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CHARACTERIZATION OF NEW BIOTYPES OF P157 STRAIN OF *Metarhizium anisopliae* var. *anisopliae*, GOT BY TREATMENT WITH GAMMA RADIATION

*(Caracterización de nuevos biotipos de la cepa P157 de **Metarhizium anisopliae** var. **anisopliae** obtenidos por tratamiento con radiación gama)*

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Key words: ***Metarhizium anisopliae***, gamma radiation, biotypes

RESUMEN

*Conidios de la cepa silvestre P157 de **Metarhizium anisopliae** var. **anisopliae** (Metsch.) Sorokin, fueron expuestos a la radiación gama para la obtención de nuevos biotipos. En la dosis de 390Gy se obtuvo 36 colonias (MaP). Cinco colonias (MaP 03, MaP 17, MaP 25, MaP 27), expresaron alteración morfológica para el color en el Medio Completo. En este, 21 colonias presentaron crecimiento micelial significativamente distinto del testigo silvestre, después de 12 días de la inoculación. La colonia MaP 25, presentó mayor valor de crecimiento micelial y la colonia MaP 11, el menor valor. Entre las colonias obtenidas, solamente la colonia MaP 28 presentó auxotrofia, identificada como una deficiencia en la síntesis del ácido paraminobenzóico. Los conidios de 29 colonias, presentaron un promedio de porcentaje de germinación significativamente destinto del respectivo aislamiento silvestre, después de 12 horas de incubación en Medio Mínimo, a 25°C. Las colonias MaP 02, MaP 21 y MaP 36, presentaron precocidad en la germinación de los conidios en relación al patrón silvestre.*

SUMMARY

*Conidia of the wild P157 strain **Metarhizium anisopliae** var. **anisopliae** (Metsch.), were exposed to gamma radiation to obtain new biotypes. At a dose of 390Gy, 36 colonies (MaP) were obtained. Five colonies (MaP 03, MaP 17, MaP 25, MaP 27 and MaP 29) showed morphological alteration to colour in the Complete Medium. In this same medium, 21 colonies showed a significantly different mycelial growth from the wild strain after 12 days of innoculation. The MaP 25 colony showed the greatest value of mycelial growth, and the MaP 11 colony, the least value. Among the colonies obtained, only MaP 28 showed auxotrophy, identified as a deficiency in the synthesis of the paraminobenzoic acid. Conidia of twentynine colonies exhibited an average germination percentage significantly different from the wild strain after a twelve hour incubation in Minimum Medium, at 25°C. The MaP 02, MaP 21 and MaP 36 colonies showed some precocity in conidial germination as regards the wild strain.*

INTRODUCTION

Some fungi reveal great possibilities of being used in insect and pest biological control programmes because they have an entomopathogenic action. Among these, ***M. anisopliae*** is one of the most studied as regards the pathogenicity and virulence on more than 200 species of insects, being many of then of agronomic interest (2, 3, 17, 25, 35). Two characteristics associated with this fungus are of great relevance for its use in the field as a biocontrol agent: it does not mean pathogenicity for man and it be obtained in large quantities at a relatively low cost (27).

In Brazil, ***M. anisopliae*** has been used very successfully in the biologic control of sugar-cane cicadas

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(***Mahanarva posticata*** Stal., ***Mahanarva fimbriolata*** Stal.) and those of pastures ***Deois flavopicta*** Stal. and ***Zulia entreriana*** Berg. This fungus is also used in the control of ***Diatraea saccharalis*** Fabr, ***Hypothenemus hamppei*** Ferrar, ***Tibraca limbativentris*** Stal. and ***Cornitermes cumulans*** Kollar(2,7,13,34).The efficiency of entomopathogenic fungi used in biological control, can be improved by means of genetic manipulation, by altering, among other things, factors like sporulation, dispersion and tolerance of the spores to stress (36). A considerable increase in the infectivity of a wild strain of ***M. anisopliae*** was obtained by Yokoyama et al. (37) after treatment of protoplasts with UV light. Tsai et al. (36) isolated samples of ***M. anisopliae*** resistant to Benomyl and Carbendazim after treatment of conidia with UV, characteristics which are desirable in integrated past control programmes.The identification of the traces which determine pathogenicity and virulence is a fundamental step for the improvement of these fungi. Riba et al. (26), point out that the virulence of a strain seems to be the result of the manifestation of different genes. Among the various factors related to virulence are the speed in germination of the conidia, production of extra-cellular enzymes related to penetration (lipases, proteases and quitinases) and production of toxins (1, 6, 23, 29, 31, 32).The speed in germination of the conidia and the formation of appressoria are crucial events in pathogenesis (9). Resistance to high temperatures and UV radiation, pH, luminosity, nutritional sources, colony and stock age are factors which act on the viability and speed germination of the conidia of entomopathogenic fungi (8, 11, 15, 18, 30).The objectives of this study were the production of new biotypes of ***M. anisopliae*** var. ***anisopliae*** using gamma radiation, through the analysis of mycelial growth, auxotrophy, conidia germination, and morphological and physiological characterization.

MATERIALS AND METHODS

The P157 strain of ***M. anisopliae*** var. ***anisopliae*** used was granted by the culture collection (Micoteca - URM), of the Department of Mycology of the Federal University of Pernambuco, Brazil; this strain was isolated from ***Mahanarva posticata*** Stal., and maintained in solid Complete Medium (MC) (24), modified by Azevedo and Costa (5).

Conidia from colonies of 8-day old monosporic cultures (12), were separately suspended in 3ml of Tween 80 (0,1% v/v) solution and disaggregated in a vortex agitator. Appropriate (1:10) dilutions were made and distributed to five test tubes, each containing 5ml of suspension with 10^6 conidia/ml, estimated quantitatively in a Neubauer Chamber. The suspensions were submitted to different doses of gamma radiation Cobalt 60 type GAMMABEAN 650 - Atomic Energy of Canada, (50, 100, 150 and 200 Gy). One of the suspensions not treated with radiation was used as a control. After this procedure, the suspensions were proportionally diluted (1:10) in a saline solution (0,85%v/v) up to a concentration of 10^2 conidia/ml. They were placed in triplicates on Petri dishes containing solid MC and 0,1ml of these suspensions was spread with a Drigalsky ring and then incubated at 25°C for 48 hours. After the incubation period, the surviving colonies were counted, taking into consideration the number of colonies obtained in the control as a 100% survival. Data obtained served as a basis for constructing a survival curve and for calculating the dose necessary to obtain 5% survival by means of linear regression.

A suspension of conidia with 10^6 conidia/ml was submitted to a dose of gamma radiation of 390 Gy, aiming at a survival rate lesser than 5%. The suspension was diluted proportionally (1:10) to a concentration of 10^2 conidia/ml and aliquots of this suspension were placed on 10 Petri dishes containing solid MC. The dishes were incubated for 48 hours at 25°C. The colonies arising were transferred to test tubes containing solid MC and incubated at room temperature and luminosity for 8 days. These colonies were designated as MaP.

The micromorphologic characters were analyzed by means of smearing conidia separately on Petri dishes containing solid MC at three different points and covering them with cover glass. The dishes were incubated for five days at 25°C. The cover glass was removed from the cultures and placed on slides with a drop of Amann blue, and observed under light microscope.

For the analysis of the macroscopic characters, 7 day-old mycelium discs from the MaP colonies, cut with the aid of a 5mm diameter cork drill, were transferred separately to the center of the Petri dishes containing solid MC, in triplicates, with the help of a platinum ring, and then, incubated at 25°C. Growth was accompanied for a period of 12 days with observation intervals on the 6th, 9th and 12th days. In this process the colour and diameter parameters of the colonies were considered.

The auxotrophy of the MaP colonies was verified by means of inoculating conidia in Petri dishes containing solid Minimum Medium (MM) (24), and incubated at 25°C for 12 days. The dishes were marked in four diameters of 2.25cm, to estimate the percentage growth. At the end of this period, conidia which had not formed colonies, were transferred to Petri dishes containing solid MM to which was added, separately, hydrolyzed casein, a solution of yeast nucleic acid, and a solution of vitamins, in order to gain specific knowledge of the nutritional deficiency.

A prior synchronization of conidia (10) was undertaken to analyze germination, thereby obtaining

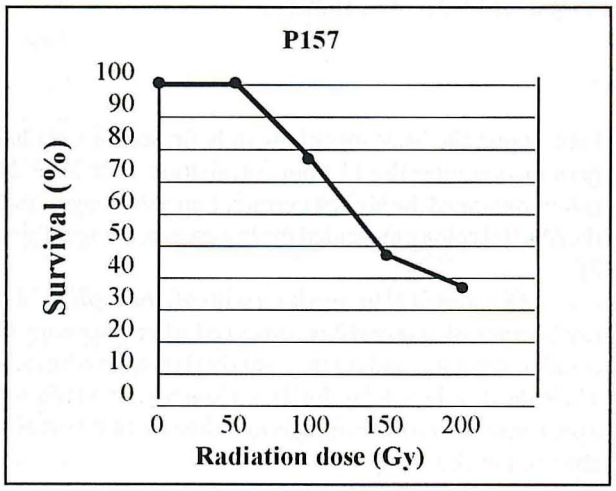
conidia of the same age and physiological state. Conidia of the MaP and wild P157 colonies, arising from the synchronization process were put in test tubes containing 9ml of liquid MM in a concentration of 10^6 conidia/ml. Aliquots of the medium were with drawn at intervals of one hour, and observed under the light microscope. The percentage of germination was calculated by observing after 12 h the presence or absence of germination tubes on 500 conidia using a Neubauer Chamber.

Turkey's test was employed to compare the mean growth of the different colonies.

RESULTS AND DISCUSSION

The results obtained as to the survival in gamma radiation, of conidia of the P157 strain of ***M. anisopliae*** var. ***anisopliae,*** were used to construct a survival curve (Figure 1). The extrapolated dose to get a 5% survival was 390 Gy.

Figure 1 - Conidia survival curve of the P'57 strain of ***M. anisopliae*** var. ***anisopliae*** submitted to gamma radiation



After exposing the conidia suspension to a 390 Gy dosis , 36 colonies were obtained. The colonies showed that the morphology of the hiphae, conidiphores and conidia were preserved. Among these, 5 showed morphological alteration in colour: 4 colonies a yellow colour (MaP 03, MaP 25, MaP 27, MaP 29) and one a white colour (MaP 17).

Of the 36 colonies obtained after treatment/dose with gamma radiation, 26 colonies showed a mycelial growth in diameter, significantly different from the wild strain according to the data appearing in Table 1.

Only one colony, MaP 28, showed auxotrophy and

Table 1

Mean mycelial growth (diameter) of MaP colonies of *M. anisopliae* var. *anisopliae* on the 12th day of growth on solid MC, at 25°C.

Colonies	Growth Average[1] (mm)
MaP 25	67.0 a[2]
MaP 08	57.3 b
MaP 34	56.6 b c
MaP 33	56.3 b c
MaP 02	55.6 b c d
MaP 20	54.0 c d e
MaP 09	54.0 c d e
MaP 35	52.6 d e f
MaP 15	52.0 e f g
MaP 22	52.0 e f g
MaP 36	52.0 e f g
MaP 28	51.0 e f g h
MaP 21	50.3 f g h
MaP 38	50.3 f g h
MaP 04	50.0 f g h
MaP 18	49.3 g h i
MaP 10	48.0 h i j
MaP 30	46.3 i j k
MaP 23	46.0 j k l
MaP 24	46.0 j k l
MaP 31	46.0 j k l
MaP 12	46.0 j k l
MaP 13	45.5 j k l
MaP 19	45.0 j k l
MaP 05	44.6 k l
MaP 01	44.3 k l
MaP 27	44.3 k l
Control3	43.0 l
MaP 14	39.0 m
MaP 37	38.0 m
MaP 29	29.0 n
MaP 07	28.0 n o
MaP 06	26.0 n o
MaP 32	25.0 o
MaP 03	20.3 p
MaP 17	18.0 p
MaP 11	10.3 q

1 Average of three samples.

2 Values followed by the same letter are not statistically different at the 5% level of probability, according to the Tukey's test.

3 Wild strain, P157.

Table 2

Growth of MaP colonies of *M. anisopliae* var. *anisopliae* on the 12th day of incubation on solid MM, at 25°C.

Colonies	Growth[1]	Colonies	Growth	Colonies	Growth
MaP 01	++	MaP14	++	MaP29	+
MaP 02	++	MaP15	+++	MaP30	++
MaP 03[2]	--	MaP17	+	MaP31	++
MaP 04	+++	MaP18	++	MaP32	++
MaP 05	++	MaP19	++	MaP33	+++
MaP 06	++	MaP20	++	MaP34	++
MaP 07	++	MaP21	+++	MaP35	++
MaP 08	++	MaP22	++	MaP36	+++
MaP 09	++	MaP23	++	MaP37	++
MaP 10	++	MaP24	++	MaP38	++
MaP 11	++	MaP25[2]	--	Control[3]	+++
MaP 12	+	MaP27	+		
MaP 13	++	MaP28	--		

1 + low growth, relative to 25% of the Petri dish diameter.
++ regular growth, relative to 50% of the Petri dish diameter.
+++ normal growth, relative to 75% of the Petri dish diameter.
-- no growth.
2 The colonies MaP 03 and MaP 25 developed on MM only after 25 days of incubation.
3 Wild strain, P157.

in the complementary tests the colony showed deficiency in paraminobenzoic acid. The MaP 03 and MaP 25 colonies did not show growth in solid MM during the twelve days trial period, but grew 25 days later, which probably indicates that the gamma radiation caused auxotrophy and a reversal of this condition occurred (Table 2).

To analyze the germination percentage the MaP colonies that showed an average of mycelial diameter of a significantly greater value than that of the wild strain, in addition to the some colonies having an average diameter of lesser value were chosen. The germination percentage of the analyzed colonies is to be found in Table 3. Among the tested colonies,17 showed an average germination percentage significantly minor from the wild strain (Table 4). The MaP 01, MaP 02, MaP 04, MaP 05, MaP 13, MaP 15, MaP 19, MaP 21, MaP 22, MaP 36 and MaP 38 colonies, showed an average germination percentage, after 12 hours incubation, significantly greater than that of the wild strain. Three colonies, MaP 02, MaP 21 e MaP 36, began germination of the conidia after a 6 h incubation; the colonies MaP 06, MaP 14, MaP 22, MaP 30, MaP 32 and MaP 37 after 8 hour; the colonies MaP 08, MaP 11, MaP 20, MaP 33, MaP 34 and MaP 35 after 9 hour; while the MaP 09 colony only presented conidial germination after the 11 hour incubation. The MaP 21 colony presented the highest germination percentage while the MaP 09 colony presented the lowest percentage(Table 3).

Okino et al.(19), working with ***M. anisopliae*** and two species of ***Aspergillus,*** observed after exposure of conidia to gamma radiation, a survival of approximately 1% in the dose of 348 Gy for ***M. anisopliae,*** and reported a great sensibility of this fungus to radiation, that was also observed in this study.

Luna-Alves Lima (12), points out that different chemical and physical agents are used in the induction of mutation in organisms, while gamma radiation is little used as a mutagenic agent in entomopathogenic fungi. One of the most widely used physical agents for induction of mutation in filamentous fungi is ultraviolet light (14, 33, 37). In the improvement programmes for fungi used in biolocal control, ultraviolet light is also used more frequently due to the case of manipulation. Azevedo (4), points that gamma radiation can be considered as an excellent mutagenic inductor in some cases.

The induction of morphological mutations in filamentous fungi may vary in frequency depending on the

Table 3

Conidial germination percentage of MaP colonies of *M. anisopliae* var. *anisopliae*, on liquid MM, at 25°C.

Colonies	Germination percentage[1]							
	Incubation period (hours)							
	5	6	7	8	9	10	11	12
Control 2	-	-	1.4	4.0	11.8	15.8	37.4	53.6
MaP 01	-	-	2.2	4.6	12.4	17.4	39.2	60.2
MaP 02	-	1.2	3.0	7.6	12.4	25.8	35.8	59.0
MaP 04	-	-	4.2	10.0	27.0	33.8	36.8	61.0
MaP 05	-	-	3.0	5.8	12.0	19.2	40.2	59.8
MaP 06	-	-	-	2.2	6.4	11.8	15.2	36.2
MaP 07	-	-	1.0	3.6	10.2	14.4	38.6	48.6
MaP 08	-	-	-	-	6.0	11.8	15.8	38.0
MaP 09	-	-	-	-	-	-	2.6	5.8
MaP 10	-	-	2.4	5.0	17.0	24.0	39.0	50.8
MaP 11	-	-	-	-	3.4	5.0	12.2	18.4
MaP 13	-	-	8.4	18.8	32.6	40.4	51.8	60.6
MaP 14	-	-	-	1.2	3.2	11.0	15.8	35.2
MaP 15	-	-	3.6	11.8	24.0	52.4	67.8	71.2
MaP 18	-	-	3.2	6.0	12.0	18.4	26.2	33.4
MaP 19	-	-	7.8	17.6	31.8	38.4	49.2	58.4
MaP 20	-	-	-	-	6.0	19.0	29.6	38.2
MaP 21	-	5.6	12.8	23.8	31.4	50.2	63.2	71.6
MaP 22	-	-	-	6.2	21.0	32.0	53.0	64.6
MaP 23	-	-	5.4	9.8	16.0	18.6	28.2	34.4
MaP 24	-	-	5.8	9.2	15.8	19.6	30.2	36.6
MaP 30	-	-	-	1.8	4.2	20.8	3.4	37.8
MaP 31	-	-	4.8	8.6	15.6	19.0	26.0	33.2
MaP 32	-	-	-	1.2	4.4	7.8	12.2	27.2
MaP 33	-	-	-	-	4.6	13.0	24.6	41.8
MaP 34	-	-	-	-	3.8	11.8	18.2	35.2
MaP 35	-	-	-	-	7.8	15.0	33.8	53.4
MaP 36	-	6.1	14.0	18.0	28.0	46.2	55.0	67.2
MaP 37	-	-	-	1.8	4.01	2.21	6.8	36.8
MaP 38	-	-	13.6	24.4	31.8	49.2	53.8	67.8

1 Counting of 500 germinated and ungerminated conidia
2 Wild strain, P157

Table 4

Conidial germination average of MaP colonies of *M. anisopliae* var. *anisopliae* on the 12th hour, on liquid MM, at 25°C.

Colonies	Growth Average[1] (mm)	
MaP 21	71.5	a[2]
MaP 15	71.1	a
MaP 38	68.1	a b
MaP 36	67.1	a b c
MaP 22	64.5	b c d
MaP 04	61.1	c d e
MaP 13	60.6	d e
MaP 01	60.2	d e
MaP 05	59.7	d e
MaP 02	59.0	d e f
MaP 19	58.4	e f
Control[3]	53.6	f g
MaP 35	53.3	f g
MaP 10	50.7	g
MaP 07	48.5	g
MaP 33	41.7	h
MaP 20	38.1	h i
MaP 08	38.0	h i
MaP 30	37.8	h i
MaP 37	36.8	h i
MaP 06	36.2	h i
MaP 24	36.1	h i
MaP 14	35.1	i
MaP 34	35.1	i
MaP 23	34.3	i
MaP 18	33.4	i
MaP 31	33.2	i j
MaP 32	27.1	j
MaP 11	18.3	k
MaP 09	5.7	l

1 Average of three samples.

2 Values followed by the same letter are not statistically different at the 5% level of probability, according to the Tukey 's test.

3 Wild strain, P157.

type of mutagenic agent used for induction (12, 21, 22). Using UV as a mutation inductor in ***Beauveria bassiana,*** Paccola-Meirelles (21), points out that it was not obtained any stable morphological mutant, while it were obtained 22 morphological mutants to colour by using gamma radiation as an agent.

Morphological mutants of entomopathogenic fungi, obtained by artificial processes, are of the highest importance, because they may facilitate the recognition of recombinants in crossings via the parasexual cycle which increases their possibilities of being in a single genome, genes potentially favourable to entomopathogenic action (12). Oliveira et al .(20), obtained morphological mutants to colour of conidia in ***M. anisopliae*** var. ***minus*** and ***M. anisopliae*** var.***majus,***using as mutagenic agents 8-metoxipsolarein associated with long UV.

Luna-Alves Lima (12), working with ***M. anisopliae*** var. ***anisopliae*** obtained by using Ultraviolet light 14 simple auxotrophic mutants and 5 doubles; Paccola-Meirelles (21), obained after treatment of conidia of an isolate of ***Beauveria bassiana*** with gamma radiation, 5 simple mutants and 3 with double auxotrophic marks.

One of the determining factors in the pathogenicity of entomopathogenic fungi is the germination of conidia, this being the first step for host infection (16). Samuels et al. (28), stated that parameters like germination speed and growth are correlated with the high virulence of some strains of ***M. anisopliae*** for ***Nilaparvata lugens.*** The age of the colony can be considered as an interaction factor in a conidial germination. Hall et al. (8), in a comparative analysis of different species of entomopathogenic fungi claim that conidia coming from young cultures show more rapid germination than those of old cultures. In this study the synchronization of the conidia from the wild strain and from the MaP colonies was reached in such a way that the conidia used in the germination trial possessed the same physilogical conditions, thus eliminating variation due to the age of the conidia.

It was observed that the MaP 15, MaP 21, MaP 33 and MaP 36 colony, showed accordingly a high germination percentage, good growth in MM and MC and in addition to this, MaP 21 began germination well in advance. Considering that characteristics like high percentage and speed of germination, apart from good mycelial growth, are desirable in entomopathogenic fungi, the colonies which showed such characteristics may be promising for use in genetic crossing aiming the incorporation of these characteristics into strains with recognized good performance in biological control.

The biotypes obtained in this study need further studies to analyze other changes arising from the exposure to gamma radiation which may well contribute to the knowledge of the genetic bases associated with the most appropriate phenotypes for biological control programmes.

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