table 2).

The Ma 140 strain appears to be highly virulent for all the insect species tested (Table 2). The effects of auxotrophy in reducing virulence of this strain seem to manifest themselves for all the insect species investigated. If the reduced virulence reflects the relative unavailability of the required nutrient (arginine or thiamine), then all the insect species tested must present to the pathogen with the same relative deficiency. The fact that the segregants 1–7 (Table 2) all exhibit about the same degree of reduced virulence suggests that other possible genes affecting virulence have not been recombined in any major way. For *M. anisopliae* variety *minor,* virulence of entomopathogenic fungi seems to be a manifestation of the interaction of many genes, as it is the case for some phytopathogenic fungi (Boccas, 1973; Yaegashi, 1978). For this reason a continuous variation of aggressiveness has been observed among the segregates.

Furthermore, according to the concept of aggressiveness and nonspecific resistance

TABLE 2

RELATIVE AGGRESSIVENESS OF WILD STRAIN, PARENTAL AUXOTROPHIC MUTANTS, DIPLOIDS, AND SEGREGANTS OF *Metarhizium anisopliae* VAR. *anisopliae* TOWARD MOSQUITOES AND *O. nubilalis* LARVAE

Strains	Spore color	Conidia size	Auxotrophic mutation	*Aedes aegypti* Mortality (%)[a]	*Aedes aegypti* LT_{50}	*Anopheles stephensi* Mortality (%)[a]	*Anopheles stephensi* LT_{50}	*Culex pipiens* Mortality (%)[a]	*Culex pipiens* LT_{50}	*Ostrinia nubilalis* Mortality (%)[b]	*Ostrinia nubilalis* LT_{50}
140	green	8	Prototroph	93.4^{a}	2.2	93.1^{a}	1.9	95.7^{a}	1.6	100^{a}	7.1
140 Arg	yellow	8	Arginine	$76.6^{a,b}$	3.1	83.0^{b}	2.6	94.6^{a}	2.1	72.4^{b}	8.3
140 Thi	white	8	Thiamine	68.1^{b}	4.5	76.1^{b}	3.7	86.6^{a}	2.9	$61.3^{b,c}$	9.8
diploïd 1	green	8	Prototroph	89.3^{a}	2.4	92.2^{a}	1.9	98.3^{a}	1.6	100^{a}	7.6
diploïd 2	green	8	Prototroph	96.0^{a}	2.2	87.6^{a}	2.0	100^{a}	1.7	95.9^{a}	7.3
diploïd 3	green	8	Prototroph	95.8^{a}	1.9	97.8^{a}	1.7	93.2^{a}	1.4	99.1^{a}	6.9
diploïd 4	green	8	Prototroph	97.6^{a}	2.3	93.7^{a}	1.9	100^{a}	1.6	91.8^{a}	7.6
segregant 1	yellow	8	Arginine	65.4^{b}	3.5	71.8^{b}	2.8	87.4^{a}	2.5	68.7^{b}	8.6
segregant 2	yellow	8	Arginine	58.3^{b}	4.8	$62.9^{b,c}$	3.9	88.1^{a}	3.4	$58.3^{b,c}$	9.6
segregant 3	white	8	Thiamine	58.2^{b}	4.1	$66.4^{b,c}$	3.4	84.5^{a}	2.8	66.5^{b}	9.6
segregant 4	white	8	Arginine	43.9^{c}	5.7	76.7^{b}	4.2	71.5^{a}	3.9	53.4^{c}	11.4
segregant 5	yellow	8	Thiamine	61.1^{b}	3.8	81.7^{b}	3.0	79.7^{a}	2.5	68.2^{b}	8.4
segregant 6	yellow	8	Arginine	67.6^{b}	3.6	81.6^{b}	3.0	86.9^{a}	2.4	65.7^{b}	8.7
segregant 7	white	8	Thiamine	72.7^{b}	4.3	$84.3^{a,b}$	3.2	90.6^{a}	2.5	$61.9^{b,c}$	9.1
segregant 8	yellow	8	Prototroph	88.4^{a}	2.3	95.2^{a}	2.1	97.0^{a}	1.8	88.1^{a}	7.8

Note. Numbers with the same letter are not significantly different (5%).

[a] Percentage of mortality calculated on the 5th day following treatment at 10^6 sp/ml.

[b] Percentage of mortality calculated on the 12th day following treatment at 10^7 sp/ml.

TABLE 3
VIRULENCE OF WILD STRAINS, AND INTERMEDIARY HYBRIDS TO *Oryctes rhinoceros*, *Ostrinia nubilalis* AND MOSQUITO LARVAE

Strain	Spore size	*Ostrinia nubilalis*[a]		Mortality (%)			
		Mortality	LT_{50}	*Oryctes rhinoceros*[b]	*Aedes aegypti*[c]	*Anopheles stephensi*[c]	*Culex pipiens*[c]
51 M	12	0	—	76 ± 4	0	0	0
91 m	8	32	21.2	0	23	20	33
5191 A i	10	0	—	0	0	0	0
5191 B i	10	0	—	0	0	0	0
5191 C i	10	0	—	0	0	0	0
5191 D i	10	0	—	0	0	0	0
5191 E i	10	0	—	0	0	0	0
5191 F i	10	0	—	0	0	0	0

Note. M, major conidia; m, minor conidia; i, intermediate conidia.
[a] Percentage of mortality evaluated on the 12th day following treatment at 10^7 sp/ml.
[b] Percentage of mortality evaluated on the 25th day following treatment at 10^7 sp/ml.
[c] Percentage of mortality evaluated on the 5th day following treatment at 10^6 sp/ml.

(Van der Plank, 1975), we noted that *Culex pipiens* is very sensitive to *M. anisopliae* var. *minor*, *A. stephensi* larvae are more resistant, and those of *Aedes aegypti* are the most resistant to this variety. But, the most aggressive strains of *M. anisopliae minor* toward one host are also the most aggressive toward the others (Table 2).

Similar relationships have been described between *Beauveria bassiana* strains and different races of *Bombyx mori* (Riba et al., 1982).

As observed by others (Ferron et al., 1972; Fargues, 1976), *M. anisopliae* var. *major* and *minor* strains do exhibit host specificity (Table 3). The putative hybrids between the varieties selected on the basis of conidial size proved to be avirulent (Table 3). The results parallel those obtained by Federici (1979, 1982), who also observed that interspecific hybrids in the *Coelomomyces dodgei* complex were avirulent. Assuming that virulence is based on many genes, it follows that many biochemical pathways and end products are probably involved. It is not implausible that strains of the same species which have evolved sufficiently to yield a morphologically distinguishable phenotype (conidial size) might produce biochemical intermediates which mutually interfere with the process of pathogenesis. In other words, when evolving varieties of a species are forced to hybridize, reciprocal incompatibilities may preclude pathogenesis by the hybrid. If pathogenesis does involve many genes and gene products, it may prove difficult to identify the biochemical interactions in the hybrid which reciprocally inhibit pathogenesis.

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