ISSN 0001-3714

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Volume 27 Número 4 Out.-Dez. 1996

FICHA CATALOGRÁFICA

Preparada pela Biblioteca do
Instituto de Ciências Biomédicas da Universidade de São Paulo

Revista de Microbiologia/Sociedade Brasileira de Microbiologia.
Journal of the Brazilian Society for Microbiology
— Vol. 27, nº 4 (out/dez 1996)
— São Paulo: SBM, [1970] -
v.:il; 27 cm

Trimestral
1970 - 1996, **4-27**
ISBN 0001-3714

1. Microbiologia I. Sociedade Brasileira de Microbiologia

NLM-QW4

SCT/PR CNPq FINEP

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Publication of the Brazilian Society for Microbiology - São Paulo - Brazil

Papers published in Revista de Microbiologia (Journal of the Brazilian Society for Microbiology) are indexed in:

Current Contents (USA); CNRS - Centre de la Recherche Scientifique (France); Chemical Abstracts Service (USA); Cambridge Scientific Abstract (USA); Commonwealth Mycological Institute (England); Hamdard National Foundation (Pakistan); IMLA - Index Medicus Latino Americano (Brasil); Institut Nauchtoi Informatsii (ex-URSS); Periodica (Mexico); Sumários Correntes Brasileiros (Brasil); UMI - University Microfilms International (USA).

Financial support: FINEP, FAPESP and CNPq.

Printed by WINNER GRAPH (phone: (011) 5584.5753)

Mailing address
Av. Prof. Lineu Prestes 1374
Phone/Fax: (011) 813.9647
Cidade Universitária - USP
05508-900 - São Paulo - SP
Brasil

SECTION EDITORS

ACKNOWLEDGEMENTS

The editors are indebted to the following colleages for collaborating with Revista de Microbiologia in 1996:

Acelino Couto Alfenas
Adalberto Pessoa Junior
Adauto Ivo Milanez
Agnes Marie Sá Figueiredo
Aline A. Pizzirani-Kleiner
Allen Norton Hagler
Ana Maria A. Coelho
Antonio Carlos Monteiro
Antonio F. Pestana de Castro
Antonio José P. Ferreira
Arlete Emily Cury
Armando Morais Ventura
Augusto Schrank
Beatriz E. C. Guth
Beatriz V. Kilikian
Benedito Corrêa
Caio M. F. Mendes
Carlos Augusto Rosa
Carlos Emílio Levy
Carlos Gil Turnes
Celeste Fava Netto
Celuta Sales Alviano
Christine C. Gaylarde
Clarisse Weis Aros
Clarisse Zaitz
Claudete Rodrigues Paula
Claudio Luiz Messias
Daison Olsany Silva
Décio Scholles
Deise Pasetto Falcão
Edison Durigon
Elba Bon
Elisa Yoko Hirooka
Ely Nahas
Elza A. Luna Alves Lima
Elza Masae Mamizuka
Fernando de Avila
Flavio Alterthum
Francisca P. França
Fumio Honma Ito
Glaci T. Zancan
Glaucia M. Pastore
Heloiza Ramos Barbosa
Henrique V. Amorim
Heriberto Fernandez
Homero Fonseca
Igor Mimica
Iracema O. Moraes
Iracema H. Schoenlein Crusius
Irene S. Schrank
Irma Gutierrez Rivera
Itamar Antonio Piffer
Jacques Nicoli
Janete Magali de Araújo
João Ramos da Costa Andrade
João Lúcio de Azevedo
João Ruy Jardim Freire
Jorge Luiz Cavalcante Coelho
José Domingos Fontana
José Geraldo Pradela
José Luiz Pereira
José M. Cabral de Souza Dias
Josias Rodrigues
Kinue Irino
Leda Cristina Mendonça Hagler
Leila Fonseca
Leonardo José Richtzenhain
Leon Rabinovitch
Lilian R. M. Marques
Lucia Maria Valente Soares
Lucia Martins Teixeira
Lucila Costallat Ricci
Luiz Carlos de Almeida
Luiz Carlos S. Ferreira
Luiz Carlos Urenha
Luiz Fernando Laranja
Luzia D. Paccola Meirelles
Manuel Portugal
Margareth Genovês
Margaretti S. Oliveira
Maria Angela Hungria
Maria Aparecida de Resende
Maria Candida R. Facciotti
Maria Cristina Dantas Vanetti
Maria Ligia Carvalhal
Maria Lúcia Pereira
Maria Lucia Racz
Maria Teresa Destro
Marina Baquerizo Martinez
Mariza Landgraf
Marisa Vieira de Queiroz
Marly Nunes
Marta Hiromi Taniwaki
Mauro Faber de Freitas Leitão
Michele Vitolo
Milton de Uzeda
Moisés Palaci
Myrna Sabino
Natalino H. Yoshinari
Neiva Monteiro de Barros
Olga F. Gompertz
Paulo H. A. Silva
Paulo S. Minami
Pedro Magalhães Lacava
Pedro Rodrigues Peres Neto
Regina T.R. Monteiro
Regine Helena S. F. Vieira
Robert W. Barreto
Roberto da Silva
Roberto F. Novaes
Rogélio Lopes Brandão
Rosa Luchese
Rosa Maria Silva
Rosa M. Castro Muchovej
Rosana F. Vazoller
Rosane Freitas Schwan
Samia Maria Tauk Tornisielo
Sandra F. Botelho Trufem
Selma Gomes
Sergio E. L. Fracalanzza
Sergio Olavo P. da Costa
Silvia Cardoso Leão
Sunao Sato
Tania A. Tardelli Gomes
Tomaz Lagenbach
Terezinha I. Estivalet Swidzinski
Valter Roberto Linardi
Vanderlei P. Canhos
Vanessa Sperandio
Vera Lucia Ramos Bonomi
Vera Lucia Garcia Calich
Virginia Maria Chaves Alves
Vivian Helena Pelizari
Walderez Gambale
Waldir Pereira Elias Junior
Walter Borzani
Walter Vieira Guimarães
Willibaldo Schmidell Netto
Yong Kun Park
Zoilo Pires de Camargo

Author Index

REVISTA DE MICROBIOLOGIA

Volume 27 Number 4 October-December 1996

ENVIRONMENTAL MICROBIOLOGY/SOIL MICROBIOLOGY

MICROBIAL PHISIOLOGY/GENETICS

INDUSTRIAL MICROBIOLOGY

Revista de Microbiologia (1996) 27:213-217
ISSN 0001-3714

SCREENING AND ISOLATION OF ANTI-*FUSARIUM MONILIFORME* COMPOUNDS PRODUCING MICROORGANISMS FROM SOIL AND CORN

Marisa Motomura[1]; Carolina E. Lourenço[1]; Danielle Venturini[1]; Yoshio Ueno[2]*, Elisa Y. Hirooka[1]

[1]Universidade Estadual de Londrina, Departamento de Tecnologia de Alimentos e Medicamentos, CCA, Londrina, PR, Brasil. [2]Faculty of Pharmaceutical Sciences, Science University of Tokyo, Shinjuku-ku, Tokyo, Japan.

ABSTRACT

Fusarium moniliforme, a worldwide corn phytopathogen, is the agent responsible for production of the most recently discovered mycotoxin denominated fumonisin. With intention to evaluate antifungal activity on *F. moniliforme*, 60 soil and 20 corn samples were screened for active microorganisms, using fumonisin producing *F. moniliforme* strain 113F. Crude extract was prepared with brain heart infusion-(BHI) culture of selected microorganisms, and concentrated with addition of ethanol at 1:1 ratio. The procedure screened 29 soil samples with positive inhibitory activity on *F. moniliforme*, with isolation of 36 antagonistic microorganisms. Fifteen corn samples also showed positive screening results, with isolation of 15 antagonists. The 51 isolates were distributed in 5 yeasts, 3 Gram-positive cocci, 3 Gram-negative cocci and 40 Gram-positive bacilli. All the 51 strains showed inhibitory activity on *F. moniliforme* 113F, demonstrated by whole culture method, while only 34 strains showed activity, when culture supernatant of same strains were assayed by antibiogram test. The sensitivity range of 5 selected antagonist strains was also compared using 10 toxigenic *F. moniliforme*. Considering the extracellular characteristic of these principles, the analysis of crude extract concentrate selected two Gram-positive cocci and 3 sporulated Gram-positive bacilli, with perspectives of bioactive substance production, for *F. moniliforme* control.

Key words: *F. moniliforme*, antagonist, antifungal, biological control, fumonisin.

INTRODUCTION

Fusarium moniliforme, a worldwide corn fungi, occurs in a pathogenic or assymptomatic form and is incriminated as main fumonisin producing *Fusarium* spp. (2,7,18). The evidence of carcinogenicity of fumonisins, with comproved hepatocarcinogenicity in rats, caused international impact, leading as one of main research topic in actual mycotoxicology (7).

At the moment, in view of difficulties to obtain corn cultivars resistant to fusaria attack, an alternative solution is succeeded through chemical fungicide treatment of seeds (13,16). With same purpose, the diversity of biologycal control emerges as promising procedure, with advantage in avoiding chemical pollution of ecosystem (1,3,4,5,11,14,17,18,19,23).

With intention to obtain new biologycal products, many microorganisms with antimicrobial activity has been isolated and identified (4).

Bacillus spp. is the main antimicrobial producing bacteria isolated from soil, easily cultivated in low cost culture medium. Its spore forming characteristic confers better subsistence in adverse conditions, and resistance to many physico-chemical factors (20).

* Corresponding author. Mailing address: Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12. Ichigaya, Funagawara-Machi, Shinjuku-ku, Tokyo, Japan.

Continuous research on antimicrobial substance of *B. subtilis* demonstrated their importance on the control of phytopathogens both bacteria and fungi (17,23). Emphasis is given to iturin, isolated from *B. subtilis* and extensively studied for agricultural application. Kimura and Hirano (9) and Ono and Kimura (15) extracted iturin A from *B. subtilis* NK 330 with inhibitory effect against *Aspergillus parasiticus* NRRL 2999 and *A. flavus* NRRL 3357, which completely controlled aflatoxin production.

This research purposes isolation of microorganisms with antifungal activity from soil and corn. Soil was chosen as material of study, based on great microbial diversity. Corn was also appointed, as the close association between pathogen and component of normal microflora promoves competition, which propiciate selection of antagonist to fusaria.

MATERIALS AND METHODS

Screening of antifungal producers:

Soil. Soil samples were collected from forest reserve of the State University of Londrina, delimited in 114 areas of 20x20m, according to the mapping prepared for ecology study purpose. A total of 60 soil samples (50 surface soil plus 10 samples of 10 cm depth from same place), in amount of 50g each were collected, and immediately cooled to 4°C before plating. One ml of soil diluited at 10^{-1} to 10^{-9}, and 1 ml of *F. moniliforme* 113F suspension with 10^4 propagules in 0,1% Tween 80 were pour-plated simultaneously, with 20 ml of potato dextrose agar (PDA). The plates were incubated at 25°C and observed daily, during 6 days. Any inhibitory activity was considered as positive reaction, and isolated colonies submitted to Gram and Wirtz staining.

Corn. Total of 20 corn samples adquired from 10 cooperatives of the State of Paraná, with positive results to *F. moniliforme* and fumonisin FB1 and FB2 (8), were used to isolate antagonists. Ten grams of corn was blended with 90 ml of water, diluted to a series of 10^{-7} and 1 ml of each dilution pour-plated with 20 ml of potato dextrose agar. Microorganisms with inhibitory activity against *F. moniliforme*, present on corn's own microflora were isolated after one week of incubation at 25°C, transferred to Brain Heart Infusion (BHI) agar tube, and submitted to Gram and Wirtz staining .

Test microorganisms. Ten single spore isolated strains, identified as *F. moniliforme* (12) were selected, comparing 43 toxigenic species previously isolated from feeds involved in animal intoxications (8). Fumonisin was determined according to Ueno *et al.* (24). Among these strains, *F. moniliforme* 113F was used as test organism of choice, based on its high toxigenic characteristic (54,21 mg/g of FB1 and 87,31 mg/g of FB2).

Assessment of antifungal activity. Two methods were compared:

Method of Bettiol *et al.* (5) transferred 0,5cm diameter disk of PDA with 72 hours culture of *F. moniliforme* 113F, on a center of PDA plate and incubated at 25°C for 48 hours. Screened antagonists were inoculated at four points, 3,5cm distance from center. The plate was incubated for more 72 hours, and evaluated for inhibition halo.

Method of Mckeen *et al.* (11) used 100µl of BHI culture supernatant of screened antagonists in the center well on PDA agar, previously inoculated with *F. moniliforme* 113F.

Culture and crude extract. Thirty-four antifungal substance producing organisms were reactivated, cultivating the strains two times in BHI broth at 35°C. Inoculum consisted of a culture with absorbance of 0,3 at 600nm. Volume equivalent to 5% of total culture of reactivated organism was transferred to 25 ml of BHI broth, and incubated at 35°C for 48 hours. Culture supernatant was concentrated essentialy as described by Abdel-Bar *et al.* (1). Supernatant added with ethanol at proportion of 1:1 was centrifuged, and alcoholic supernatant dried at 45°C. The material was suspensed with destilled water, to a 1/5 volume of initial culture and denominated crude extract.

Antibiogram. Inhibitory activity of crude extracts on *F. moniliforme* 113F was tested by method of Mckeen *et al.* (11), with some modifications. One ml of *Fusarium* suspension with 10^4 propagules/ml was pour-plated in 20 ml of PDA and after solidification, 100µl of crude extract, introduced to central well with 10mm diameter. Plates incubated at 25°C was observed for inhibition halo, at the intervals of 3 to 40 days.

For analysis of sensitivity variation among fungal strains, five selected antagonists (3 sporulated bacilli and 2 Gram-positive cocci) were tested on 10 *F. moniliforme* strains.

RESULTS AND DISCUSSION

Screening procedure for antagonist isolation from soil and corn detected 51 microorganisms with

anti-fusarium activity, distributed in 5 yeasts, 3 Gram-positive cocci, 3 Gram-negative cocci and 40 Gram-positive bacilli.

Analysis of 20 corn samples demonstrated 75% positivity for presence of microorganism with anti-fungal activity, with isolation of 15 strains. The same procedure screened 29 (48,3%) positive soil samples, with isolation of 36 strains (data not shown). The result confirms occurrence of antagonistic inhabitant, which allows simultaneous subsistence of host, pathogen and different kinds of organisms (4). In addition, the constant presence of *F. moniliforme* in corn(6), directed some degree of pre-selection.

Method of Bettiol *et al.* (5) confirmed inhibitory activity of all the 51 isolates, while the use of culture supernatant by method of Mckeen *et al.* (11) reduced the positivity to 34 strains. Extracellular characteristic of bioactive component of these 34 strains is a peculiar advantage, as the scale up of procedure is favoured essentialy by simplification of extraction process (1), and concerned to 25 soil isolates and 9 corn origin organisms (Table 1). The data confirms the theory of competivity (19), as well as the selection by survivence of pathogen together with microflora, in same plant substrate (4).

The Table 1 shows morphological characteristics of corn and soil isolated microorganisms, and stability-activity response of respective extracts on *F. moniliforme* 113F. Inhibition halo diameter produced by crude extract concentrate ranged from 18 to 30mm,

Table 1. Inhibition of *F. moniliforme* strain 113F by microoganisms isolated from corn and soil.

Microorganisms	Morphology	Inhibition halo (mm)	Stability at 25°C (days)
Corn M1	Gram positive bacilli	18	10
M2	sporulated Gram positive bacilli	18	10
M3	sporulated Gram positive bacilli	18	3
M5	sporulated Gram positive bacilli	18	15
M8	Gram positive bacilli	20	10
M9	Sporulated Gram positive bacilli	20	10
M10	Yeast	20	15
M17	Gram positive bacilli	21	10
M18	Sporulated Gram positive bacilli	22	15
Soil M4	Sporulated Gram positive bacilli	19	15
M6	Sporulated Gram positive bacilli	19	20
M7	Sporulated Gram positive bacilli	20	30
M11	Sporulated Gram positive bacilli	20	40
M12	Sporulated Gram positive bacilli	20	10
M13	Gram positive bacilli	20	3
M14	Sporulated Gram positive bacilli	22	3
M15	Sporulated Gram positive bacilli	22	15
M16	Sporulated Gram positive bacilli	23	15
M19	Sporulated Gram positive bacilli	23	30
M20	Gram negative cocci	23	10
M21	Sporulated Gram positive bacilli	23	10
M22	Sporulated Gram positive bacilli	24	15
M23	Sporulated Gram positive bacilli	24	20
M24	Gram positive bacilli	24	35
M25	Sporulated Gram positive bacilli	24	10
M26	Sporulated Gram positive bacilli	24	10
M27	Gram positive bacilli	24	3
M28	Sporulated Gram positive bacilli	25	35
M29	Sporulated Gram positive bacilli	25	10
M30	Sporulated Gram positive bacilli	28	5
M31	Gram positive bacilli	28	30
M32	Sporulated Gram positive bacilli	28	10
M33	Sporulated Gram positive bacilli	28	40
M34	Gram positive cocci	30	30

well diameter:10mm

with average on 24mm, which persisted for 3 to 40 days, i.e, total analysis time (Table 1). The corn isolated group was composed by 8 Gram-positive bacilli and 1 yeast. Inhibition halo ranged from 18 to 22mm, and except one strain, evidenced stability of 10 to 15 days (Table 1). The soil isolated group was composed by 22 Gram-positive bacilli, 2 Gram-positive cocci and 1 Gram-negative cocci, with active diameter of 19 to 30mm. The effect persisted from 10 to 40 days (end point of evaluation) in 21 (84%) strains, of which two strains demonstrated persistance of inhibition halo for 40 days, another 2 strains for 35 days and 4 for 30 days (Table 1).

The data clearly indicated superiority of soil isolated organism (8), reforcing competivity theory in adverse environment (25). Isolation of organisms with high antimicrobial potential, more resistant and easily cultivated is increased in soil, as these requirements are indispensable for own survivance. Obtention of high antifungal activity microorganisms from soil is related by Baker *et al.* (3), Kobayashi and Yamaguchi (10) and Omura *et al.* (14).

Otherwise, corn is also an important substrate intended to isolate microorganisms with antifungal activity, as *F. moniliforme* is the most frequent pathogen of corn (22). Despite of lower activity, 9 corn isolates produced extracellular active component (Table 1), in addition to the positivity of 75%, during sample screening. Corn isolates may serve as progenitor for new strains, to develop improvement through genetic techniques.

Five soil isolates were selected based on its activity-stability response, and antifungal effect tested on 10 fumonisin producing *F. moniliforme* (Table 2). Table 2 shows diverse sensitivity of particular fungal strains, but better results were obtained with sporulated Gram-positive bacilli (M11, M28 and M33). Identification procedure, proceeding 30 biochemical tests described in Bergey's Manual of Systematic Bacteriology (21), with emphasis on *Bacillus* spp., indicated M11 as *B. subtilis*, M28 as *B. bradius* and M33 as *B. lactesporus*.

Evidence of Gram-positive bacilli as main antifungal compound producer is constantly related in literature (20). Diversity in stability displayed by organisms isolated in this study suggests that the substance produced by each may be biochemically distinct, from one another. Isolation of yeast (Table 1) among promissor biocontrol organism is interesting, as Walker *et al.* (26) related Killer toxins as potent novel antimycotic agents.

Although the datas indicated potential of soil bacteria metabolites on *Fusarium* spp. biocontrol, the simultaneous presence of antagonist and pathogen in corn suggested application of natural microbial balance, on *Fusarium* spp. and fumonisin control in the field.

ACKNOWLEDGEMENTS

The authors are grateful to CNPq, CAPES and JICA for the fellowships.

RESUMO

Triagem e isolamento de microrganismos produtores de substâncias anti-*Fusarium moniliforme* de solo e milho

Fusarium moniliforme, fitopatógeno cosmopolita de milho, é responsável pela produção de micotoxina recentemente descoberta, denominada fumonisina.

Table 2. Effect of crude extract concentrate of five selected antagonists on fumonisin producing *F. moniliforme*.

F. moniliforme	Fumonisins (mg/g)		Inhibition halo (mm) Antagonists				
Strains	FB1	FB2	M11	M28	M31	M33	M34
97I	35.82	33.39	25	22	23	25	42
164H	12.09	14.59	23	25	18	23	R
103A	32.31	25.17	30	24	R	23	R
118BR	23.53	25.49	25	21	R	24	R
104B	27.55	25.93	20	R	35	21	15
113B	29.49	24.28	26	35	17	15	R
113F	54.21	87.31	20	25	28	28	30
118F	37.93	39.64	23	26	22	22	35
119E	37.10	29.00	23	25	R	R	30
118A	39.11	43.73	R	22	20	25	25

R: Resistant
halo diameter: 10 mm

Com a finalidade de avaliar a inibição de *F. moniliforme*, 34 antagonistas isolados de 60 amostras de solo e de 20 amostras de milho foram testados contra *F. moniliforme* 113F, produtor de fumonisina. O extrato bruto foi preparado com a cultura de microrganismos selecionados em caldo infuso de cérebro e coração (BHI) e concentrados, adicionando-se etanol na proporção 1:1. A presença de organismos inibidores de *F. moniliforme* ocorreu em 29 amostras de solo, obtendo-se 36 microrganismos antagonistas. Referente ao milho, 15 amostras apresentaram microrganismos inibidores, permitindo o isolamento de 15 antagonistas. A caracterização destes 51 isolados demonstrou que 5 consistiram de leveduras, 3 cocos Gram-positivos, 3 cocos Gram-negativos e 40 bacilos Gram-positivos, destacando-se a predominância do último grupo. Todos os 51 isolados apresentaram atividade inibidora sobre *F. moniliforme* 113F, empregando-se o método de cultura íntegra. Entretanto, a análise de sobrenadante de cultura, pelo antibiograma detectou apenas 34 linhagens positivas. A variabilidade na sensibilidade entre as linhagens foi testada, empregando-se 10 *F. moniliforme* toxigênicos, frente a 5 antagonistas selecionados. Considerando as características extracelulares destes princípios ativos, a análise dos respectivos extratos brutos concentrados, selecionou 2 cocos Gram-positivos e 3 bacilos Gram-positivos esporulados, com boas perspectivas na obtenção de produtos bioativos, para o controle de *F. moniliforme*.

Palavras-chave: *F. moniliforme*, antagonistas, antifúngico, controle biológico, fumonisinas.

REFERENCES

1. Abdel-Bar, N.; Marris, N.D.; Rill, R.L. Purification and properties of an antimicrobial substance produced by *Lactobacillus bulgaricus*. *J. Food Sci.*, 42:411-415, 1987.
2. Bacon, C.W.; Marijanovic, D.R.; Norred, W.P.; Hinton, D.M. Producing of fusarin C on cereal and soybean by *Fusarium moniliforme*. *Appl. Environ. Microbiol.*, 55:2745-2748, 1989.
3. Baker, C.J.; Stavely, J.R.; Thomas, C.A.; Sasser, M.; Macfall, J.S. Inhibitory effect of *Bacillus subtilis* on *Uramyces phaseoli* and on development of rust pustules on bean leaves. *Phytopathol.*, 73:1148-1152, 1983.
4. Bettiol, W. Controle biológico de doenças de plantas. Brasília: Embrapa, 1991.
5. Bettiol, W.; Auer, C.G.; Camargo, L.E.A.; Kimati, H. Controle da mancha foliar de *Eucalyptus grandis* e *E. verophylla* induzida por *Cylindrocladium scoparium* com *Bacillus* sp. *Sum. Phytopathol.*, 14:210-218, 1988.
6. Bullerman, L.B.; Draughon, F.A. *Fusarium moniliforme* and fumonisin symposium. *J. Food Protect.*, 57:523, 1994.
7. Gelderblom, W.C.A.; Jaskiewicz, K.; Marasas, W.F.O.; Thiel, P.G.; Horak, R.M.; Vleggaar, R.; Kriek, N.P.J. Fumonisins: novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl. Environ. Microbiol.*, 54:1806-1811, 1988.
8. Hirooka, E.Y.; Yamaguchi, M.M.; Aoyama, S.; Sugiura, Y.; Ueno, Y. Natural occurrence of fumonisins in Brazilian corn Kernels. *Food Addit. Contam.*, 13:173-183,1996.
9. Kimura, N.; Hirano, S. Inhibitory strain of *Bacillus subtilis* for growth and aflatoxin-production of aflatoxigenic fungi. *Agric. Biol. Chem.*, 52:1173-1179, 1988.
10. Kobayashi, Y.; Ko, K.; Yamaguchi, I. A new antibiotic, tautomycetin. *J. Antib.*, 42:141-144, 1988.
11. McKeen, C.D.; Reilly, C.C.; Pusey, P.L. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola from Bacillus subtilis*. *Ecol. Epidem.*, 76:136-139, 1986.
12. Nelson, P.E.; Toussoun, T.A.; Marasas, W.F.O. *Fusarium* species - An illustrated manual for identification. *The Pennsylvania State University Press*, Pennsilvania, 1983. 193p.
13. Norred, W.P.; Bacon, C.W.; Plattner, R.D.; Vesonder, R.F. Differential cytotoxicity and mycotoxin content among isolates of *Fusarium moniliforme*. *Mycopathol.*, 115:37-43, 1991.
14. Omura, S.; Tanaka, Y.; Hisatome, K.; Miura, S.; Takahashi, Y.; Nakagawa, A. Phthoronycin, a new antibiotic active against a plant pathogen, *Phytophthora* sp. *J. Antib.*, 41:1910-1912, 1988.
15. Ono, M.; Kimura, N. Antifungal peptides produced by *Bacillus subtilis* for the biological control of aflatoxin contamination. *Proc. Jpn. Assoc. Mycotoxicol.*, 34:23-28, 1991.
16. Park, D.L.; Rua, Jr. S.M.; Mirocha, C.J. Abd-Alla, E.A.M.; Weng, C.Y. Mutagenic potentials of fumonisins contaminated corn following ammonic decontamination procedure. *Mycopathol.*, 117:105-108, 1992.
17. Phae, C.G.; Sasaki, M.; Shoda, M.; Kubota, H. Characteristics of *Bacillus subtilis* isolated from composts supressing phytopathogenic microorganisms. *Soil Sci. Plant Nutr.*, 36:576-586, 1990.
18. Ray, L.L.; Bullerman, L.B. Preventing growth of potentially toxic moulds using antifungal agents. *J. Food Protect.*, 45:953-963, 1982.
19. Rose, A.H. Production and industrial importance of secondary products of metabolism. In: Rose, A.H. (ed.). Secondary Products of Metabolism. *Economic Microbiology*. Academic, v.3, New York, 1979, p.1-33.
20. Silo-Suh, L.A.; Lethbridge, B.J.; Raffel, S.J.; He, H.; Clardy, J.; Handelsman, J. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW 85. *Appl. Environ. Microbiol.*, 60:2023-2030, 1994.
21. Sneath, P.M.A. Endospore-forming Gram-positive rods and cocci. In: Sneath, P.H.A.; Mair, N.S.; Smarpe, M.E.; Holt, J.G. (eds). *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins, v.2, Baltimore,1988, p.1104-1207.
22. Sydenham, E.W.; Shephard, G.S.; Thiel, P.G.; Marasas, W.F.O.; Stockenström, S. Fumonisin contamination of corn-based human foodstuffs. *J. Agric. Food Chem.*, 39:2014-2018, 1991.
23. Turner, J.T.; Backman, P.A. Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. *Plant Dis.*, 75:347-353, 1991.
24. Ueno, Y.; Aoyama, S.; Sugiura, Y.; Wang, D.S. Lee, U.S.; Hirooka, E.Y.; Hara, S.; Karki, T.; Chen, G.; Yu, S.Y. A limited survey of fumonisins in corn and corn-based products in Asia Countries. *Mycot. Res.*, 9:27-34, 1993.
25. Vining, L.C. Functions of secondary metabolites. In: Balows, A.; Greenberg, P.G. (eds.). *Ann. Rev. Microbiol. Ann. Reviews Inc.*, v.44, California, 1990, p. 395-427.
26. Walker, G.M.; Mcleod, A.H.; Hodgson, V.J. Interactions between killer yeast and pathogenic fungi. *FEMS Microbiol. Let., 127:213-222, 1995.*

Revista de Microbiologia (1996) 27:218-222
ISSN 0001-3714

REMOVAL OF GOLD, SILVER AND COPPER BY LIVING AND NONLIVING FUNGI FROM LEACH LIQUOR OBTAINED FROM THE GOLD MINING INDUSTRY

Newton Carlos Marcial Gomes[1,2]; Valter Roberto Linardi[2]*

[1]Laboratório de Taxonomia e Ecologia Microbiana, Departamento de Microbiologia, IM, CCS, UFRJ, Rio de Janeiro, RJ, Brasil; [2]Laboratório de Fermentação, Departamento de Microbiologia, ICB, UFMG, Belo Horizonte, MG, Brasil.

ABSTRACT

Strains of *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger* isolated from samples obtained from the gold extraction plant of "Mineração Morro Velho" (Nova Lima, Brazil) were tested for removal of heavy metals in a solution containing cyanide obtained from the gold extraction plant (leach liquor). The ability of these fungi to remove gold, silver and copper through metabolism-independent processes (dried biomass) and after growth in leach liquor was investigated. The dried fungal biomass resulted in weak biosorbents, a fact probably due to the high cyanide concentration in leach liquor, whereas metabolically active cells of *A. fumigatus* and *A. niger* presented greater ability to remove metals. The metabolic activity should be considered for the removal of heavy metals from cyanide-containing solutions obtained from the gold mining industry.

Key words: *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, biosorption, cyanide, heavy metals.

INTRODUCTION

Some fungi are found in aquatic environments contaminated with high metal concentrations and may present tolerance to and ability to accumulate metal ions (2). Fungi may accumulate metals by biosorption, a process through which metals are rapidly accumulated via physico-chemical mechanisms that do not depend on metabolism, and by bioaccumulation that is a slower metabolic process and may result in more accumulation than biosorption (3). These metal-accumulative processes by microorganisms are of current industrial interest, mainly because they may provide the development of bioprocesses for the reduction of toxic metal levels or even for the recovery of valuable metals from liquid wastes (10).

Various studies have proved that biosorbents have efficient and potent capacity of accumulation of metals present in synthetic solutions (11). However, relatively little is known about biosorption of metals in real solutions (1, 5). Solutions originating from industrial activity are not simple and homogeneous metal solutions, but usually include various ions and organic and inorganic ligands. Cyanide is a strong ligand of metals and solutions containing this substance are commonly used in the extraction of gold from ores. These solutions contain various anionic cyano-metallic complexes and sulfur and arsenic compounds after ore treatment (leach liquor) (9).

In this study we evaluated the ability of three species of *Aspergillus*, isolated from a gold extraction plant, to remove gold, silver and copper from leach liquor in the form of dried biomass (metabolism-independent process - biosorption) and living fungi (after growth in the leach liquor). This strain of *Aspergillus niger* has been previously

* Corresponding author. Mailing address: Laboratório de Fermentação, Departamento de Microbiologia, ICB UFMG, Caixa Postal: 486, CEP 31270-970, Belo Horizonte, MG, Brasil. Fax number: (55 31 441-1412).

investigated by Gomes (4). In the present study it was tested for biosorptive ability and compared with the other two fungi in the experiments that evaluated the ability of the fungi to remove metals after growth in the leach liquor.

MATERIALS AND METHODS

Microorganisms. The fungal strains were isolated from samples obtained from a gold extraction plant as described previously (4). The strains were cultivated at 30°C and maintained at 4°C on modified Sabouraud agar medium (0.5 % yeast extract, 1 % peptone, 2 % glucose and 2 % agar).

Leach liquor. The leach liquor sample used in all experiments was obtained from the gold extraction plant of "Mineração Morro Velho" (Nova Lima, Brazil) which showed the following composition: 198 mg/l CN^- ,as total cyanide; 2.76 mg/l Au; 0.63 mg/l Ag; 25.0 mg/l Cu; 23.0 mg/l Fe and 1.68 mg/l Zn. All experiments used a solution of leach liquor originating from the same sample.

Metal removal by fungi after growth in the leach liquor. A spore suspension of the fungi was inoculated in 50 ml of filter-sterilized leach liquor enriched with 0.5% peptone and 1% glucose (LLE medium). The fungi were incubated in 250 ml shaker flasks at an inoculum level of 1.5×10^4 spores ml^{-1}, for 84 hours under constant shaking (80 rpm) at 30°C (4). After this period the fungal biomass was filtered using 0.45 µm Millipore filter membrane and dry weight was determined. The pH and concentration of metals (Ag, Au and Cu) were measured in the supernatant. Controls consisted of the incubation of LLE medium without fungi to determine the removal of metals by abiotic factors. The control (uninoculated LLE medium) was acidified by the addition of 1N HCl and filtered after 12 hours of incubation, and metals were determined in the supernatant. The amount of metals removed was determined by atomic absorption spectrophotometry with a Varian air-acetylene flame model AA-475 and considered as the difference between the initial concentration and the concentration of metals after fungal growth.

Metal biosorption by dried biomass. The biomass for biosorption experiments was obtained by growing the fungi in modified Sabouraud broth for 72 hours under constant shaking (80 rpm) at 30°C, the resulting growth being harvested by filtration through Whatman # 42 filter paper. Fungal biomass samples were washed three times with distilled deionized water, dried at 60°C to constant weight and ground with a mortar and pestile prior to determination of metal biosorption. Activated carbon was tested for comparison with the fungal biosorbents. Experiments were done using 0.05g of fungal biomass or activated carbon powder in 50 ml of leach liquor at different pH values (4.0, 6.0, 8.0 and 10). The contact of the sorbents and metals in solution was performed in 250 ml Erlenmeyers flasks for 3 hours under constant shaking (160 rpm) at 30°C. The pH was controlled by the addition of 1N HCl or 1N NaOH until stabilization. At the end of the incubation time the sorbents were passed through a 0.45 µm Millipore filter membrane and the supernatant was analyzed by atomic absorption spectrophotometry. Controls consisted of the incubation of leach liquor without sorbents under the conditions described above. The amount of metal removed was determined as the difference between the initial concentration and the concentration of metals after contact with the sorbent. The glassware used in the experiments was washed with detergent (Alconox-INLAB) and 3N HNO_3 and rinsed several times with distilled deionized water prior to use to avoid contamination with metals.

RESULTS AND DISCUSSION

The fungi tested for the ability to grow and remove metals from a solution containing cyanide and metals enriched with nutrients (LLE) presented distinct characteristics, in terms of growth, medium acidification and metal removal (Table 1). *Aspergillus fumigatus* presented highest growth (2.7 mg dry $wt.ml^{-1}$) and removed 35% of gold, 67% of silver and 87% of copper. *Aspergillus niger* presented the lowest growth (0.42 mg dry $wt.ml^{-1}$), promoted the most intense acidification of the medium and was the most efficient in terms of gold and silver removal (37% and 100% respectively). The ability of *A. niger* to remove metals from leach liquor during growth has been previously characterized and the present results were similar to those described by Gomes (4). *Aspergillus flavus* grew twice as much as *A. niger*, but presented a lower removal of gold and silver, about 1% and 3% respectively, and removed about 62% of copper present in LLE medium. Most of the copper removal from LLE medium was probably due to the acidification caused by fungal growth. Due to the presence of cyanide the metals form cyano-metallic complexes in the leach liquor. Cyano-metallic complexes become less soluble as the acidity of the

Table 1 - Fungal growth, medium pH and percentage of gold, silver and copper removed by *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger* after 84 hours of incubation in leach liquor.

Strains	Growth (mg dry wt.ml^{-1})	pH (Culture medium)	Au (%) (IC = 2.76 mg.L^{-1})	Ag (%) (IC = 0.63mg.L^{-1})	Cu (%) (IC = 25 mg.L^{-1})
A. flavus	0.85	5.20	1.50	3.18	62.18
A. fumigatus	2.70	4.53	35.45	66.67	86.91
A. niger	0.42	3.60	36.97	100	81.49
Control 1	–	7.90	–	1.00	26.08
Control 2	–	5.00	–	0.80	40.00
Control 3	–	3.5	–	1.00	45.20

Controls (1, 2 e 3) - Correspond to fungus-free LLE medium to determine the removal of metals by pH acidification.
(IC) - Corresponds to the initial concentration of the metal in the medium.
(–) - Absence of growth or metal removal.
The values are means of duplicate experiments.

medium increases, differing from the usual behaviour of metallic cations in solution (9). Almost 45 % of copper was removed due to precipitation after acidification of the uninoculated LLE medium (Table 1). Therefore, the acidification caused by fungi in LLE medium may have resulted in the precipitation of copper at the same rate as observed in the uninoculated LLE.

In the biosorption experiments the amount of copper removed by dried biomass at pH 4.0, 6.0 and 8.0 (Fig. 1 A, B, C), was similar to the amount of copper removed in the control flasks (without biomass) (Fig. 1 E). Probably, the copper was removed by precipitation at this pH range. At pH 10 the concentration of copper in control flasks did not change and the biomasses of *A. flavus*, *A. fumigatus* and *A. niger* were responsible for the removal of 9, 11 and 15 % of the metal, respectively. In control experiments gold and silver were the most stable metals and their removal due to precipitation or adsorption to glass was lower than 1.5%. About 20% of silver was accumulated by dried biomass of *A. flavus* at pH 10, and the biomass of *A. niger* accumulated 11.1 % at pH 8.0 and 10.8% at pH 10. The dried biomass of all the fungi tested presented biosorptive ability at pH 10 and the biomass of *A. niger* also removed silver at pH 8.0. *Aspergillus flavus* was the only strain that removed gold by biosorption (about 4% at pH 10). The pH can affect the charges of metal complexes and the adsorbent surface, being one of the main components regulating the biosorption of metals. The dependence on pH for metal removal by dried mycelia in the leach liquor may have occurred due to electrostatic interactions between the fungal surface and the cyano-metallic complexes.

The pH also affected the removal of copper and silver by activated carbon. Copper removal was lower and silver removal increased when the pH rose from 4.0 to 10.0. Gold was efficiently removed from leach liquor at all pH values tested. These results are in agreement with the literature (6, 7). The removal of copper by activated carbon was higher than in control experiments at all pH values tested (Fig. 1 D). The removal of gold, silver and copper by the dried biomass of all fungi studied was lower than that of activated carbon at all pH values tested.

Among the fungi tested, *A. niger* had been previously evaluated for biosorbent ability in synthetic solutions. Kuyucak and Volesky (5) reported that *A. niger* biomass had the ability to accumulate approximately 170 mg gold per g biomass. Mullen *et al.* (8) observed that *A. niger* biomass was able to remove approximately 90% of silver in a solution containing 0.1 mM of the metal. However, even though the ability of *A. niger* to remove gold and silver was proved by these investigators, the results obtained here with this fungal species were divergent. This was due to the fact that we evaluated the biosorbent ability of *A. niger* biomass in a real solution originating from a gold extraction plant which contained other metals and complex-forming agents that commonly interfere with metal biosorption removal.

In the present study the dried biomass of the fungal strains investigated presented a low capacity to biosorbe metals from leach liquor, which was much lower than the capacity of activated carbon. The living fungi *A. niger* and *A. fumigatus* accumulated more metals after growth in leach liquor than when they were metabolically inactive. This may be explained by the ability of living fungi to exhibit different mechanisms of resistance which are induced by metal ions or intrinsic properties that could enable them to accumulate free or complexed metals. The removal of metals in industrial solutions using technologies based

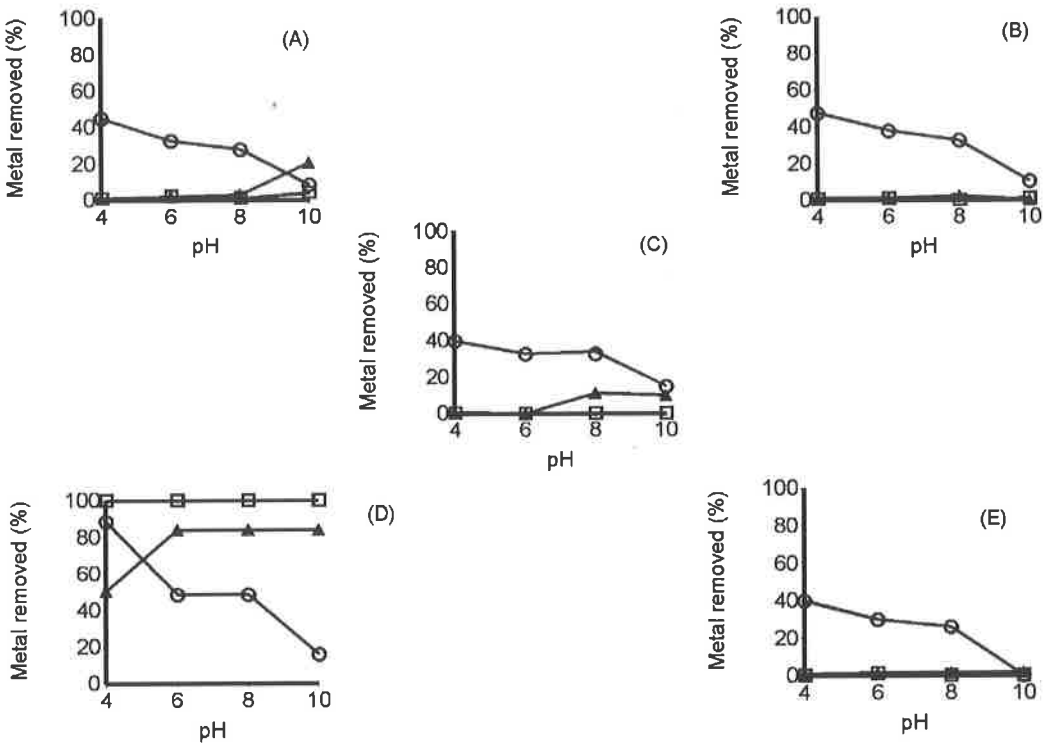

Figure 1 - Gold (❑ IC = 2.76 mg.L^{-1}), silver (▲ IC = 0.63 mg.L^{-1}) and copper (○ IC = 25 mg.L^{-1}) removal from leach liquor at different pH values by dried biomass of *Aspergillus flavus* (A), *Aspergillus fumigatus* (B), *Aspergillus niger* (C), activated carbon (D) and in control (without biomass) (E). IC corresponds to the initial concentration of the metal in solution. The values are the means of duplicate experiments.

on the action of microorganisms provides an alternative or an additional method for the removal or recovery of these elements. However, tests using real solutions and evaluation of the advantages and disavantages of the use of live or dead microorganisms are an absolute necessity.

ACKNOWLEDGMENTS

We thank the "Mineração Morro Velho" for the laboratory and field facilities. This work was supported by CNPq, FAPEMIG and PRPq/UFMG. We thank Dr. Paula B. Morais, ICB UFMG, for suggestions to the manuscript.

RESUMO

Remoção de ouro, prata e cobre por fungos viáveis e inviáveis em um liquor de lixiviação obtido da indústria de mineração de ouro.

Três linhagens de fungos, identificadas como *Aspergillus flavus*, *Aspergillus fumigatus* e *Aspergillus niger*, isoladas de amostras da planta de extração de ouro da Mineração Morro Velho (Nova Lima, Brasil), foram testadas quanto à capacidade de remover metais pesados em uma solução obtida do circuito de beneficiamento de minério de ouro da mineração (liquor de lixiviação). Foi investida a habilidade desses fungos em remover ouro, prata e cobre via processos independentes do metabolismo (biomassa seca) e células cultivadas no liquor de lixiviação. A biomassa seca das três linhagens estudadas apresentou uma baixa capacidade de bissorção de metais, provavelmente devido a elevada concentração de cianeto no liquor de lixiviação. Os fungos *A. fumigatus* e *A. niger*, quando metabolicamente ativos, apresentaram uma elevada habilidade de remoção desses metais. A presença de atividade metabólica nas células fúngicas foi considerada como um fator importante para a remoção de metais pesados de soluções contendo cianeto provenientes da indústria de mineração do ouro.

Palavras-chave: *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, biossorção, cianeto, metais preciosos.

REFERENCES

1. Brierley, J.A.; Vance, D.B. Recovery of precious metals by microbial biomass. *In*: Norris, P.R.; Kelly, D.P. (eds). *Biohydrometallurgy symposium* Warwick, England, 1988, p.477-485.
2. Gadd, G.M. Fungal responses towards heavy metals, *In*: Herbert, R.A.; Codd, G.A. (eds). *Microbes in extreme environments*. Academic. Press, London, 1986, p.83-110.
3. Gadd, G.M. Heavy metal accumulation by bacteria and other microorganisms. *Experientia.*, 46:834-840, 1990.
4. Gomes, N.C.M. Acumulação e recuperação de metais por uma linhagem de *Aspergillus niger* em solução cianetada de uma mineração de ouro. Belo Horizonte, 1995, 72p. (M.Sc. Dissertation. Instituto de Ciências Biológicas. UFMG).
5. Kuyucak, N.; Volesky, B. New algal biosorbent for a gold recovery process. *In*: Norris, P.R.; Kelly, D.P. (eds). *Biohydrometallurgy symposium*. Warwick, England, 1988, p.453-463.
6. McDougall, G.J.; Hancock, R.D. Activated carbons and gold - a literature survey. *Minerals Sci. Engng.*, 12: 85-99, 1980.
7. Merello, R.P.; Zárate, G.C. Aplication de carbon activado en la metalurgia extractiva del oro. *Minerales.*, 42: 25-38, 1987.
8. Mullen, M.D.; Wolf, D.C.; Beveridge, T.J.; Bailey, G.W. Sorption of heavy metals by the soil fungi *Aspergillus niger* and *Mucor rouxii*. *Soil Biol. Biochem.*, 24: 129-135, 1992.
9. Osseo-Asare, K.; Xue, T.; Ciminelli, V.S.T. Solution chemistry of cyanide leaching systems. *In*: Kudryk, V.; Corrigan, D.A.; Liang, W.W. (eds). *Precious metals: mining, extraction, and processing. Proceedings of an international symposium.* The Metallurgical Society of AIME, Los Angeles, 1984 p.173-197.
10. Tobin, J.M.; White, C.; Gadd, G.M. Metal accumulation by fungi: applications in environmental biotechnology. *J. Ind. Microbiol.*, 13: 126-130, 1994.
11. Volesky, B. Removal and recovery of heavy metals by biosorption. *In*: Volesky, B. (ed). *Biosorption of Heavy Metals*. CRC Press, Boca Raton, 1990, p.7-43.

Revista de Microbiologia (1996) 27:223-227
ISSN 0001-3714

A THEORETICAL MODEL DESCRIBING THE INFLUENCE OF DIFFUSION ON MICROBICIDAL ACTION IN BIOFILMS

Peter M. Gaylarde, Christine C. Gaylarde*

Departamento de Solos, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre - RS.

ABSTRACT

One of the standard methods for the determination of microbicidal activity is to measure the rate of suspended (planktonic) and/or sessile cell death. The role of diffusion within the biofilm on the kinetics of sessile cell death is generally not considered. It is known that, in the majority of cases, sessile cells are more resistant to microbicidal substances than are planktonic cells. The reasons for this increased resistance have been considered to be altered cell physiology or failure of the microbicide to penetrate the biofilms. A model is presented which demonstrates how the rate of diffusion of microbicides through a biofilm influences the rate of cell death. The calculations may be performed using a simple spreadsheet package. The results show a biofilm will decrease the rate of cell death when microbial physiology in the biofilm is unaltered, especially where the concentration exponent of the microbicide is high. Using this model, the microbicide concentrations required to kill equivalent numbers of sessile and planktonic cells in the same physiological state in a given time may be estimated if diffusion rates are known. The effects of altered cell metabolism may be considered only once the effect of diffusional time-lag on the rate of cell death within the biofilm is known.

Key words: Biocide; biofilm; microbicide; model; diffusion

INTRODUCTION

Increasingly, the scientific literature in the areas of medical, food and industrial microbiology has reported the problems concerned with microbial biofilms (21). These assemblages of cells, which form on any surface in a biologically active environment, may be the cause of failure of antibiotic treatment (e.g. in infections in the lungs (19) and on the surfaces of implanted prostheses (2)), corrosion of metal structures in industry (13) and tooth decay (15). Biofilms are also important in other industrial problems such as blocked pipes and filters (25) and in transfer of microbial contaminants via food preparation equipment (3, 18). In addition they may act as reservoirs of infection in clinical, industrial and domestic environments (5, 17, 21).

It is well known that microorganisms present in biofilms are in some way protected from the action of antimicrobial agents (10, 16), either by lack of penetration of the chemicals through the polysaccharide matrix (6) and/or because of an altered cell sensitivity (11, 12). An accepted method for measuring microbicidal activity in the laboratory is to determine a time-kill relationship with respect to microbicide concentration. At any given temperature and microbicide concentration, the rate of cell death is usually an approximately first order process (20,22). The physiological state of the microorganisms is important and factors such as nutrients, ionic strength,

* Corresponding author. Mailing address: Departamento de Solos, Faculdade de Agronomia, UFRGS, Av. Bento Goncalves 7712, Porto Alegre - RS. CEP 90001-970. Tel. (051) 316 6026 Fax: (051) 316-6029 ou (051) 336 1211

pH and the age of the culture modify the rate of cell death (8, 11, 12). Microorganisms present in biofilms often show marked differences in their metabolism and metabolic rate compared to suspended (planktonic) organisms of the same strain and species grown in identical media (1) and this could affect their sensitivity to inhibitory and cidal agents (14).

The most common methods used to assay microbicides against sessile microorganisms determine minimum cidal concentrations (25), or reduction in cell numbers after a given time (17). In this type of assay, the notion of innate microbial resistance in a system in which diffusional time-lag must occur is nonsensical. The model presented here was developed in an attempt to determine the effect of microbicide diffusion through the biofilm on the rate of sessile cell death and hence to be able to assess the relevance of other factors, such as altered physiology, on cell death.

For the purpose of the development of the model, it has been assumed that the rate of cell death is dependent solely on microbicide concentration and is identical in both the suspended cells and the biofilm; that is, that the planktonic and sessile cells are equally sensitive to the microbicide. Only when the interaction between the kinetics of diffusion and cell death are known can one attempt to assess the possible effects of microbial physiology in biofilms in a meaningful manner.

MATERIALS AND METHODS

Description of the kinetic model. In the presence of many microbicides, the number of viable cells declines exponentially with time when temperature and concentration of the chemical agent remain constant. Diffusion rates of chemicals through a membrane (or biofilm) are normally proportional to the concentration gradient in the direction of the measured flux. Although both of these statements refer to phenomenological relationships, these processes are readily treated mathematically as having first order kinetics. The effct of microbicide concentration on the rate of cell death varies with the power of microbicide concentration. The exponent varies between 0.5 and 19 for different chemicals (2, 23).

In this model, the following assumptions are made:

- cell death is a first order process determined by microbicide concentration and a concentration exponent
- the diffusional resistance of the unstirred surface layer is both constant and small compared to the diffusional resistance of the biofilm
- the rate of cell death is proportional to microbicide concentration for cells both in the biofilm and in suspension
- the activity coefficient of the microbicide at any concentration is identical both in the biofilm and in solution and does not vary with concentration
- the half-life of the cells in the presence of microbicide ($Bt_{1/2}$) is one time unit when the concentration of the microbicide is unity.

This system can easily be modelled on many standard spreadsheets; these graphs were prepared and the calculations performed on a Lotus-123 package. Standard spreadsheet programs do not allow the use of all the memory available in most computers. This memory limitation does not allow the steps to be sufficiently small to avoid errors accumulating in repeated calculations, unless care is taken. To avoid such errors, the number of sequential steps must be limited, or small steps may be included and the move funcion may be used to complete a series of repeated calculations.

Spreadsheet calculations. The spreadsheet calculations are shown here. For those unused to spreadsheets, the details of all the column entries required (including column headings) are shown in the second paragraph of each instruction (beginning "**Enter**....").

1. Column A: Create a time series in column A (0, 0+dt, dt+dt....).
   Enter 'Time in [A;1], 0 in [A;5], then +A5+dt in [A;6] and use the copy control to enter this value in as many rows as desired.
2. Column B: Create a first order regression line {6-([A;n] x (log2))}, where 6 is the logarithm of the initial cell number, A is the column and n is the row number. This is the rate of cell death when the microbicide at unit concentration kills 50% of the cells in 1 time unit ($Bt_{1/2} = 1$ when $c = 1$).
   Enter 'log in [B;1], 'Cell No. in [B;2], 'Suspended in [B;3] and 6-A5*@LOG(2) in [B;5]. Copy [B;5] as many times as required.
3. Column C: Calulate how the microbicide concentration at any arbitrary point increases from $t = 0$ assuming a first order process. The diffusional half-life constant ($Dt_{1/2}$) may be any chosen value.
   Enter 'Concentration in [C;1] and (1-(10^(-A5*(@LOG(2)/z))))*c in [C;5] and copy to the other rows. z is the ratio $(Dt_{1/2})/(Bt_{1/2})$ and c

is the microbicide concentration relative to the selected concentration. Note, when entering formulae, there must be no gaps between characters.

N.B. In the model used here, ($Bt_{1/2}$) = 1, c = 1 and z = 100.

4. Column D: Using the value [C;n], calculate a value using the power exponent (x) for the chosen microbicide.

   Enter +C5^x in [D;5] and then copy to the other rows.

5a. Column E: Calculate the rate of cell death from the values in column D. Use the geometric mean of the values in [D;n] and [D;n-1], except when [D;n-1] = 0, when [D;n1/2] should be used.

5b. Calculate the remaining viable cell number at each time.

5c. Calculate the logarithm of the number of cells remaining at each time.

   Enter 'log in [E;1], 'Cell No in [E;2],'sessile in [E;3], 6 in [E;5],

   +E5-(((D5+D6)/2)*@LOG(2)) in [E;6],

   +E6-(((D6*D7)^0.5)*@LOG(2)) in [E;7], then copy [E;7] to the remaining rows.

Columns C and D may be combined and the copying may be carried out in all columns below row 7 simultaneously.

To display the results graphically against time, dt = 1 was selected here.

RESULTS AND DISCUSSION

Smith (22) shows the effect which diffusion time-lag of a biocide into cells has on the rate of cell death when the concentration exponent is unity. This, however, is a rare occurrence and this simplified model has not been used here.

Figs. 1 and 2 show the theoretical time-kill curves for two microbicides with very different kinetics. The examples chosen are a phenolic agent, such as might be used as a disinfectant, and a heavy metal of the type frequently used in industry or as a topical application in the clinical situation. The phenolic agent (Fig. 1) is assumed to have a concentration exponent of 6 and the heavy metal (Fig. 2) one of 0.7. This signifies that, for a twofold increase in concentration of each agent, the rate of cell death increases by 2^6 and $2^{0.7}$ respectively. These figures are in accord with those reported in the literature (2, 23).

For both microbicides, line A represents the time-kill curve for suspended cells at a nominal active agent concentration of 1 unit weight per unit volume and a $Bt_{1/2}$ value of 1 time unit. The other lines show the curves for sessile cells at various multiples of this microbicide concentration. The diffusion rate of the microbicide into the biofilm is assumed to be such that time taken to reach half maximum concentration ($Dt_{1/2}$) is 100 times that of the cell half-life ($Bt_{1/2}$). This ratio ($Dt_{1/2}$:$Bt_{1/2}$) has been exaggerated in order to show more clearly the effect of the diffusional lag. It is immediately obvious that for similar microbicide concentrations the reduction in cell numbers in the biofilm will never equal that in the suspension (line A will never cross the line representing sessile cell death for c = 1). Indeed, for the phenolic agent, the line for sessile cell death rate at c = 1 is almost horizontal, indicating that the cells within the biofilm are killed extremely slowly up to the time shown in Fig. 1. The slow initial rate of sessile cell death may be seen at all concentrations. Agents with a high concentration exponent, such as phenolics and penicillin (6, 7), will tend to show this effect, as their reduced concentration in the biofilm will affect their cidal action to a much greater extent than chemicals with a low concentration exponent, such as the heavy metal used in this example.

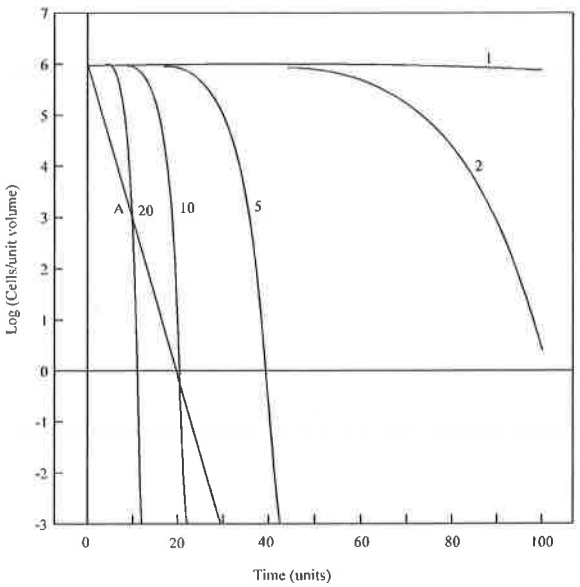

Figure 1. Logarithm of sessile cell numbers against time for a phenolic agent with a concentration exponent of 6. Cell half-life ($Bt_{1/2}$) is 1 time unit when microbiocide concentration is 1 unit weight/unit volume and time taken for the concentration in the biofilm to attain half the maximum value ($Dt_{1/2}$) is 100 time units. Line A represents death of suspended cells ($Dt_{1/2}$ = 0) at a concentration of unity. Numbers on the other lines indicate microbicide concentrations external to the biofilm.

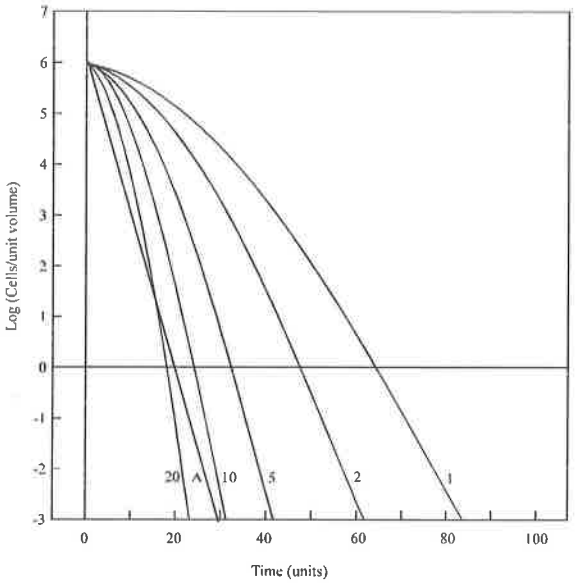

Figura 2. Logarithm of sessile cell numbers against time for a heavy metal agent with a concentration exponent of 0.7. Conditions and notations are the same as in Figure 1.

A 12-fold increase in microbicide concentration is required to produce a 10^6-fold reduction in sessile cells in the same time interval as for suspended cells when the $t_{1/2}$ value for diffusion within the biofilm ($Dt_{1/2}$) is 100 times the $t_{1/2}$ value for microbicide-induced cell death ($Bt_{1/2}$) and the concentration exponent is 6 (the phenolic agent). When the concentration exponent is 0.7 (the heavy metal), the equivalent increase in concentration is 17 times.

The effect of diffusion rate on the rate of cell death is complex, since the factors determining the rate of increase of the "free" microbicide in the biofilm (i.e. that available in the aqueous phase to act against microbial cells) are also complex. We have reported that the partition coefficient for the uptake of a simple biocide (a copper salt) by a model biofilm is concentration-dependent; we inferred that the biocide was held in the biofilm in three ways: by ionic forces, as a complex and in solution (9). With other microbicidal agents, hydrogen bonding, covalent bonding and Van der Waals forces may also be important. When the chemical produces aggregates or complexes in solution or in the biofilm, the diffusion constants for each of the chemical species produced will vary; formaldehyde and surfactants are two examples of substances that tend to have this property of auto-association. The rate of formation of these various complexes may be a simple first order process, or may be complex. Even when the concentration of a microbicide in a biofilm may be determined by experiment, much of it may be held in inactive forms. Most such chemicals are reactive and will bind to the abiotic components of a biofilm. Simple diffusion (if it is indeed simple) will act together with other rate constants to determine the "free" concentration of microbicide. These time-dependent processes will all ensure that the rate of cell death departs from a first order relationship. When diffusion or other factors cause the concentration of "free" microbicide to vary with time, a semi-logarithmic plot of cell number against time will not be linear. This departure from linearity shows that diffusional delays are an important factor in determining microbicide activity in biofilms. Until this aspect of the activity of antimicrobial agents on sessile organisms is better understood, any suggestion about the role of the physiological state of the sessile cells in resistance is unfounded.

Application of microbicides for short periods, even at high concentrations, will be ineffective if a sufficient cidal level acting for sufficient time is not attained in the biofilm. As shown in Fig. 1, when the agent has a high concentrtion exponent, no detectable microbicide-induced death will occur in the biofilm for a long period even though the concentration in the environment outside is many times that required to kill cells efficiently in suspension.

In practice, then, inhibitory or cidal substances that are unstable or which may penetrate biofilms slowly should be avoided as much as possible, especially if they have a high concentration exponent. Prolonged exposure and/or high concentrations of the chemicals are needed to treat organisms within biofilms.

RESUMO

Um modelo teórico que descreve a influência da difusão na ação de microbicidas em biofilmes

Um dos métodos padrões para avaliar a atividade de um microbicida é a medida da taxa de morte de células sésseis ou em suspensão (planctônicas). Sabe-se que, na maioria dos casos, células sésseis são mais resistentes às substâncias inibidoras do que as células planctônicas. Este aumento de resistência pode ser devido à mudança na fisiologia das células ou à falta de penetração do microbicida no biofilme. Um modelo que demonstra a influência da taxa de penetração de microbicidas sobre a morte das células

é apresentado. Os cálculos podem ser feitos por computador utilizando-se programas de planilhas eletrônicas. Os resultados mostram que o biofilme reduz a taxa de morte das células, quando a fisiologia microbiana não é alterada. Pelo modelo, pode-se estimar a concentração de microbicida necessária para matar tanto células sésseis quanto células em suspensão, no mesmo estado fisiológico, num tempo determinado, se as taxas de difusão forem conhecidas. Os efeitos de mudanças do metabolismo celular podem ser considerados somente quando o efeito da difusão sobre a taxa de morte for conhecido.

Palavras-chave: Biocida; biofilme; microbicida; modelo; difusão

REFERENCES

1. Anwar, H., Strap, J.L., Costerton, J.W. Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrob. Ags. Chemoth.*, 36: 1347-1351, 1992.
2. Bisno, A.L., Waldvogel, F.A. (eds.) *Infections associated with Indwelling Medical Devices*, ASM Press, Washington, D.C., 1989.
3. Chamberlain, A.H.L., Johal, S. Biofilms of meat processing surfaces. *In* Houghton, D.R., Smith, R.N. and Eggins, H.O.W. (eds.), *Biodeterioration 7*. Elsevier Appl. Sci., Barking, 1987, p. 57-61.
4. Chick, H. The theory of disinfection. *In A System of Bacteriology in Relation to Medicine, vol.1*, H.M. Stationery Office, London, 1930, p. 179.
5. Cook, P.E., Gaylarde, C.C. Microbial biofilms in the light engineering industry. *In* Denyer, S.P.and Gorman, S.P. (eds.), *Microbial Biofilms: Formation and Control*. Blackwell Scientific Publications, Oxford, 1993, p. 267-283.
6. Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J. Bacterial biofilms in nature and disease. *Ann. Rev. Microbiol.*, 41: 435-464, 1987.
7. Eagle, H., Musselman, A.D. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. *J. Exper. Med.*, 88: 99-101, 1948.
8. Evans, D.J., Brown, M.R.W., Alison, D.G., Gilbert, P. Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. *J. Antimicrob. Chemoth.*, 25: 585-591, 1991.
9. Gaylarde, P.M., Gaylarde, C.C. The kinetics of biocide uptake in a model biofilm. *Internat. Biodeter. Biodeg.*, 29: 273-283, 1992.
10. Gaylarde, C.C., Johnston, J.M. The effect of some environmental factors on biocide sensitivity in *Desulfovibrio*: implications for biocide testing. *In Microbial Corrosion*, The Metals Society, London, 1983, p. 91-97.
11. Gilbert, P., Brown, M.R.W. Cell-wall mediated changes in the sensitivity of *Bacillus megaterium* to chlorhexidine and 2-phenoxyethanol, associated with growth rate and nutrient limitation. *J. Appl. Bacteriol.*, 48: 223-230, 1980.
12. Gilbert, P., Collier, P.J., Brown, M.R.W. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy and stringent response. *Antimicrob. Ags. Chemoth.*, 34: 1865-1868, 1990.
13. Hamilton, W.A. Sulphate-reducing bacteria and the off-shore oil industry. *Trends in Biotechnol.*, 39: 195-217, 1983.
14. Hoyle, B.D., Jass, J., Costerton, J.W. The biofilm glycocalyx as a resistance factor. *J. Antimicrob. Chemoth.*, 26: 1-6, 1990.
15. Kolenbrander, P.E. Coaggregation of human oral bacteria: potential role in the accretion of dental plaque. *In* Quesnel, L.B., Gilbert, P. and Handley, P.S. (eds.), *Microbial Cell Envelopes: Interactions and Biofilms, J. Appl. Bacteriol.*, 74: 795-865, 1993.
16. Keevil, C.W., Mackerness, C.W., Colbourne, J.S. Biocide treatment of biofilms. *Internat. Biodeter. Biodeg.*, 26: 169-180, 1990.
17. Lee, J.V., West, A.A. Survival and growth of *Legionella* species in the environment. *In* Lund, B.M., Sussman, M. and Stringer, M.F. (eds.), *Enterobacteriaceae in the Environment, J. Appl. Bacteriol.*, 70: 1215-1305, 1991.
18. Mattila-Sandholm, T., Wirtanen, G. Biofilm formation in the industry: a review. *Food Rev. Internat.*, 8: 573-603, 1992.
19. May, T.B., Shinabarger, D., Maharaj, R. et al. Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. *Clin. Microbiol. Rev.*, 4: 191-206, 1991.
20. Meynell, G.G., Meynell, E. *Theory and Practice in Experimental Bacteriology*. Cambridge University Press, Cambridge, 1970.
21. Morton, L.H.G., Surman, S.B. The involvement of biofilms in biodeterioration processes. *In* Gaylarde, C.C., Sa, E.L.S., Gaylarde, P.M. (eds.) *LABS 2 - Biodegradation and Biodeterioration in Latin America*. MIRCEN, UNEP/UNESCO/ICRO, Porto Alegre, RS, 1996, p. 85-90.
22. Smith, R.N. Kinetics of biocide kill. *Internat. Biodeter. Biodeg.*, 26: 111-126, 1990.
23. Tilley, F.W. An experimental study of the relation between concentration of disinfectants and the time required for disinfection. *J. Bacteriol.*, 38: 499, 1939.
24. Whitham, T.S., Gilbert, P.D. Evaluation of a model biofilm for the ranking of biocide performance against sulphate-reducing bacteria. *J. Appl. Bacteriol.*, 75: 529-535, 1993.
25. Williams, E.E., Knox Holmes, B. Marine biofouling solutions for closed seawater systems. *Sea Technol.*, 30: 17-18, 1989.

Revista de Microbiologia (1996) 27:228-236
ISSN 0001-3714

MORPHOLOGICAL VARIABILITY OF *COLLETOTRICHUM GLOEOSPORIOIDES* ISOLATES FROM AVOCADO TREES FROM NORTHEASTERN BRAZIL

Maria Menezes[1]*, Richard T. Hanlin[2]

[1]Departamento de Agronomia, Universidade Federal Rural de Pernambuco, Recife, PE, Brasil. [2]Department of Plant Pathology, University of Georgia, Athens, GA, USA.

ABSTRACT

Colletotrichum gloeosporioides isolates obtained from avocado trees grown in Northeastern Brazil were studied in monoconidial cultures. Most isolates produced concentric rings of conidial masses on PDA in response to alternating fluorescent light and darkness. Some monoconidial cultures formed perithecia and low conidial production, whereas the others have given high conidial production without perithecia. In general, the sclerotia were small, black, and round in shape. Conidia were produced in succession from the tips of phialidic or non phialidic conidiophores or in some cases, from the tips of setae, which were straight, septate, dark, usually lighter at the apex All monoconidial isolates within or between each geographical area showed variation in relation to conidia and appressoria size. Probably, differences in size encountered to conidia of *C. gloeosporioides* isolates studied were due in part to the type production.

Key words: anthracnose, avocado, *Colletotrichum gloeosporioides,* cultural and morphological characterization.

INTRODUCTION

Colletotrichum gloeosporioides (Penz.) Sacc., teleomorph: *Glomerella cingulata* (Stonem.) Spauld and Schrenk, causal agent of the anthracnose diseases, occurs on numerous crops in tropical and subtropical regions, especially in warm, humid areas. The disease symptoms induced by the pathogen are characterized by necrotic leaf spots, blight and flower drop, fruit rot and fruit drop, cankers and dieback of the branches, and, in some cases, damping-off.

Avocado anthracnose is one of the most important diseases of this crop in many countries because it may cause postharvest fruit-rotting. According to Binyamini and Schffmann-Nadel (5), germinated spores of *C. gloeosporioides* persist on unripe fruits of avocado (*Persea gratissima* L.) through appressoria, but fungal development occurs only during fruit softening. Prusky *et al.* (20) have provided evidence that infection remains latent until the fruit ripens because of the presence of an inhibitory compound contained in unripe fruits. As fruits ripen the concentration of the compound decreases, allowing infection to proceed. The fungus then produces conidia arranged concentrically or scattered in dark-colored spots or in slightly sunken lesions. Under conditions favorable for sporulation the lesions are soon covered with masses of pink colored conidia which spread the disease. The pathogen survives and reproduces on many perenial plants in the same way (12).

The anthracnose symptoms in general are similar in all avocado hosts from different areas, but the causal agent varies in its cultural and morphological characters. Shear and Wood (22) studied isolates of *G. cingulata* from many plants collected over a wide geographical range and found extreme variability within as well as among isolates from a given host.

* Corresponding author. Mailing address: Área de Fitossanidade, Departamento de Agronomia, Universidade Federal Rural de Pernambuco, Av. Dom Manoel de Medeiros, s/n. Dois Irmãos, CEP 52171-900, Recife, PE, Brasil.

They referred isolates from 34 different hosts to *G. cingulata*. Arx (4) considered the *C. gloeosporioides* anamorph of *G. cingulata* as composed of a heterogeneous group of types which he separated into nine subspecific forms. Sutton (24) regarded the variation in *C. gloeosporioides* as so great that the species could not be effectively circumscribed. Andes and Keit (3) concluded that the genetic constitution of the fungus is the major factor responsible for the wide variability of *C. gloeosporioides* in vitro. Nelson (18) emphasized that the stability of morphological characters under variable environmental conditions is fundamental to taxonomic studies and the development of stable species concepts. Snyder (23) observed that genotypic and phenotypic variability must be taken into account in delimiting species. Such variability can be detected experimentally using the single-spore method of culturing isolates and comparing them.

The aims of the present study were to compare the isolates of *C. gloeosporioides* from avocado trees growing in different geographics areas of Northeastern Brazil, and to determine the variability based on morphological and cultural characteristics using monoconidial cultures. Such data should provide researchers in the region with greater confidence in identifying these pathogens to species.

MATERIALS AND METHODS

Sources of isolates. Plant material was collected on July, 1984 from six different geographical areas in five coastal states in Northeastern Brazil, and the isolates were obtained from avocado leaf spots showing typical symptoms of anthracnose diseases. Leaves were first washed with soap and water, then small pieces were removed from the margins of lesions and surface sterilized in 1.5% sodium hypochlorite solution, rinsed twice in sterile distilled water, and placed in Petri dishes containing potato dextrose agar (PDA). The material was incubated at room temperature (28°C), for five days and the fungal colonies that developed were transferred to test tubes for storage and further study, on August in the same year.

Cultural characteristics. Studies were conducted on cultural characteristics of the isolates on PDA. Pure cultures were grown on 13 ml medium in Petri dishes at 25°C, for 10 days, for inoculum. For each original isolate, 15 monoconidial subcultures were made, and replicated three times. A conidial suspension in 10 ml sterile water was adjusted to contain one to ten conidia under the low power (10x) microscope field (26). One ml of the conidial suspension was placed in the center of a solidified agar plate and spread with a sterile bent glass rod. The dishes were incubated for 24 hours in an inclined position at room temperature, then they were examined under a dissecting microscope. Small bits of agar contained a single conidium were transferred to the center of fresh PDA dishes, one conidium per dish. Monoconidial cultures were grown under a daily alternating 12 hr light/12 hr dark regime ("cool white" fluorescent light) for 15 days, at 25°C. For each monoconidial culture, colony color, presence of sclerotia, setae, perithecia, sector formation, and conidial production, were observed.

Sporulation. For conidial production studies, conidial suspensions were prepared from each monoconidial isolate by flooding 15-day-old cultures with 20 ml of sterile distilled water, and then scraping the agar surface with a soft brush. The conidial suspensions were filtered through two layers of cheesecloth, and concentrations were determined with an improved Neubauer hemacytometer (25, 27).

Morphological characteristics. Studies were made to determine the color, shape, and size of conidia, appressoria and other structures of *C. gloeosporioides* isolates, using monoconidial cultures.

Measurements of conidium size were made using samples of 50 units for each of the 90 monoconidial cultures. The conidial suspension was prepared as described above for the sporulation studies. Averages for length and width, and the size range of conidia were determined. Means of conidia between locations were compared by Duncan's multiple range test.

For appressoria studies, samples of 25 units for each of the 90 monoconidial cultures were made by the microculture technique, which consists of inoculating the four sides of a small square of agar ($1cm^2$) placed on a microscope slide and covering it with a coverslip. (24). This slide culture was maintained in a Petri dish moist chamber at room temperature, for 24-48 hours. After the incubation period, the size and shape of the appressoria were determined. Averages for length, width, and range were established and the means between locations were compared by Duncan's multiple range test.

RESULTS

Isolations made from avocado leaves showing typical lesions and symptoms of anthracnose from

different geographic areas of Northeastern Brazil consistently yielded cultures that fit the broad concept of *C. gloeosporioides* (4, 24). When cultured on PDA, the original isolates from different areas showed variation in their cultural and morphological characteristics. To further characterize these differences, a total of 90 monoconidial cultures were obtained from the original isolates and compared.

Cultural characteristics. In general, monoconidial cultures within the same original isolate showed similar behavior in relation to characters such as colony color, mycelial growth, and conidial distribution on the agar surface. However, variations among the original isolates were observed (Table 1).

Most monoconidial cultures were similar in appearance on PDA, forming a light gray colony, with a greenish tone, but the isolates from RN and SE were typically darker gray, also with a greenish tone. All of the isolates except those from AR-PB had a predominance of submersed mycelium. Variation in the color of the conidial masses also was noted among the monoconidial isolates. Those from AR-PB and PE

Table 1. Colony characteristics of avocado monoconidial isolates grown on PDA (Frequency in %).

Characteristics	AVO-isolates from					
	CE*	RN	AR-PB	BAN-PB	PE	SE
Colony color						
light-gray (greenish)	100	0	100	100	100	0
dark-gray (greenish)	0	100	0	0	0	100
Mycelial growth						
submersed	100	100	0	100	100	100
aerial	0	0	100	0	0	0
Conidial mass color						
orange	0	0	100	0	100	0
dark/orange	100	100	0	0	0	0
dark-cream	0	0	0	100	0	0
dark	0	0	0	0	0	100
Conidial distribution						
concentric rings	100	0	0	100	80	100
center of colony	0	100	100	0	20	0
Sclerotia	67	0	0	0	100	100
Sector formation	20	0	0	0	100	100
Setae	0	0	0	0	20	0
Perithecia	0	0	30	0	6	20

*CE = Ceara; RN = Rio Grande do Norte; AR-PB = Arei-Paraíba; BAN-PB = Bananeiras-Paraíba; PE = Pernambuco; SE = Sergipe

Table 2. Sporulation and size (µm) of conidia and appressoria of *Colletotrichum gloeosporioides* from avocado.

Isolate code[1]	Sporulation	Conidium				Appressorium			
		Length		Width		Length		Width	
	(x10^{5}con/ml)	range	mean	range	mean	range	mean	range	mean
AVO-CE	230	8.5-18.0	14.8b	3.5-5.0	4.0a	10.2-12.2	11.4a	5.5-7.3	6.3a
AVO-RN	24	9.0-16.0	14.3c	3.0-5.5	3.6b	9.6-11.7	10.6b	5.5-6.4	6.0b
AVO-AR-PB	25	12.0-20.0	15.7a	2.5-5.0	3.6b	9.4-11.4	10.4b	5.5-7.0	6.1b
AVO-BAN-PB	224	10.0-15.0	14.9b	3.0-5.0	3.4c	6.9-12.3	10.5b	5.7-8.0	6.6a
AVO-PE	182	10.0-18.0	15.1b	3.0-4.5	3.6b	9.1-11.0	10.1b	4.9-7.0	6.1b
AVO-SE	5	9.0-20.0	15.8a	3.0-5.0	3.9a	8.6-13.1	10.5b	5.6-8.2	6.1b

Means followed by the same letter in the same column do not differ significantly at P= 0.05, according to Duncan's multiple range test.
[1]From: Ceará (AVO-CE), Rio Grande do Norte (AVO-RN), Areia-Paraíba (AVO-AR-PB), Bananeira-Paraíba (AVO-BAN-PB), Pernambuco (AVO-PE), Sergipe (AVO-SE).

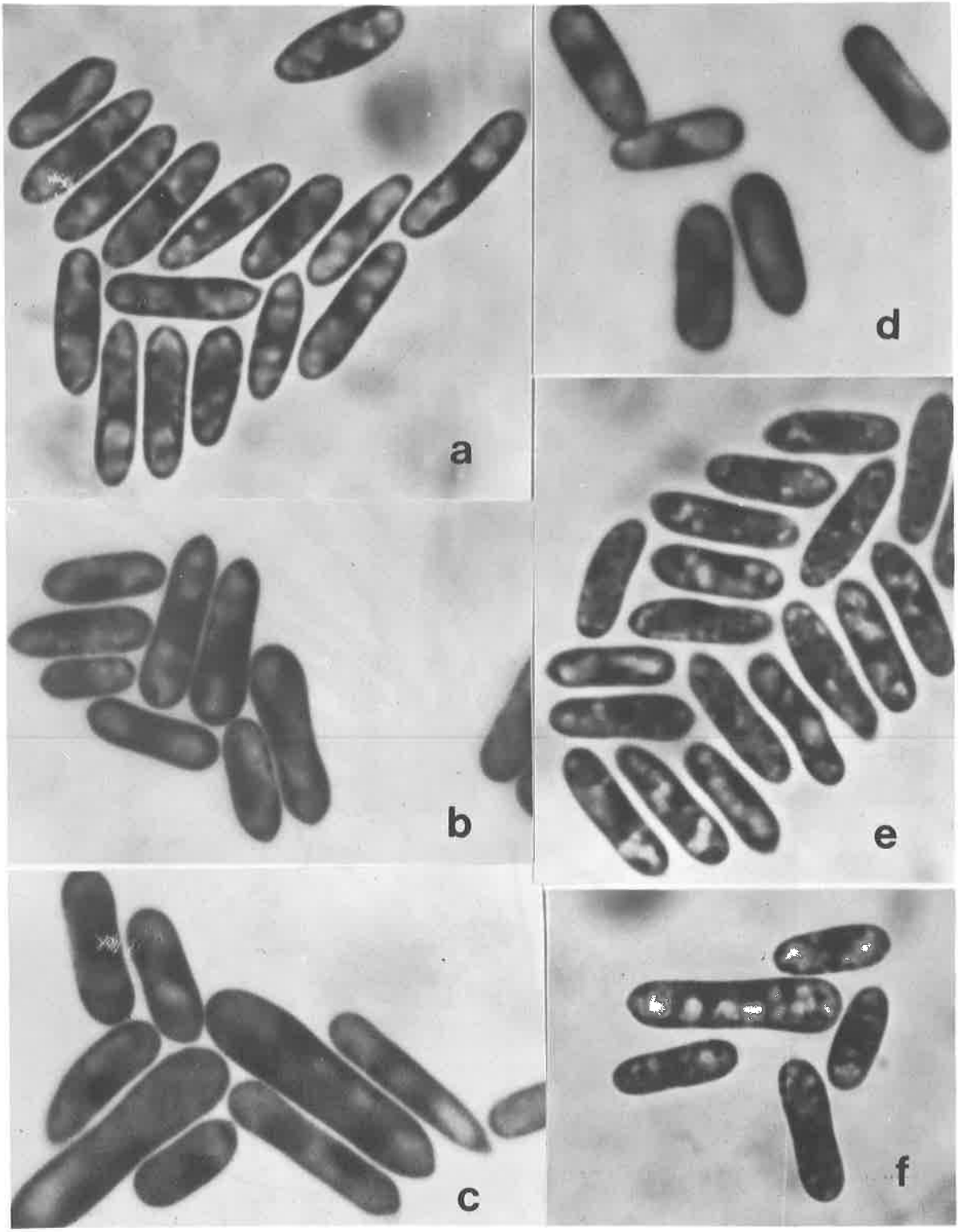

Figure 1. Conidia produced by *Colletotrichum gloeosporioides* isolates from avocado: **a.** AVO-CE, **b.** AVO-RN, **c.** AVO-AR-PB, **d.** AVO-BAN-PB, **e.** AVO-PE, **f.** AVO-SE.

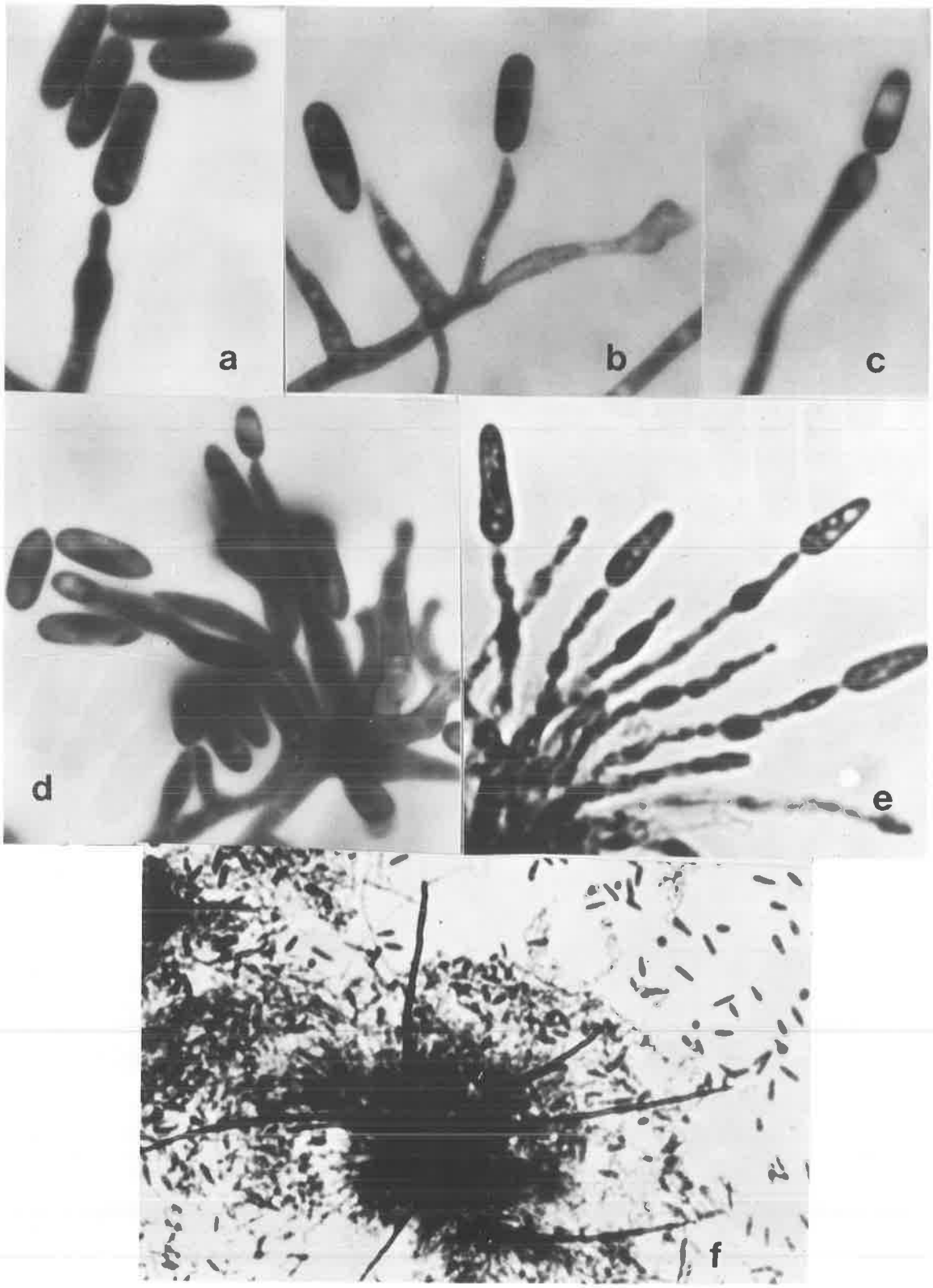

Figure 2. Conidia produced by *Colletotrichum gloeosporioides* isolates in phialidic conidiophores: **a.** AVO-CE, **b.** AVO-RN, **c.** AVO-AR-PB, **d.** AVO-BAN-PB, **e.** AVO-PE, **f.** AVO-SE.

were uniformly bright orange, whereas those from CE, RN, and BAN-PB, had alternating concentric rings of dark and orange or dark and cream color on the colony surface. Isolates from SE were uniformly dark in conidial color.

The monoconidial cultures from CE, PE, and SE isolates produced scattered black sclerotia. Generally, these were small, round and were either immersed in the agar or on the agar surface. Some sclerotia from PE isolate formed a tuft of setae on the top; these setae were dark, usually lighter at the apex, and ranged from non-septate when young to four-septate with age. Sector formation was observed in only a few monoconidial cultures from the CE and PE isolates. Most monoconidial isolates remained conidial; only a low percentage of those from AR-PB, PE, and SE formed perithecia. When formed, perithecia contained well developed asci and ascospores.

Sporulation. The results for conidial production showed variation between monoconidial cultures from the same original isolate, and also among isolates from different areas (Table 2). AVO-CE and AVO-BAN-PB exhibited the best sporulation, 230 x 10^5 and 224 x 10^5 conidia/ml of distiled water, respectively.

Morphological characteristics. ***Conidia*** - conidia produced from avocado isolates were straight, cylindrical to subcylindrical or oval shape, sometimes slightly narower in the middle, hyaline, unicellular, and variable in length (Fig. 1); often become bicellular

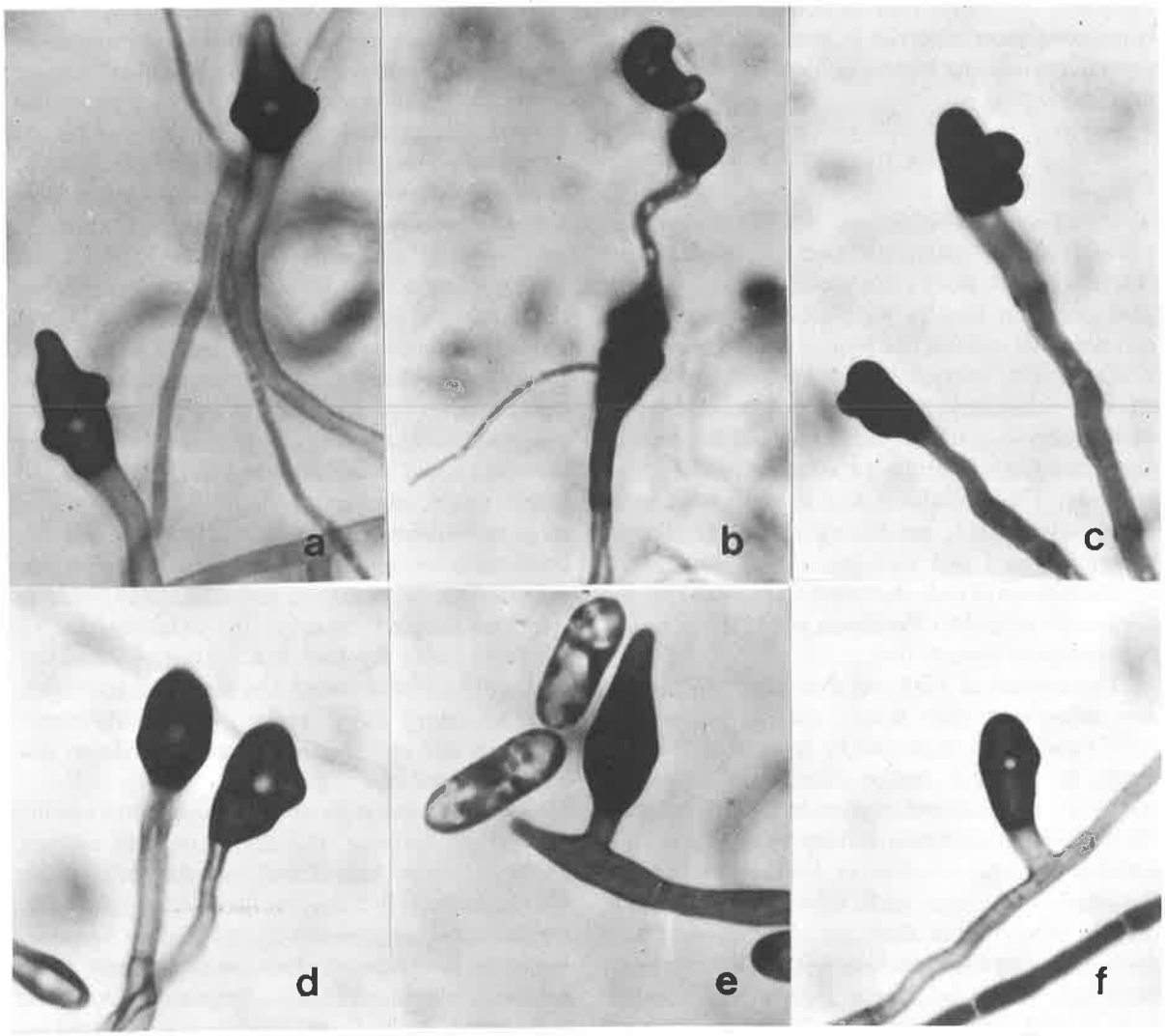

Figure 3. Appressoria shapes produced by *Colletotrichum gloeosporioides* isolates: **a.** AVO-CE, **b.** AVO-RN, **c.** AVO-AR-PB, **d.** AVO-BAN-PB, **e.** AVO-PE, **f.** AVO-SE.

when germinating. Conidia were produced in succession from phialidic conidiophores, on side branches of the mycelium, acervuli, or from the tips of setae (Fig. 2). The conidial dimensions are presented in Table 2. Among isolates, AVO-SE (15.8 x 3.9 μm) and AVO-AR-PB (15.7 x 3.6 μm) were significantly larger, compared with the others listed in the same Table.

Appressoria - all of the monoconidial cultures produced appressoria, which were irregular in shape and variable in size (Table 2), and the number formed. They varied from light brown to dark brown in color and were thick-walled, with a hyaline germpore. Appressoria were formed by germinating conidia, as well as on side branches of hyphae. Appressoria from mycelium were larger than those from conidia. Some appressoria were observed to germinate by forming germtube producing conidia or more frequently other appressoria (Fig. 3).

DISCUSSION

Under studied conditions, the isolates showed variations in cultural and morphological characteristics. Colony color varied from light-gray to dark-gray, but usually with greenish tone. Some monoconidial cultures that formed perithecia, such as AVO-AR-PB, AVO-SE, and AVO-PE presented colonies color darker with tendency to dark-green when observed against light. The two former isolates produced fewer conidia in relation to the other cultures. These isolates had a behavior like a homothallic fungi producing fertile perithecia containind asci and ascospores well developed, similar to those already described by Mordue (16) for *Glomerella cingulata*. Perithecia were formed singly or in groups of three to five.

The amount of PDA per Petri dish (13 ml), in association with other factors, such as temperature (25°C) and light conditions (12 hr light/12 hr dark) seems to increase the production of submerse mycelium, and to stimulate sporulation. The color of the conidial masses was influenced by light. The conidial masses, whether in concentric rings or dispersed on the agar surface, were at first bright orange, changing to dark orange with age, and developing a hard surface. Generally, dishes receiving more light had a brighter orange-colored conidial mass, than those that received less light due to shading, in which the conidial mass was darker-colored. Possibly, the germination of the outer conidia of that mass had ocurred, and by anastomosis of germ tubes a stroma was formed. This supposition is based on the fact that under microscope conidial anastomosis between two or more conidia was observed, resulting in structures similar to prosenchyma tissue. This phenomenon may increase the chance of major variability for *C. gloeosporioides*. According to Nelson (18) hybridization is an important biological process conditioning the variation in many fungi. Latham (14) observed different behavior among monoconidial isolates from apples, and the change of color in the conidial masses from orange to black with age.

In relation to size of conidia, significant differences among isolates were observed. Also, within the same monoconidial culture it was detected presence of some conidia larger than those commonly produced in colony, suggesting a natural genetic segregation in 3:1 proportion (three normal size to one longer). Chilton and Wheeler (7) reported on the effects of gene pairs producing a greater variety of cultural forms, but nothing was mentioned on conidia size. Cox and Irwin (8) compared conidia produced in conidiomata with those from free hyphae, and observed that conidia from hyphae were larger than those from conidiomata from the same isolate. Ogle et al. (18) observed that conidial size varied when formed by hyphal conidiophores, which gave origin to longer conidia (up to 42 μm) that never were observed from conidiomata. The production of conidia of different sizes was found in this study and that was due in part to their origin, whether from phialidic conidiophores, setae, or conidiomata. The form in which conidia are produced is very important for taxonomic studies. This may explain the variations and differences found in literature, particularly in relation to *C. gloeosporioides*. Agostini *et al.* (1) distinguished two strains of *C. gloeosporioides* by some characteristics, such as colony color, growth rate, morphology, conidium size and shape, appressorium shape, and also pathogenicity.

All of the cultures formed appressoria from conidia or hyphae, variable in size, shape, and amount produced. Those formed from conidia were smaller and more regular in shape than those from hyphae. The appressoria when germinating can produce new ones singly, or in sympodial chain, or in "bouquet" due proximity and fusion between appressoria. A similar fact was reported by Emmett and Parbery (10), and the importance of these structures was pointed out by Hasselbring (11), Kubo *et al.* (13), Muirhead and

Deverall (17). As is well known, appressoria formation is a prerequisite for invasion of host, and they possess capacity to survival under adverse environmental conditions. Denham and Waller (9), Prusky and Plumbley (21) observed that *C. gloeosporioides* produces appressoria and quiescent infections on tropical and subtropical crops. Also, Menezes and Hanlin (15) verified that appressoria may propagate *C. gloeosporioides* by germination producing new conidia from germtube apex. These findings are similar to those obtained by Agostini and Timmer (2) inoculating detached citrus leaves with *C. gloeosporioides* .

From the results herein reported on causal agent of avocado anthracnose, it was admitted that variations observed in size of *C. gloeosporioides* structures within each monoconidial culture from the same isolate should be due to genetic factors, as well the origen of conidia: from phialid, hyphae, setae, or conidiomata. Then, it is important to make measurements on conidia from structure that give relatively uniforme size to reduce variation. Probably, differences in size encountered to *C. gloeosporioides* isolates studied were due in part to the type prodution.

On the base of morphological analysis, the results suggest the need to use molecular markers, such as RAPD (Random Amplified Polymorphic DNA) for comparing morphological and molecular data obtained from avocado isolates, and to determine the differences and similarities among them.

RESUMO

Variabilidade morfológica de isolados de *Colletotrichum gloeosporioides* obtidos de abacateiros no nordeste do Brasil

Isolados de *Colletotrichum gloeosporioides* de abacateiros cultivados no Nordeste do Brasil foram estudados em culturas monoconidiais. A maioria dos isolados produziram massas de conídios distribuidas em anéis concêntricos na superfície do BDA em resposta a alternância de luz. Algumas culturas monoconidiais formaram peritécios, porém baixa produção de conídios, enquanto que outras produziram conídios em abundância, mas não formaram peritécios. Em geral, houve produção de esclerócios escuros, pequenos e de formato esférico. Os conídios foram produzidos em sucessão na extremidade de conidióforos fialídicos ou no ápice de setas. Estas, apresentaram-se retas, septadas, escuras, com ápice mais claro e, em alguns casos, férteis. Todos os isolados monoconidiais dentro de uma mesma área geográfica ou entre elas mostraram variação em relação a tamanho de conídios e apressórios. A diferença encontrada mais frequentemente, em relação a tamanho de conídios de *C. gloeosporioides*, pareceu estar muito associada ao tipo de produção.

Palavras-chave: antracnose, abacate, *Colletotrichum gloeosporioides*, caracterização cultural e morfológica.

REFERENCES

1. Agostini, J.P.; Timmer, L.W.; Mitchell, D.J. Morphological and pathological characteristics of strains of *Colletotrichum gloeosporioides* from citrus. *Phytopathol.*, 82: 1377-1382, 1992.
2. Agostini, J.P.; Timmer, L.W. Population dynamics and survival of strains of *Colletotrichum gloeosporioides* on citrus in Florida. *Phytopathol.*, 84: 420-425, 1994.
3. Andes, J.O.; Keitt, G.W. Variability of *Glomerella cingulata* (Stonem.) Spauld ad Schenk from apples. *Phytopathol.*, 40: 915-924, 1950.
4. Arx, J. A. von. Die Arten der Gattung *ColletotrIchum* Cda. *Phytopathol. Zeitsch.*, 29: 413-468, 1957.
5. Byniamini, N.; Schffmann-Nadel, M. Latent infection in avocado fruit due to *Colletotrichum gloeosporioides*. *Phytopathol.*, 62: 592-594, 1972.
6. Baxter, A.P.; Westhuizen, G.C.A. van der; Eicker, A. Morphology and taxonomy of South African isolates of *Colletotrichum*. S. Afr. *J. Bot.* 2: 259-289, 1983.
7. Chilton, S.J.P.; Wheeler, H.E. Genetics of *Glomerella*. VI. Linkage. *Amer. J. Bot.*, 36: 270-273, 1949.
8. Cox, M.L.; Irwin, J.A.G. Conidium and appressoria variation in Australian isolates of the *Colletotrichum gloeosporioides* group and closely related species. *Aust. Syst. Bot.* 1: 139-149, 1988.
9. Denham, T.G.; Waller, J.M. Some epidemiological aspects of postbloom fruit drop disease (*Colletotrichum gloeosporioides)* in citrus. *Ann. Appl. Biol.* 98: 65-77, 1981.
10. Emmett, R.W.; Parbery, D.G. Appressoria. *Ann. Rev. Phytopathol.*, 13: 147-167, 1975.
11. Hasselbring, H. The appressoria of the anthracnosis contributions from the Hull Bot. Labora-tory. *Bot. Gaz.*, 42: 129-142, 1906.
12. Jeffries, P.; Dodd, J.C.; Jeger, M.J.; Plumbley, R.A. The biology and control of *Colletotrichum* species on tropical fruit crops. *Plant Pathol.*, 39: 343-366, 1990.
13. Kubo, Y.; Suzuki, K.; Furusawa, I.; Ishida, N.; Yamamoto, M. Relation of appressorium pigmentation and penetration of nitrocellulose membranes. *Phytopathol.*, 72: 498-501, 1982
14. Latham, A.J.; Williams, J. Cultural characteristics and pathogenicity of *Glomerella cingulata* isolates of apples in Alabama. *Plant Dis.*, 57: 1065-1068, 1983.
15. Menezes, M.; Hanlin, R.T. Appressoria of Brazilian isolates of *Colletrotrichum gloeosporioides* causal agent of anthracnosis diseases. Fifth International Mycological Congress, Vancouver, Canada, 1994. Abstract.
16. Mordue, J.E.M. *Glomerella cingulata.* Comm. Mycological Institute, C.M.I. n. 315, 1971.
17. Muirhead, I.F.; Deverall, B.J. Role of appressoria in latent infection of banana fruits by *Colletotrichum musae*. *Physiol. Plant Pathol.*, 19: 77-84. 1981.

18. Nelson, R.R. Interspecific hybridization in the fungi. *Mycologia,* 55: 104-121. 1963.
19. Ogle, H.J.; Irwin, J.A.G.; Cameron, D.F. Biology of *Colletotrichum gloeosporioides* isolates from tropical pasture legumes. *Aust. J. Bot.,* 34: 537-550, 1986.
20. Prusky, D.; Keen, N.T.; Sims, J.J.; Midland, S.L. Possible involvement of an antifungal diene in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. *Phytopathol.,* 72:1578-1582. 1982.
21. Prusky, D.; Plumbley, R.A. Quiescent infections of *Colletotrichum gloeosporioides* in tropical and subtropical fruits. In: *Colletotrichum* - Biology, pathology and control, p. 289-307. J. A. Bailey and M.J.Jeger, Eds. C.A.B. International, Wallingford, England. 1992.
22. Shear, C.L.; Wood, A.K. Studies of fungous parasites belonging to the genus *Glomerella.* U.S.D.A. Bur., *Plant Indust. Bull.,* 252: 1-110, 1913.
23. Snyder, W.C. Introduction. In: Nelson, P.E.; Toussoun, T.A.; Cook, R.J. *Fusarium* diseases, biology and taxonomy, p. 3-8. The Pennsylvania State Univ. Press, Univ. Park, PA. 1981.
24. Sutton, B.C. The Coelomycetes. Comm. Mycol. Institute. Kew, Surrey, England. 695 p., 1980.
25. Sutton, T.B.; Shane, W.W. Epidemiology of the perfect stage of *Glomerella cingulata* on apples. *Phytopathol.,* 73: 1179-1183.
26. Toussoun, T.A.; Nelson, P.E. A pictorial guide to the identification of *Fusarium* species according to the taxonomy system of Snyder and Hansen. The Pennsylvania State Univ. Press, Univ. Park, PA. 1968.
27. Tu, J.C. Anthracnose (*Colletotrichum lindemuthianum)* on white bean (Phaseolus vulgaris L.) in Southern Ontario: spread of the disease from an infection focus. *Plant Dis.,* 65: 477-480. 1981.

Revista de Microbiologia (1996) 27:237-241
ISSN 0001-3714

PRODUCTION OF AMYLASES AND PROTEASES BY WILD-TYPE AND MUTANT STRAINS OF *METARHIZIUM ANISOPLIAE* VAR. *ANISOPLIAE*

Maria Cléria Cordeiro Valadares[1]*; João Lúcio Azevedo[2]

[1]EMBRAPA/CENARGEN, Brasiília, DF, Brasil; [2]Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP, Brasil

ABSTRACT

Wild-type, auxotrophic and colour mutant strains of *Metarhizium anisopliae* var. *anisopliae* were assayed for the secretion of amylases and proteases on solid media. No differences in protease and amylase secretion were observed between monosporic colonies originating from the same strain. Some mutant strains with auxotrophic and colour markers secreted more protease and amylase than their respective parentals, demonstrating a pleiotrophic effect of one or more mutations in these strains. On the two amylase activity media used, one containing only starch and the other with starch and glucose as carbon source, it was shown that wild type strains had a reduced amylase secretion in the presence of glucose as compared to the mutant strains used.

Key words: *Metarhizium anisopliae*, auxotrophic mutants, proteases, amylases.

INTRODUCTION

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, is an entomopathogenic fungus, widely used to control insects pest in agriculture. In Brasil, *M. anisopliae* has been employed to control *Mahanarva posticata* and *M. fimbriolata* (Homoptera: Cercopidae), pests of sugarcane (2). *M. anisopliae* is also a promising agent in the control species of *Deois flavopicta*, *D. incompleta* and *Zulia entreriana* (Homoptera:Cercopidae), and pests of pasture in different areas of Brasil (13). Other species of *Metarhizium*, such as *M. flavoviride* (11) are used to control locusts in several tropical countries.

The disease process involves the adhesion of spores on the surface of the insect, germination, differentiation, penetration, and invasion of the insect cuticle followed by growth and emergence of hyphae producing external conidia (18). Penetration of the insect cuticle involves mechanical pressure and enzymatic digestion of proteins, chitins and lipids. Enzymes produced by entomopathogenic fungi have been studied with the aim of understanding their relationship with the disease process (4, 5, 8, 9, 16, 17, 20, 21, 25, 28). Enzymes such as amylases, proteases, lipases and chitinases are produced *in vitro* by entomopathogenic fungi, and in some systems, proteases have been shown to be important in insect protein digestion (27). Semi-quantitative assays of enzymes have been carried out, using solid media, allowing the screening of variation occurring in some species (7) and studies of the relationship of the enzymes to the infection process (6, 16). Also, exoenzymes produced by *M. anisopliae* on solid media have been used to characterize strains from diverse geographical areas and from different hosts (10, 19, 21, 26, 30).

Most of the studies involving enzymes in *M. anisopliae* were carried out with wild-type strains. However, to facilitate genetic studies using this fungus, mutants are usually necessary to provide genetic markers and as a means to use parasexuality

* Corresponding author. Mailing address: CENARGEN/EMBRAPA SAIN, Parque Rural, Caixa Postal 02372, CEP 70770-900 Brasília, DF, Brasil, Fax: (061) 340 3624. E.mail: cleria@cenargen.embrapa.br

to produce recombinants (12, 22, 23, 24). The influence of auxotrophy and other mutations on enzyme production is not known. Also, little is understood about the variability of enzyme production from subcultures of monosporic colonies. In the present work, monosporic colonies from wild-type strains, and the auxotrophic and colour mutants derived from them were analysed for production of amylases and proteases in an attempt to detect pleiotrophic effects of the mutations on enzyme production.

MATERIAL AND METHODS

Strains. Strains of *Metarhizium anisopliae* used in this work were obtained from Laboratório de Genética de Microrganismos (ESALQ/USP). The wild-type strains E_6 and E_9 were isolated in Espírito Santo State, from *Deois flavopicta* (Homoptera: Cercopidae). Mutant strains $E_{6\text{-}7}$ (*vio met⁻ bio⁻*) and $E_{6\text{-}8}$ (*ylo pyr⁻ lis⁻*) were produced by Silveira and Azevedo (23) and the mutant strains $E_{9\text{-}19}$ (*ylo leu⁻rib⁻*) and $E_{9\text{-}20}$ (*ylo leu⁻ade⁻*) isolated by Bagalhi (3). The *vio* and *ylo* mutations refer to pale vinaceous and yellow mutant colours as opposed to the wild-type colour (green). The notations *ade*, *bio*, *leu*, *lys*, *met*, *pyr* and *rib* are used to designate nutritional requirements for adenine, biotin, leucine, lysine, methionine, pyridoxine and riboflavin, respectively.

Media. The media used was prepared as described below: Solid minimal medium (MM): $NaNO_3$ 6g; KH_2PO_4 1.5g; KCl 0.5g; $MgSO_4.7H_2O$ 0.5g; $FeSO_4$ 0.001g; $ZnSO_4$ 0.001g; glucose 10g; agar 15g l^{-1}; pH 6.8. Solid complete medium (CM): solid minimal medium containing yeast extract 0.5g; peptone 2g; hydrolysed casein 1.5g; vitamin solution 1ml, yeast nucleic acid hydrolysate, 2.5g; l^{-1}, pH 6.8 (15). Activity media: Media for amylolytic activity: MM with or without glucose (MMSG and MMS media respectively) containing 0.2% soluble starch; pH 6.0. Media for proteolytic activity: (a) MM plus 1% of gelatin (MMPG), (b) MM without $NaNO_3$, plus 1% solution of skimmed milk (10% w/v) pH 6.0 (MMPS).

Vitamin solution. Vitamin solution was prepared with nicotinic acid 100mg, *p*-aminobenzoic acid 10mg; thiamine 50mg, pyridoxine 50mg, riboflavin 100mg, biotin 0.2mg, sterile distilled water to 100ml. The solution was steam sterilised and stored in a light proof bottle at 4°C, over chloroform.

Hydrolysed nucleic acid (HNA). The hydrolysed nucleic acid solution was prepared by adding 2g of HNA in 15 ml HCl 1N and 2g of HNA in 15ml NaOH 1N. The solutions were both heated at 100°C for 15min, mixed and the pH adjusted to 6.0. The mixture was filtered while still hot and the volume adjusted to 40ml. The preparation was stored at 4°C over chloroform.

Supplements added to the activity media. According to the strains used, the following supplements were added per litre of medium: adenine: 70mg; biotin: 0.02mg; leucine: 50mg; lysine: 50mg; methionine: 50mg; pyridoxine: 0,05mg and riboflavine: 0.05mg.

Exoenzyme activity. Conidia were suspended in Tween 80 solution (0.1% v/v) and plated using appropriate dilutions on CM. After 7 days incubation at 28°C conidia of the monosporic derived colonies were transferred to the activity media. For auxotrophic mutants, the respective nutritional requirements were supplemented. Analysis of the enzyme activities were carried out after 5 days incubation at 28°C and the enzymatic index calculated by measurement of the diameter of the degradative zones produced, divided by the diameter of the colonies. To reveal activities, iodine (1% v/v in alcohol) and protein stain solution were used for detection of amylases and proteases secreted by colonies, respectively.

Solution for proteins stain. The proteins stain was carried out by using fixer PAGE: methanol 22.5ml; acetic acid 5ml and distilled water 22.5ml. Stain: Comassie blue (Brilliant Blue R 250) 50mg (14).

RESULTS AND DISCUSSION

Monosporic colonies isolated from wild-type and auxtrophic mutants were analysed for secretion of amylases and proteases. No variation was observed among colonies derived from subcultures of the same strain (Tables 1 and 2). These results have shown that these strains are mitotically stable. Comparative analysis of strains, however, demonstrated, that the mutant $E_{9\text{-}20}$ is the best amylase producing strain on MMS-medium and that the mutant strain $E_{6\text{-}7}$ produces more protease than the respective wild-type. An increase in enzyme secretion by auxotrophic mutants could be correlated to the specific mutation for auxotrophy, suggesting a possible pleiotrophic effect. However, since no tests have been made with individual mutants, it is not yet possible to conclude if one or more mutation are responsible for these variations. Another effect of mutation could involve the rate of growth of the mutants in the media used for

Table 1. Protease secretion in wild-type and mutant strains of *Metarhizium anisopliae* on media containing milk as nitrogen source. The values of enzymatic index are means of triplicate determinations. Values followed by different letters are statistically different by Tukey test at the 5% level (dms=8.660).

Strain	Enzymatic index/colony*										Mean ± SD
	1	2	3	4	5	6	7	8	9	10	
$E_{6\text{-}7}$	1.680	1.640	1.597	1.680	1.837	1.687	1.723	1.747	1.770	1.757	1.712 ± 0.069 a
$E_{9\text{-}20}$	1.493	1.510	1.570	1.647	1.593	1.667	1.567	1.610	1.620	1.557	1.584 ± 0.056 b
$E_{6\text{-}8}$	1.523	1.620	1.580	1.540	1.563	1.407	1.413	1.510	1.560	1.520	1.530 ± 0.058 b
$E_{9\text{-}19}$	1.463	1.360	1.433	1.370	1.500	1.537	1.503	1.523	1.280	1.220	1.419 ± 0.108 c
E_6	1.397	1.383	1.433	1.367	1.417	1.363	1.447	1.483	1.403	1.467	1.417 ± 0.041 c
E_9	1.160	1.097	1.127	1.100	1.203	1.200	1.210	1.190	1.210	1.159	1.159 ± 0.049 d

* Monosporic colonies were isolated from each strain and assayed for enzyme secretion in triplicate. The enzymatic index was calculated by measurement of the diameter of the degradative zones divided by the diameter of the colonies.

Table 2. Amylase secretion in wild-type and mutant strains of *Metarhizium anisopliae*, in presence of starch and starch plus glucose. Values of enzymatic index are the means of triplicate determinations. Values followed by different letters are statistically different at Tukey test at the 5% level (dms=0.152).

Media	Strain	Enzymatic index/colony*										Mean ± SD
		1	2	3	4	5	6	7	8	9	10	
	E_9	1.363	1.217	1.270	1.237	1.227	1.243	1.153	1.230	1.123	1.133	1.220 ± 0.071 a
Starch	E_6	1.147	1.200	1.093	1.083	1.210	1.257	1.127	1.260	1.157	1.137	1.167 ± 0.063 ab
+	$E_{9\text{-}20}$	1.123	1.217	1.127	1.123	1.103	1.127	1.217	1.310	1.103	1.177	1.163 ± 0.067 ab
Glucose	$E_{6\text{-}7}$	1.103	1.090	1.070	1.097	1.140	1.160	1.143	1.140	1.160	1.125	1.125 ± 0.031 abc
(MMSG)	$E_{6\text{-}8}$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.230	1.030 ± 0.072 bc
	$E_{9\text{-}19}$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000 ± 0.000 c
	$E_{9\text{-}20}$	2.093	2.090	1.943	1.943	2.347	2.103	2.370	2.380	2.447	2.450	2.217 ± 0.202 a
	$E_{9\text{-}19}$	1.927	2.077	1.923	2.047	2.047	2.117	2.113	1.887	1.637	1.853	1.963 ± 0.149 b
	$E_{6\text{-}7}$	1.767	1.680	1.873	1.763	1.973	1.863	1.980	1.843	1.863	1.343	1.795 ± 0.183 c
Starch	$E_{6\text{-}8}$	1.920	1.830	1.430	1.430	1.710	1.750	1.600	1.670	1.730	1.620	1.669 ± 0.157 cd
(MMS)	E_6	1.557	1.650	1.483	1.620	1.493	1.567	1.400	1.503	1.490	1.623	1.539 ± 0.078 de
	E_9	1.750	1.447	1.600	1.570	1.457	1.323	1.433	1.410	1.430	1.463	1.488 ± 0.121 e

* Monosporic colonies were isolated from each strain and assayed for enzyme secretion, in triplicate. The enzymatic index was calculated by measurement of the diameter of the degradative zones divided by the diameter of the colonies.

enzyme production, despite the enzymatic index calculation being based on secretion ability related to colony size. Pleiotrophic effects of mutations for amylases correlated with increasing hypervirulence against *Culex pipiens* was reported by Robert and Messing-Al-Aidroos (16) in strains of *M. anisopliae*.

For amylolytic activity, medium containing glucose and starch as carbon source (MMSG) produced lower levels of secretion compared to media with starch only (MMS). Wild-type strains have demonstrated a reduced effect of repression on amylase secretion in the presence of glucose and starch when compared to the mutants. Mutants produced higher activity on the starch-only medium (MMS) as compared to wild-type strains (Table 2). Amylases are reported in wild-type strains of *M. anisopliae* as being repressible by sugars and inducible by starch (1, 29). The same was found in the present work but we have found distinctions when mutants and the parental wild-type strains are compared. There might also be explained by a pleiotrophic effect of mutations affecting the regulatory system of amylase secretion.

No degradative zones were produced on the protease activity medium containing gelatin (MMPG). As a consequence, the enzymatic index was 1.0 for all strains analysed. The effect of the gelatin or milk on protease secretion could suggest specialization of the

strains in terms of substrate specificity, or be related to the nature of the proteases produced. Gabriel (5) suggested that protease activity is related to substrate, acting as inducer or repressor. Al-Aidross and Seifert (1) obtained mutants negative for gelatin degradation (E^-) which reatined activity on milk (M^+). Strains negative for milk (M^-) were positive for gelatin (E^+). Similar correlation was observed by Roberts and Messing-Al-Aidross (16) where mutants assayed for proteases demonstrated high activity in gelatin and low activity in milk media. All these authors used wild-type strains only.

The results presented here show that genetic variability in terms of protease and amylase secretion does exist in wild-types and mutants of *M. anisopliae*. The isolation of mutants with auxotrophic or morphological markers in wild-type strains does not guarantee that enzyme production will be the same. Also, high secretion levels of an enzyme in mutant strains could be reduced when a recombinant with a wild-type phenotype is derived from it.

ACKNOWLEDGMENTS

We thank Dr. Bonifácio Magalhães for the valuable suggestions and Dr. Peter Inglis for correction of the manuscript.

RESUMO

Produção de amilases e proteases por linhagens selvagens e mutantes auxotróficos de *Metarhizium anisopliae* var. *anisopliae*

Linhagens parentais, mutantes auxotroficos e morfológicos do fungo entomopatogênico *Metarhizium anisopliae* var. *anisopliae* foram analisados para secreção de amilases e proteases, em meio sólido. Não foram observadas diferenças na secreção de proteases e amilases entre colônias monospóricas, originadas da mesma linhagem. Algumas das linhagens com marcas auxotróficas e morfológicas secretaram mais proteases e amilases, quando comparadas aos seus respectivos parentais, demonstrando efeito pleiotrópico para uma ou mais mutações nestas linhagens. Nos dois meios usados para atividade de amilases, um contendo somente amido e outro contendo amido e glicose, linhagens parentais demonstraram menor secreção de amilases em presença de glicose em comparação com as linhagens mutantes usadas.

Palavras-chave: *Metarhizium anisopliae*, mutantes auxotróficos, proteases, amilases.

REFERENCES

1. Al-Aidroos, K.M.; Seifert, A.M. Polysaccharide and protein degradation, germination, and virulence against mosquitoes in the entomopathogenic fungus *Metarhizium anisopliae*. *J.Inv.Pathol. 36*: 29-34, 1980.
2. Alves, S.B. Fungos entomopatogênicos. In: *Controle Microbiano de Insetos* (Alves, S.B. Coord.). São Paulo, Editora Manole Ltda., 1986, p.73-126.
3. Bagagli, E. Parameiose em *Metarhizium anisopliae* (Metsch.) Sorokin. Piracicaba, 1986, 123 p. (Master Thesis. Piracicaba. ESALQ/USP)
4. Bajan, C.; Kalalova, S.; Kmitowa, K.; Samsinakova, A.; Mojuechouska, M. The relationship between infectious activities of entomophagous fungi *Beauveria bassiana* andtheir productions of enzymes for the biological control of the honeybee pest *Galleria mellonella*. *Bull.Acad.Pol.Sci. 27*:963-968, 1979.
5. Gabriel, B.P. Enzymatic activities of some entomophthorous fungi. *J.Inv.Pathol. 11*: 70-81, 1968.
6. Gupta, S.C.; Leathers, T.D.; Al-Sayed, G.N.; Ignoffo, C.M. Production of degradative enzymes by *Metarhizium anisopliae* during growth on defined media and insect cuticle. *Exp. Mycol. 15*: 310-315, 1991.
7. Hankin, L.; Anagnostakis, S.L. The use of solid media for detection of enzyme productionby fungi. *Mycology. 67*: 597-607, 1975.
8. Kücera, M. Partial purification and properties of *Galleria mellonella* larvae proteolytic inhibitors acting on *Metarhizium anisopliae* toxic protease. *J.Inv.Pathol. 43*: 190-196, 1984.
9. Kücera, M.; Samsinakova, A. Proteases from the fungus *Metarhizium anisopliae* toxic for *Galleria mellonella* larvae. *J.Inv.Pathol. 35*: 304-310, 1980.
10. Leite, B. Variabilidade natural de linhagens de *Metarhizium anisopliae* (Metsch) Sorokin quanto à resistência ao ultravioleta, atividade enzimática e virulência ao vetor da doença de chagas *Panstrogylus megistus*. Campinas, 1984 , 136p. (Master Thesis, UNICAMP).
11. Magalhães, B.P.; Faria, M.; Tigano, M.S. Characterization and virulence of a Brazilianisolate of *Metarhizium flavoviride* Gams and Rozsypal (Hyphomycete). *Can. J. Entomol.*00: 00-00, *in press.*
12. Messias, C.L.; Azevedo, J.L. Parasexuality in the Deuteromycete *Metarhizium anisopliae*. *Trans. Brit. Mycol. Soc.* 75: 473-477, 1980.
13. Naves, M.A. As cigarrinhas das pastagens e sugestões para o seu controle.*CircularTécnica* Brasília, *EMBRAPA 3*: 5-27, 1980.
14. Paccola-Meirelles, L.D.; Valarini, M.J.; Azevedo, J.L.; Alfenas, A.C. Manual de técnicas eletroforéticas em microrganismos. FEALQ-USP, São Paulo, 1988, 54p.
15. Pontecorvo, G.; Roper, J.A.; McDonald, K.D.; Bufton, A.J.W. The genetics of *Aspergillus nidulans*. *Advances in Genetics* 5: 138-245, 1953.
16. Robert, A.; Messing-Al-Aidroos, K. Acid production by *Metarhizium anisopliae*, effects on virulence against mosquitoes and on detection of *in vitro* amylases, protease and lipase activities. *J.Inv.Pathol. 45*: 9-15, 1985.
17. Roberts, D.W. Toxins. In: *Microbial Control of Pest and Plant Disease*. (Ed. Burges, H.D.). Academic Press, New York, 1981, p.441-464.
18. Roberts, D.W.; Humber, R.A. Entomopathogenic fungi. In: *Conference on infection processes of fungi*. Rockfeller

Foundation Study and Conference Centre (Ed) RockefelerFoundation, Bellagio, 1983, p1-6.
19. Rosato, Y.B.; Messias, C.L.; Azevedo, C.L. Production of extracellular enzymes inisolates of *Metarhizium anisopliae*. *J.Inv.Pathol. 38*: 1-3, 1981.
20. Samsinakova, A.; Misikova, S.; Leopold, J. Action of enzymatic systems of *Beauveriabassiana* on the cuticle of the greater wax moth larvae, (*Galleria mellonella*). *J.Inv.Pathol. 18*: 302-320, 1971.
21. Silva, J.C.; Messias, C.L. Virulência de mutantes e revertentes de exoenzimas de *Metarhizium anisopliae* em *Rhodnius prolixus*. *Ciência e Cultura 38*: 814, 1985.
22. Silveira, W.D. Obtenção e fusão de protoplastos em *Metarhizium anisopliae* (Metsch.)Sorokin. Piracicaba, 1983, 153p. (PhD Thesis. ESALQ-USP).
23. Silveira, W.D.; Azevedo, J.L. Isolation of auxotrophic mutants of *Metarhizium anisopliae*by the filtration enrichment technique. *Rev. Bras. Gen.* 7: 1-8, 1984.
24. Silveira, W.D.; Azevedo, J.L. Protoplast fusion and genetic recombination in *Metarhizium anisopliae*. *Enz. Microbiol. Technol.* 9: 149-152, 1987.
25. Smith, R.J.; Perkul, S.; Grula, E.A. Requirement for sequential enzymatic activities for penetration of the integument of the corn earworm (*Heliothis zea*). *J. Inv. Pathol. 38*: 335-344, 1981.
26. Soza-Gomes, D.R.; Alves, S.B. Caracterización de once aislamientos de *Metarhizium anisopliae* (Metsch.) Sorok.; Estandartización, virulência y actividad enzimatica. *CIRPON. Rev.Invest. 1*: 83-102, 1983.
27. St Leger, R.J.; Charnley, A.K.; Cooper, R.M. Kinetics of the digestion of insect cuticles by a protease (PR1) from *Metarhizium anisopliae*. *J.Inv.Pathol.* 57: 146-147, 1991.
28. St Leger, R.J.; Cooper, R.M.; Charnley, A.K. Cuticle-degrading enzymes of entompathogenic fungi: cuticle degradation *in vitro* by enzymes from entomopathogens.*J. Inv. Pathol. 47*: 167-177, 1986.
29. Turcotte, D.; Al-Aidroos, K. Production d'activité amylolitique par le champignon entomopathogène, *Metarhizium anisopliae*. In: *Comptesrendus du congrès annuel de l'Association canadienne-francaise pour l'avancement des sciences.* Montreal. 1981, 185p..
30. Valadares, M.C.C. Genética e produção de exoenzimas em linhagens de *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin. Piracicaba, 1989, 160p.(Master Thesis, ESALQ/USP).

Revista de Microbiologia (1996) 27:242-246
ISSN 0001-3714

EFFECTS OF TEMPERATURE, WATER CONTENT AND SUBSTRATE ON CONIDIAL PRODUCTION OF *METARHIZIUM FLAVOVIRIDE*

Bonifácio Peixoto Magalhães*, Heloisa da Silva Frazão

Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia, CENARGEN/EMBRAPA, Brasília, DF, Brasil

ABSTRACT

The optimization of the conidial production by *Metarhizium flavoviride* (CG 423) was investigated under conditions of varying temperature, water content and substrates to develop this pathogen as a mycoinsecticide against grasshoppers, a serious agricultural pest in Brazil. Conidial production was highly affected by temperature and optimized when the fungus was grown at 27°C. The amount of water present in the substrate also influenced conidial production. The optimal water content was between 30 and 120% (v/w) depending on the substrate. The substrates tested were common rice, rice husk + bran rice, broken rice and parboiled rice. The best substrates for conidial production were rice husk + bran rice and parboiled rice. Growth of *M. flavoviride* - CG 366 (a standard isolate) produced in the different substrates did not present a significant change in virulence against the grasshopper *Rhammatocerus schistocercoides*.

Key words: Solid fermentation, entomopathogenic fungi, mass production, microbial control, grasshopper.

INTRODUCTION

Metarhizium flavoviride (Hyphomycetes), a pathogen of several species of grasshoppers, is under study as a bioinsecticide in Brazil (7) and Africa (2). *Metarhizium flavoviride* was recently found in Northeast Brazil infecting the grasshopper *Schistocerca pallens* (11), a serious pest of many crops including corn, sugarcane, pasture and rice (3). This pathogen has been studied regarding cytology (13) molecular characterization (8), infection process (14), pathogenicity (8) and bait formulation (6).

Fungal growth, including conidial production, is affected by the type of substrate and also by environmental factors such as water content and temperature. The type of response to these factors depends on the species (5). Elucidation of the factors encouraging optimization of conidial production by entomopathogenic fungi may facilitate their development as bioinsecticides. *Metarhizium anisopliae*, a pathogen of sugarcane froghopper in Brazil, is produced in several substrates such as corn, potato, common beans, sorghum, and soybean (10). However, the best yields are obtained when the fungus is grown in common rice (1), broken rice (12), parboiled rice (12) and husk + rice bran (4). For *M. flavoviride*, there is no information on the conidial production as affected by the substrate, water content and temperature. We report here on the performance of *M. flavoviride* regarding conidial production on common rice, rice husk + bran rice, broken rice and parboiled rice at different water contents and temperatures.

MATERIALS AND METHODS

Fungal strain. *Metarhizium flavoviride*, CG 423 (Collection of Entomopathogenic Fungi,

* Corresponding author. Mailing address: CENARGEN/EMBRAPA, C.P. 02372, 70770-000 - Brasília, DF, Brazil, Fax: +55613403573 - Email: boni@cenargen.embrapa.br

CENARGEN/EMBRAPA, Brazil), isolated from the grasshopper *S. pallens*, was removed from liquid nitrogen and grown for 10 days at 28°C on complete medium agar plate (0.4 g $NaHPO_4$, 0.6 g $MgSO_4$, 1 g KCl, 0.7 NH_4NO_3, 10 g glucose, 5 g yeast extract, 15 g agar, 1 liter of distilled water). Only cultures of up to two weeks in age were used. The initial inoculum added to each flask, produced in complete solid medium, was a suspension of 5 ml (10^7 conidia/ml) prepared in an aqueous solution containing Tween 80 (0.1%). The conidial suspension was homogenized by mechanical agitation (Vortex, 1 min). There were three replicates per treatment in all experiments unless stated otherwise.

Temperature. The effects of temperature in conidial production of *M. flavoviride* grown on common rice, husk + rice bran, broken rice and parboiled rice were tested. Cultures were incubated in growth chambers (B.O.D.) at 18, 22, 27 and 32°C in the dark. Other cultivation procedures were as described below. In this experiment, water content (v/w), previously determined, in each substrate was as follows: common rice (30%), husk + rice bran (100%), broken rice (30%) and parboiled rice (80%). Conidial harvesting was as described below and conidial production was determined with the aid of a hemocytometer.

Water content. The effects of the initial water content in the conidial production of *M. flavoviride* were studied in common rice, husk + rice bran, broken rice and parboiled rice. Due to variation in absorption of the water by the media, a different water gradient was tested for each substrate: 20-45% (v/w) for common rice, 20-160% for rice husk + bran rice, 10-32.5% for broken rice, and 20-140% for parboiled rice. The water content of each treatment was based on volume of water and weight of the substrate. The flasks containing the substrates and water were weighed before and after autoclaving (20 minutes, 120°C) to replace an eventual loss of water during sterilization. The volume of water used in the inoculum was considered in the final adjustment of the water content. Conidia were harvested 8 days after incubation at 27°C, a temperature normally used to produce *M. anisopliae* in the Mycology Laboratory at CENARGEN.

Substrates. Conidial production of *M. flavoviride* was tested on common rice, rice husk + bran rice, broken rice and parboiled rice at the optimal water content and temperature as determined in the experiments described above. Except for rice husk + bran rice medium, 100 g of each substrate were added to each 500 ml flask. For rice husk + rice bran medium, 12.5 g of each of these ingredients were added to 500 ml flasks. The weight of this substrate (25 g) is four times less than the weight of common rice, broken rice and parboiled rice tested. However, 25 g of husk + rice bran occupy approximately the same volume as 100 g of the three other substrates tested.

Conidial harvesting. Conidia were separated from substrate by washing with pure kerosene (200 ml/flask) and sieving (50 mesh). After sedimentation of conidia, the kerosene was gently drawn with the aid of a pipette. Conidia were then formulated in soybean oil containing 5% kerosene and stored at 4°C. A similar mixture (groundnut oil and kerosene) is used in Africa to formulate and carry *M. flavoviride* in ultra low volume applications (2).

Conidial viability was determined before formulation in oil by plating the aqueous conidial suspension (Tween 80, 0.1%) onto complete agar plate medium. Due to the difficulty in spreading the oil formulation onto the medium, we did not perform the viability after formulating the fungus. To avoid a possible problem with attenuation of the fungal viability, the bioassays were run immediately after formulating the conidia.

Bioassay. This experiment was previously performed with the isolate CG 366 of *M. flavoviride* (= IMI 330189, isolated from *Austracnis guttulosa*), used as standard in the beginning of this study. The isolate CG 366 is as virulent against *R. schistocercoides* as *M. flavoviride* - CG 423 (8), and there is no difference in conidial production by these isolates in different substrates, water content, and temperature (B. Magalhães and H. Frazão, unpublished). Based in these similarities, the results with the isolate CG 366 are being reported.

The virulence of *M. flavoviride* - CG 366, produced in the four substrates, was compared by standardized laboratory bioassays using conidia formulated in soybean oil and kerosene. Suspensions were adjusted to 1.67×10^6 conidia/ml. The fungus was topically inoculated (3 µl of suspension with 5,010 conidia/insect) on the right pleural region of the adult grasshoppers (M. Faria, unpublished). After inoculation, insects were kept individually in plastic boxes (11 x 11 x 3.5 cm) containing wheat germ, textured soybean grains and fresh leaves of *Andropogon gayanus*. Diet was renewed every other day. There were 10 insects per treatment and the experiment was repeated three times. Boxes were

maintained in an incubator at 27°C, in a 12h:12h (light:dark) photoperiod, and 55% relative humidity. To confirm infection, dead insects were maintained in a wet chamber until they presented profuse sporulation.

Data analysis. The mean ± SE is indicated in the figures and tables. SigmaStat™ and SigmaPlot™ (Jandel Scientific, Corte Madera, CA, USA) were used to calculate statistics and plot data. The conidial production of *M. flavoviride* in the different substrates was compared using all pairwise multiple comparisons (Student-Newman-Keuls method), and the median values among treatment groups in the bioassay were compared using Kruskall-Wallis ANOVA on Ranks.

RESULTS AND DISCUSSION

Conidiogenesis of *M. flavoviride* (CG 423) is highly affected by temperature. For the four substrates tested, the best temperature for conidial production was 27°C (Fig. 1). There was a reduction in the number of conidia produced when the fungus was grown at temperatures lower than 22°C and higher than 27°C ($P < 0.05$). However, conidia of *M. flavoviride* produced at 30°C and formulated in oil presented a greater tolerance to high temperatures than those produced at 26°C (9). This high temperature tolerance to short periods (e.g. one week) is very important for field application of this pathogen.

The water content of the substrate was critical for conidial production of *M. flavoviride*. At the optimal water contents for sporulation, conidial production was the same whether the substrate was husk + rice bran (80%; v/w) or parboiled rice (100%) ($P = 0.23$) (Fig. 2). For husk + rice bran, however, more than 80% water content seems to inhibit conidial production. Similarly, for parboiled rice containing more than 100% water, conidial production was reduced (Fig. 2).

The external water requirement for conidial production in common and broken rice is much lower than in parboiled rice and husk + rice bran. Conidial production in these two substrates is optimized with 30% water (Fig. 2). It is possible that the higher levels of humidity required for conidial production in husk + rice bran and parboiled rice are related to a higher absorption of water by these substrates than that for common and broken rice.

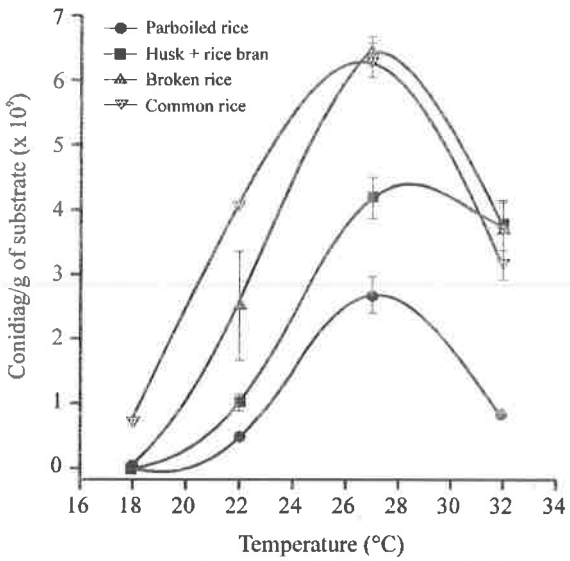

Figure 1. Conidial production (mean ± SE) of *Metarhizium flavoviride* (CG 423) in different substrates as a function of temperature. Fungus grown during 8 days. Average from three replicates.

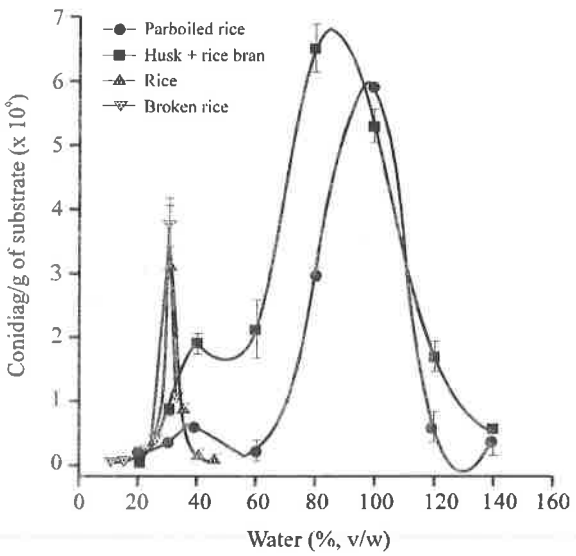

Figure 2. Conidial production (mean ± SE) of *Metarhizium flavoviride* (CG 423) in different substrates at 27°C as a function of water content. Fungus grown at 27°C during 8 days. Average from three replicates.

After determining the best temperature (27°C) and water contents (30%, common rice; 80%, rice husk + rice bran; 30%, broken rice; 100%, parboiled rice) to produce conidia of *M. flavoviride*, a final experiment was performed. The best substrates for conidial production were rice husk + rice bran and parboiled rice followed by broken rice and common rice ($P <$

Table 1. Production of conidia (mean ± SE) by *Metarhizium flavoviride* (CG 423) in different substrates at optimal temperature (27°C) and water contents (30%, common rice; 80%, rice husk + rice bran; 30%, broken rice; 100%, parboiled rice). Fungus grown during 8 days. Average from three replicates.

Substrate	Number of conidia/g of substrate (x 10^9)[1]
Husk + rice bran	65.33 ± 8.74 a
Parboiled rice	57.67 ± 2.85 a b
Broken rice	42.00 ± 1.53 b c
Common rice	26.67 ± 2.85 c

[1]Means within columns followed by the same letter are not significantly different by all pairwise multiple comparisons (Student-Newman-Keuls method) (P < 0.05).

0.05) (Table 1). The number of conidia/g of substrate produced by *M. flavoviride* in parboiled rice (5.9 ± 0.06 x 10^9) and broken rice (4.2 ± 0.15 x 10^9) is comparable to the number of conidia/g produced by *M. anisopliae* in the same media (3.5 x 10^9 and 5.0 x 10^9, respectively) (12).

The use of the substrate husk + bran rice and broken rice is a very attractive option in a bioinsecticide production system based on entomopathogenic fungi because of its low cost (4, 12). The cost of broken rice is four times lower than the cost of parboiled rice (12). However, the use of broken rice in large scale may be limited by its lack of uniformity. Thus, parboiled rice should be considered as the substrate to be used in a routine system to produce *M. flavoviride*.

A minimal effect of the type of substrates on the virulence of *M. flavoviride* is anticipated, as indicated in the bioassay against *R. schistocercoides* (Fig. 3). In all cases, the median survival was between 6 and 7 days with no significant differences among the treatments (P = 0.562) (Table 2). Ten days after inoculation there was 100% mortality with confirmed infection of the insects inoculated with conidia produced in the four substrates.

Parboiled rice has been adopted for the massal production of *M. flavoviride* in the Mycology Laboratory (CENARGEN) and of *M. anisopliae* at Biotech (Controle Biológico Ltda) (10). The reasons for chosing parboiled rice are the high yield and a better performance when harvesting conidia from this substrate. Conidia are more easily separated from parboiled rice when compared with husk + bran rice and the other culture media tested. However, the scaling up of the harvesting process of conidia will require an improvement in the use of pure kerosene. Extraction of conidia from kerosene would be facilitated by the development of a better filtration system. Another problem with the oil formulation is a correct estimation of the conidial viability in the aqueous agar plate medium. Due to the differences in polarity between the oil formulation and the aqueous medium, conidia do not contact the medium and do not germinate satisfactorily. Investigations on several processes of conidial washing with detergent to overcome this limitation are now in progress.

Table 2. Survival (mean SE) of *Rhammatocerus schistocercoides* treated with conidia of *Metarhizium flavoviride* (CG 366) produced in different substrates at 27°C and formulated in soybean oil containing 5% kerosene.

Substrate	Survival time (days)[1]
Broken rice	7.20 ± 0.34
Common rice	7.16 ± 0.23
Parboiled rice	6.66 ± 0.27
Husk + rice bran	7.10 ± 0.24

[1]There is not a statistically significant difference in the median values among treatments according to Kruskall-Wallis ANOVA on Ranks (P = 0.62); [2]Thirty insects/treatment maintained at 27°C in a 12h:12h (light:dark) photoperiod, and 55% relative humidity. Each insect represents a replicate and data a combination of three experiments.

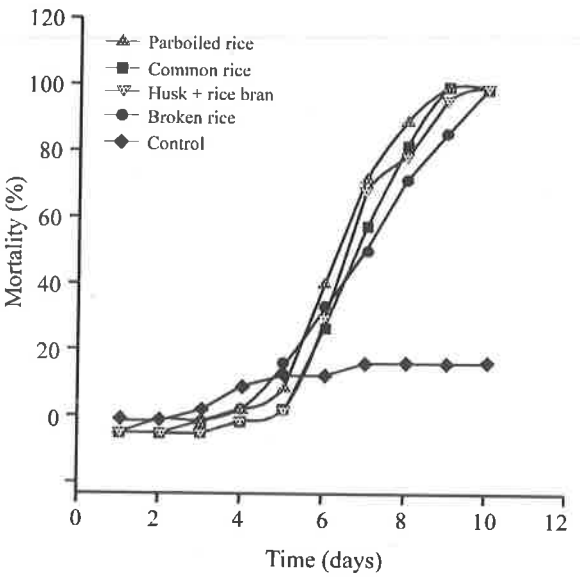

Figure 3. Mortality of *Rhammatocerus schistocercoides* caused by mycosis of *Metarhizium flavoviride* (CG 366) formulated in soybean oil containing 5% kerosene. Thirty insects/treatment maintained at 27°C in a 12h:12h (light:dark) photoperiod, and 55% relative humidity. Data represent a combination of three experiments.

ACKNOWLEDGEMENTS

We thank Dr. Myrian Tigano (CENARGEN/EMBRAPA) for critically reviewing the manuscript, Edison Sujii (CENARGEN/EMBRAPA) for advising on the statistical analysis for the bioassay data, Dr. Chris Prior (International Institute of Biological Control, UK) for kindly providing the isolate IMI 330189, and three anonymous reviewers for their criticism and helpful suggestions. This study was partially funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Food and Agriculture Organization (FAO).

RESUMO

Efeito da temperatura, teor de água e substrato na produção de conídios de *Metarhizium flavoviride*

A otimização da produção de conídios de *Metarhizium flavoviride* (CG 423) foi investigada variando a temperatura, teor inicial de água e substratos visando ao desenvolvimento deste patógeno como um bioinseticida contra gafanhotos, uma séria praga da agricultura brasileira. A produção de conídios foi altamente afetada pela temperatura, tendo sido otimizada quando o fungo foi cultivado a 27°C. O teor de água no substrato também influenciou a produção de conídios. A quantidade ótima de água ficou entre 30 e 120% (v/w) dependendo do substrato. Os substratos testados foram arroz parboilizado, arroz comum, quirela de arroz e palha + farelo de arroz. Os melhores substratos para produção de conídios de *M. flavoviride* foram palha de arroz + farelo de arroz e arroz parboilizado. O cultivo *M. flavoviride* - CG 366 (um isolado padrão), nos diferentes substratos, não alterou a virulência contra o gafanhoto *Rhammatocerus schistocercoides*.

Palavras-chave: Fermentação semi-sólida, fungos entomopatogênicos, produção massal, controle microbiano, gafanhotos.

REFERENCES

1. Aquino, M.L.N.; Cavalcanti, V.A.L.B.; Sena, R.C.; Queiros, G.F. Nova tecnologia de multiplicação do fungo *Metarhizium anisopliae*. Boletim Técnico CODECAP, Recife no. 4. 1975. 29p.
2. Bateman, R.; Godonou, I.; Kpindu, D.; Lomer, C.J.; Paraïso, A. Development of a novel field bioassay technique for assessing mycoinsecticide ULV formulations. In: Lomer, C.J.; Prior, C. (eds.) *Biological control of locusts and grasshoppers*. Wallingford, UK, 1992, p. 255-262.
3. Cosenza, G.; Ribeiro, J.G.B.; Carvalho, J.S. - Programa nacional de controle do gafanhoto: manual técnico. Ministério da Agricultura, do Abastecimento e da Reforma Agrária - Brasília: EMBRAPA/SPI, 1994, 34p.
4. Dorta, B.; Bosch, A.; Arcas, J.A.; Ertola, R.J. High level of sporulation of *Metarhizium anisopliae* in a medium containing by-products. *Appl. Microbiol. Biotechnol.* 33: 712-715, 1990.
5. Garraway, M.O.; Evans, R.C. *Fungal nutrition and physiology*. John Wiley and Sons, New York, USA, 1984, 401p.
6. Goettel, M.S.; Magalhães, B.P.; Gama, G. Palatability and efficacy of a bait formulation of *Metarhizium flavoviride* against the grasshopper *Rhammatocerus schistocercoides*. XXVIII Annual Meeting of the Society for Invertebrate Pathology, Ithaca, NY, USA, 1995, p. 23-24.
7. Magalhães, B.P. Microbial control of grasshoppers in Brazil with the use of entomopathogenic fungi. VII International Symposium on Microbial Ecology, Santos, SP, Brazil, 1995, p.28.
8. Magalhães, B.P.; Faria, M.R.; Tigano, M.S.; Sobral, B. Characterization and virulence of a Braszilian isolate of *Metarhizium flavoviride* GAMS and ROZSYPAL (Hyphomycetes). *Can. Entomol.* (In Press).
9. McClatchie, G.V.; Moore, D.; Prior, C. Temperature effects of conidia of *Metarhizium flavoviride* in oil formulations. XXVI Annual Meeting of the Society for Invertebrate Pathology, Asheville, NC, USA, 1993, p.58.
10. Mendonça, A.F. Mass production, application and formulation of *Metarhizium anisopliae* for control of sugarcane froghopper, *Mahanarva posticata*, in Brazil. In: Lomer, C.J.; Prior, C. (eds.) *Biological control of locusts and grasshoppers*. Wallingford, UK, 1992, p. 239-244.
11. Moreira, M.; Magalhães, B.P.; Valadares, M.C.C.; Chagas, M.C.M. Occurrence of *Metarhizium flavoviride* Gams and Rozsypal (Hyphomycetes) on *Schistocerca pallens* (Thunberg) (Orthoptera: Acrididae) in Rio Grande do Norte, Brazil. *An. Soc. Entomol. Brasil*. 25:359-361, 1996.
12. Quintela, E. Produção de *Metarhizium anisopliae* (Metsch.) Sor. em quirela de arroz. *An. Soc. Entomol. Brasil* 23: 557-560, 1994.
13. Xavier-Santos, S. Aspectos do comportamento cultural, citológico e entomopatogênico de *Metarhizium flavoviride*. Recife, PE, 1995, 108p. (M.Sc. Thesis. Departamento de Micologia, Universidade Federal do Recife).
14. Vicentini, S.; Magalhães, B.P. Infection of the grasshopper, *Rhammatocerus schistocercoides* Rehn by the entomopathogenic fungus, *Metarhizium flavoviride* Gams and Rozsypal. *An. Soc. Entomol. Brasil*. 25:309-314, 1996.

Revista de Microbiologia (1996) 27:247-251
ISSN 0001-3714

APPRESSORIA OF BRAZILIAN ISOLATES OF *COLLETOTRICHUM GLOEOSPORIOIDES* (PENZ.) SACC. CAUSAL AGENT OF ANTHRACNOSES DISEASES

Maria Menezes[1]*, Richard T. Hanlin[2]

[1]Departamento de Agronomia, UFRPE, Recife, PE, Brasil; [2]Department of Plant Pathology, University of Georgia, Athens, GA, USA.

ABSTRACT

Morphological and functional aspects of appressoria of *Colletotrichum gloeosporioides* from different sources and localities in the Northeastern Brazil were studied. From leaves of avocado (*Persea gratissima L.)*, cashew (*Anacardium occidentale* L.), citrus (*Citrus* sp.), guava (*Psidium guajava* L.), *Anona muricata* L., and mango (*Mangifera indica* L.) showing typical anthracnose symptoms twelve isolates were selected. From each isolate, fifteen monoconidial cultures were made, and the microculture technique was used for studies of appressorial morphology. All isolates produced appressoria, but variation in size, amount, and shape were observed among isolates. The same isolate showed unlobed or slightly lobed appressoria, which varied in color from dark to light brown, occasionally one septate showing a germ pore in each cell. In some cases, appressoria germinated and formed secondary appressoria in chain or isolated. Sometimes they formed germtube that produced conidia in phialidic way. These results suggest that appressoria play an important role in the nature, not only in the direct penetration of a host, but also in the ability to propagate species by production of conidia.

Key words: Appressorium, *Colletotrichum*, morphology, conidial production, anthracnose.

INTRODUCTION

Anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Sacc. has a worldwide geographical distribution. It is a very common and destructive disease on several crops, specially in humid and warm areas. The disease causes more severe losses in the tropical and subtropical regions. On fruit crops, the symptoms are necrotic leaf spots, blight and drop of flowers, fruit drop and rot, stem canker, dieback, and damping-off on seedlings. Under favorable conditions, affected tissues are covered with masses of pink colored spores.

The pathogen can penetrate through intact surface of a host plant, and cause latent infections in green fruits (8). An infection process of *C. gloeosporioides* on papaya fruit was showed by Chau and Alvarez (5) in which an appressorium produces an infection peg that penetrates the cuticle 3-4 days after inoculation. Some studies on latent infection caused by *C. gloeosporioides* were developed by Byniamini and Shiffmann-Hadel (3); Brown (4); Daquioag and Quimio (7); Shane and Sutton (16), and others. Muirhead and Deverall (13) suggest that latency of *Colletotrichum* in banana is mediated by factors which control appressorial dormency. They also comment that appressoria and infection pegs or subcuticular hyphae are essential for fungal penetration, and might play a role in its survival in the environment. In according to Emmett and Parbery (9), the main role of

* Corresponding author. Mailind address: Área de Fitossanidade, Depto. de Agronomia, Univ.Federal Rural de Pernambuco - Av. Dom Manoel de Medeiros, s/n. Dois Irmãos, CEP 52171-900. Recife-PE.

appressoria is the direct penetration in a host plant. Prusky and Plumbley (15) reported that *C. gloeosporioides* produces appressoria and quiescent infections on most tropical and subtropical crops. Recently, Agostin and Timmer (1) studied *C. gloeosporioides* from citrus, and based on theirs findings, They suggested that conidia onto vegetative tissues may germinate and produce appressoria, and quiescent infections are probably formed to some degree. When leaf surface provide some stimulating substance, appressoria are induced to form a few conidia.

The purpose of this research was to study some morphological and functional aspects of *C. gloeosporioides* appressoria from different sources and localities from Northeastern Brazil.

MATERIALS AND METHODS

Isolates of *C. gloeosporioides* were obtained from leaves of avocado (AVO), cashew (CAS), citrus (CITR), guava (GUA), *Anona muricata* (ANON), and mango (MANG) from the following locations: Ceará (CE), Rio Grande do Norte (RN), Paraiba (PB), Pernambuco (PE), Sergipe (SE), and Bahia (BA). Small leaf segments from infected tissue were cut, surface sterilized in 1,5% sodium hypochlorite for two minutes, and then rinsed in sterile distilled water. The material was plated onto PDA and incubated for seven days at 25°C. Twelve isolates were studied: AVO-CE, AVO-PE, CAS-PE, CAS-PB, CITR-SE, CITR-BA, GUA-CE, GUA-BA, MANG-CE, MANG-RN, MANG-PE, and ANON-BA. From each isolate, fifteen monoconidial cultures were made. An agar mycelium disk (5mm diameter) removed from the advancing margin of colony was placed on the center of PDA plate and incubated for five days at 25°C, under daily alternating light (12 h light/12 h dark).

Studies on appressoria morphology were made from 180 monoconidial cultures using a microculture technique described by Sutton (18), which consists of inoculation at the sides of a small square of agar (1 cm^2) placed on a glass slide and covered with a coverslip. This culture was maintained in moist chamber, at room temperature for 48-72 hours, when the size and shape of appressoria were determined. Samples of 25 units for each monoconidial culture were analyzed in relation to length and width. Means of appressoria size were compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

All isolates of *C. gloeosporioides* from monoconidial cultures produced appressoria, but they varied in amount, size, and shape, within the same isolate and among them (Table 1).

Morphological variation was observed, such as lobed or unlobed appressoria, one cell or rarely two cells, subglobose to irregular shape, showing a germ pore in each cell. Baxter *et al.* (1983) described the appressoria as being obovoid or subglobose, size 8.3-12.6 x 6.4-6.9 µm, similar to the results obtained in this research. The Duncan's test revealed significant difference to dimensions of appressoria produced by Citr-SE and Citr-BA in relation to other isolates listed in Table 1. Although measurements of appressoria have been made, the size did not reveal taxonomic

Table 1. Size (µm) of appressoria produced by *Colletotrichum gloeosporioides*.

Isolate code	Length (mean)	range	Width (mean)	range	Origin locality
AVO-CE	11.4 b	10.4 - 12.2	6.3 b	5.8 - 7.0	Ceará
AVO-PE	9.1 cde	9.1 - 11.0	6.1 bcd	5.4 - 7.0	Pernambuco
CAS-PB	10.8 bcd	9.8 - 11.6	6.1 bcd	5.8 - 6.6	Paraíba
CAS-PE	9.4 c	8.7 - 10.0	5.8 cde	5.5 - 6.1	Pernambuco
CITR-SE	12.8 a	11.3 - 14.9	7.3 a	6.3 - 8.3	Sergipe
CITR-BA	12.4 a	11.4 - 14.4	5.7 de	5.1 - 6.4	Bahia
GUA-CE	9.9 de	9.2 - 10.5	5.7 de	5.5 - 6.0	Ceará
GUA-BA	11.3 b	9.5 - 12.2	6.1 bcd	5.5 - 7.1	Bahia
ANON-BA	10.5 bcd	9.2 - 14.7	6.4 b	6.0 - 6.7	Bahia
MANG-CE	11.2 b	9.1 - 12.7	5.8 cde	5.4 - 6.7	Ceará
MANG-RN	10.7 bcd	9.5 - 12.0	6.2 bc	5.4 - 7.0	Rio Grande do Norte
MANG-PE	10.9 bc	9.3 - 12.1	6.3 b	5.3 - 7.0	Pernambuco

All means are averages of 15 monoconidial cultures, of which 25 appressoria were measured for each monoconidial isolate studied.

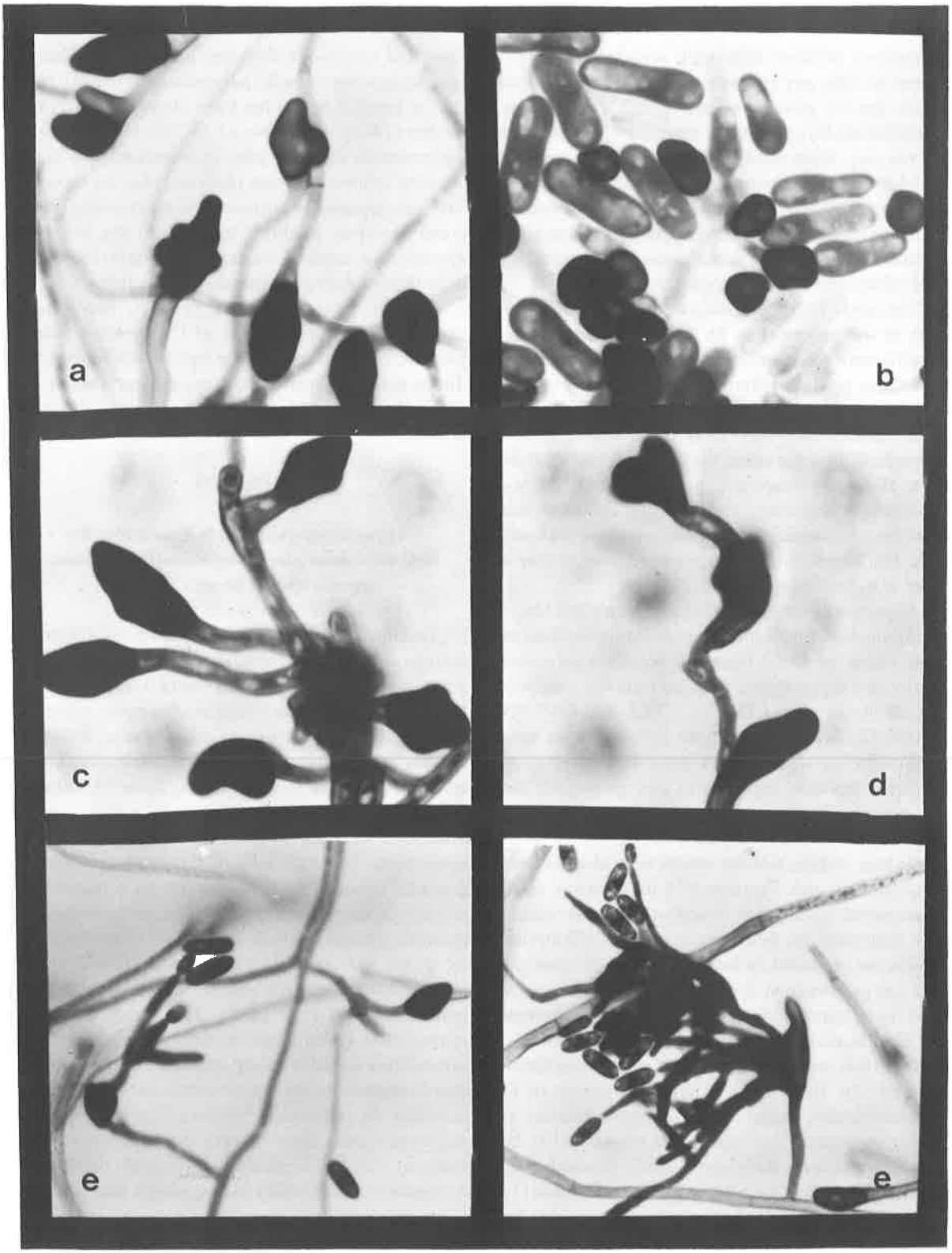

Figure 1. Appressoria of *Colletotrichum gloeosporioides:* on hyphal apex (a); from conidia (b); group of appressoria (c); chain of appressoria (d); conidia produced from germtube of appressoria (e).

importance because they vary widely and do not appear to offer any characteristics in delimiting taxa within the *C. gloeosporioides* group. Nevertheless, Cox and Irwin (6) reported that appressorial morphology, but not size, when combined with conidium size was useful to reduce the variability within identification of this species. Also, Shear and Wood (17) found an excessive variability in size and shape of appressoria, and comment its occurrence is associated to contact with a hard surface and to lack of some nutrient.

The most of the appressoria were formed on hyphal apex or sporophore (Fig. 1a.). Those produced from conidia were more uniform in size, usually smaller than those produced from tips of hyphae (Fig. 1b). Sometimes one septum was formed at the base before the full expansion of appressorium, separating it of the sporophore. In some cases, the hyphae branches at the base of an appressorium, growing around it and producing a secundary appressorium or many side branches, close each other forming a group were found (Fig. 1c), or secundary appressorium give origen to other as a chain of apressoria (Fig. 1d).

Appressorial formation was common when bits of mycelium, better than spores, grow in microculture on glass slides for 48-72 hours. Appressoria germinate readily, and the germtube emerged from the germ pore and, as observed in CITR-SE, CITR-BA, CAS-PB, MANG-CE, many conidia from germtube apex were produced (Fig. 1e). This is the principal point in evidence because appressoria can propagate and disseminate the species by conidia formation, in addition to well known capacity of penetrate directly on the host surface. Similar results were also obtained by Agostini and Timmer (1) in relation to *C. gloeosporioides* on citrus. Based on their observations they suggested the following cycle for PFD strains: conidia are produced on infected tissues of citrus and they can germinate to form appressoria. In favorable conditions, appressoria are stimulated to germinate and form conidia that are dispersed by rain-splash.

From this results the concept of appressoria can be altered. In the past, it was reported in *C. lindemuthianum*, agent of bean anthracnose, as "adhesion organs" or "spore-like organs" (10), by means of which the pathogen is firmly atached to the host surface during the early stages of infection (11). Appressoria were regarded as secondary spores, but no particular function was attributed to them, except that they were resting spores.

Then, as infection structures, appressoria are well known, and were named resting bodies because under favorable conditions they can germinate, adhere to host surface and directly penetrate on it (9), but their role in survival do not has been clearly defined (14). In according to Kubo *et al.* (12), appressoria pigmentation seems to play an important role in the capacity to penetrate host plant and also for survival. The dark appressoria are more resistent to unfavorable condition than hyaline. In general, the color of appressoria studied varied from dark-brown to light-brown, with predominance of the first.

On the basis of these results, the autors concluded that appressoria of *C. gloeosporioides* play an important role in the nature, not only in the direct penetration of a host tissue, but also in the ability to propagate the species by production of conidia.

RESUMO

Apressórios de isolados brasileiros de *Colletotrichum gloeosporioides* (Penz.) Sacc., agente causal de antracnoses

Foram estudados alguns aspectos morfológicos e funcionais de apressórios de *Colletotrichum gloeosporioides* de diferentes fontes e localidades do Nordeste do Brasil. De folhas de abacateiro, cajueiro, citros, goiabeira, gravioleira e mangueira, exibindo sintomas típicos de antracnose, foram selecionados 12 isolados. De cada isolado, foram feitas 15 culturas monoconidiais, sendo utilizada a técnica de microcultura para os estudos morfológicos dos apressórios. Todos os isolados formaram apressórios, havendo variação no tamanho, forma e quantidade produzida entre os isolados. Um mesmo isolado mostrou apressórios levemente lobados e não lobados, os quais variaram de castanho escuro a claro, ocasionalmente apresentando um septo, com um poro germinativo em cada célula. Em alguns casos, os apressórios germinaram e formaram apressórios secundários isolados ou em cadeias. Algumas vezes, eles formaram um tubo germinativo que deu origem a conídios de um modo fialídico. Estes resultados sugerem que a importância dos apressórios na natureza, não é somente a sua capacidade de penetrar diretamente na superfície do hospedeiro, mas também, a sua habilidade de propagar a espécie, através da produçao de conídios.

Palavras-chave: Apressórios, *Colletotrichum*, morfologia, produção de conídios, antracnose.

REFERENCES

1. Agostini, J.P.; Timmer, L.W. Population dynamics and survival of strains of *Colletotrichum gloeosporioides* on citrus in Florida. *Phytopathology*, 84: 420-425, 1994.
2. Baxter, A.P.; Westhuizen, G.C.A.; Eicker, A. Morphology and taxonomy of South African isolates of *Colletotrichum*. S. Afr. *J. Bot.*, 2: 260-289, 1983.
3. Byniamini, N.; Shiffmann-Hadel, M. Latent infection in avocado fruit due to *Colletotrichum gloeosporioides* . *Phytopathology*, 62: 592-594, 1972.
4. Brown, G.E. Factors affecting postharvest development of *Colletotrichum gloeosporioides* in citrus fruits. *Phytopathology*, 65: 404-409, 1975.
5. Chau, K.F.; Alvarez, A.M. A histological study of anthracnose on *Carica papaya.Phytopathology* 3: 1113-1116. 1983.
6. Cox, M.L.; Irwin, J.A.G. Conidium and appressoria variation in Australian isolates of the *Colletotrichum gloeosporioides* group and closely related species. *Aust. Syst. Bot.*, 1: 139-149. 1988.
7. Daquioag, V.R.; Quimio, T.H. Latent infection in mango caused by *Colletotrichum gloeosporioides*. *Philipp. Phytopathol.*, 15: 35-46, 1978.
8. Dickman, M.B.; Alvarez, A.M. Latent infection of papaya caused by *Colletotrichum gloeosporioides*. *Plant Disease*, 67: 748-750, 1983.
9. Emmett, R.W.; Parbery, D.G. Appressoria. *Ann. Rev.Phytopathology*, 13: 147-167. 1975.
10. Frank, A.B. Ueber einige neue und weniger bekannte Pflanzenkrankeiten. Ber. Dtsch. *Bot. Gesells.* I: 29-34. *Landw Jarhb. 12: 511-539*, 1983.
11. Hasselbring, H. The appressoria of the anthracnoses. *Bot. gaz.*, 42: 135-146, 1906.
12. Kubo, Y.; Suzuki, K.; Furusawa, I.; Ishida, N.; Yamamoto, M. Relation of appressorium pigmentation and penetration of nitrocellulose membranes by *Colletotrichum lagenarium*. *Phytopathology*, 72: 498-501, 1982.
13. Muirhead, I.F.; Deverall, B.J. Role of appressoria in latent infection of banana fruits by *Colletotrichum musae*. *Physiol. Plant Pathology*, 19: 77-84, 1981.
14. Parbery, D.G.; Emmett, R.W. Hypothesis regarding appressoria spores, survival, and phylogeny in parasitic fungi. *Revue de Mycologie*, 41: 429-447, 1977.
15. Prusky, D.; Plumbley, R.A. Quiescent infections of *Colletotrichum* in tropical and subtropical fruits. In: *Colletotrichum:* Biology, Pathology and Control, pages 289-307. J.A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, England, 1992.
16. Shane, W.W.; Sutton, T.B. Germination, appressorium formation, and infection of immature and mature apple fruit by *Glomerella cingullata*. *Phytopathology*, 71: 454-457, 1981.
17. Shear, C.L.; Wood, A.K. Studies of fungous parasites belonging to the genus *Glomerella*. Bureau of Plant Industry, *Bulletin* n. 252, 1913.
18. Sutton, B.C. The Coelomycetes. Comm. Mycol. Inst., Kew, Surrey, England. 1980, 596p.

Revista de Microbiologia (1996) 27:252-254
ISSN 0001-3714

ENVIRONMENTAL FACTORS AFFECTING CONIDIAL FORMATION IN *HUMICOLA* SP.

Roberta Hilsdorf Piccoli-Valle, Virgínia Maria Chaves-Alves, Daison Olzany Silva*

Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, MG, Brasil.

SHORT COMMUNICATION

ABSTRACT

Humicola sp. was cultured on potato-dextrose-agar (PDA) with the pH adjusted to 7.2 with K_2HPO_4 or KOH, or not (pH 5.76). The cultures were compared for their sporulation capacity in the dark and in the presence of tungsten or fluorescent lamps (6 volts/5 W and 15 volts/54 W, respectively) at 40°C (fungal growth) and 20°C.

The best sporulation was obtained in the medium with the pH adjusted with K_2HPO_4 and incubated under exposure to fluorescent light at 20°C.

Key words : *Humicola* sp., sporulation, pH, light, temperature

The fungus *Humicola* sp. produces several extracellular enzymes, namely, cellulases (5), xylanase (9), amylases (15), trehalase (17), and ß-glucosidase (1, 10).

The genus *Humicola* is characterized by the production of simple or extensively branched conidiophores with unicellular globose or subglobose apical conidia called aleuriospores. Some species also produce simple phialids and phialospore chains (2).

Genetic and physiological studies of *Humicola* sp. require a good production of spores. Though mycelial growth is uniform in the species of this genus, conidial formation presents some problems.

In the present investigation we determined the effects of temperature, light and pH on conidial formation in *Humicola* sp. isolated from compost (4).

Microorganism. Initial inoculum was obtained by cultivating *Humicola* sp. on potato-dextrose-agar (PDA/DIFCO) for 5 days at 40°C.

Procedure. Screw-capped tubes (10 x 50 mm) containing 1.0 ml PDA/DIFCO were inoculated with *Humicola* sp. at levels ranging from 3.6×10^6 to 10^7 spores/ml.

Spores counts were performed using a hemocytometer (Neubauer chamber) at 3-day intervals for 12 days. Spores were scraped off the tubes with a glass rod after adding 1.5 ml of 0.3% (v/v) Tween 80 prepared with distilled water.

To study the effects of light and temperature on sporulation, the pH of PDA was adjusted to 7.2 with KH_2PO_4. All experiments were done in triplicate.

Effect of Light. Three sets of inoculated tubes were incubated for 12 days at 40^0C and, then, exposed to either a fluorescent lamp (15 volts/54 W), a tungsten lamp (6 volts/5 W) or left in the dark at 40^0C.

Effect of Temperature. Another set of inoculated tubes were incubated for 3 days at 40^0C in the absence of light and, then, maintained at 20^0C. Half the tubes were exposed to fluorescent light and half were left in the dark.

Effect of pH. The pH of PDA was adjusted to 7.2 with K_2HPO_4 or KOH, to a final concentration of 0.01M, or was left without any adjustment (pH 5.76). Inoculated tubes were incubated in the dark at 40^0C for 3 days. After this time, incubation temperature was

* Corresponding author. Mailing address: Departamento de Microbiologia, Universidade Federal de Viçosa, 36571000, Viçosa, MG, Brasil. Telefone: (031)899-2553 - FAX: (031)899-2573

changed to 20°C and the tubes were exposed to fluorescent light.

Fluorescent light alone had little or no effect on the sporulation of *Humicola* sp. (Fig. 1A) but increased spore production when combined with the reduction of the temperature to 20°C (Fig. 1B). Although light is not required for fungal growth, it is essential for sporulation in many fungal species (6). How light

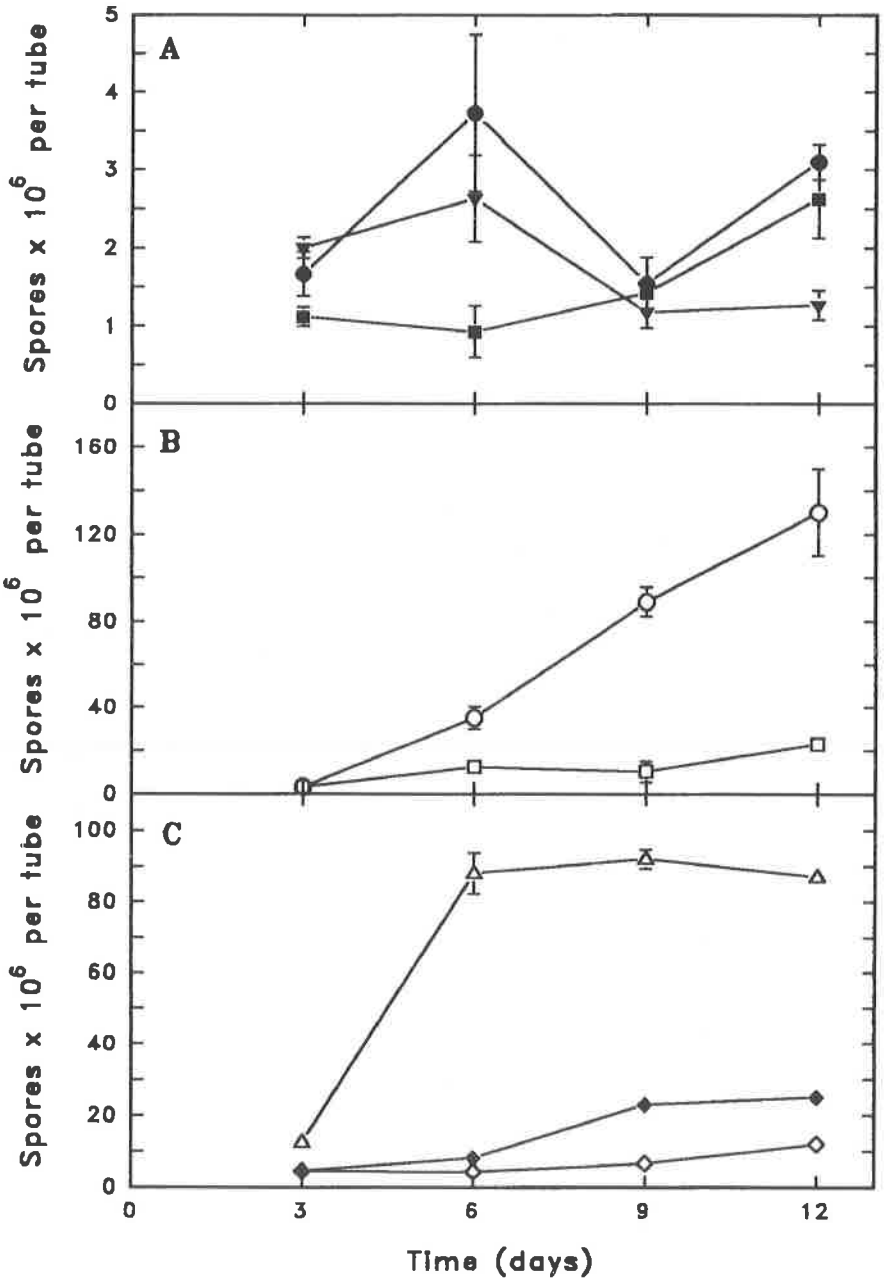

Figure 1: Spore formation by *Humicola* sp. under difrent growth conditions:
A - Sporulation on PDA (pH 7.2/K_2HPO_4) after incubation for 12 days at 40°C in the presence of tungsten (▼) or fluorescent (●) light, and in the dark (■).
B - Sporulation on PDA (pH 7.2/K_2HPO_4) at 20°C in the presence of fluorescent light (○) and in the dark (□) after previous incubation for 3 days at 40°C.
C - Sporulation on PDA at pH 7.2 adjusted with K_2HPO_4 (Δ) or KOH (◆), and at pH 5.76 (◇), at 20°C in the presence of fluorescent light after previous incubation for 3 days at 40°C.

promotes sporulation in fungi has not yet been determined. It has been hypothesized that light may interfere with growth and start the chain of events that ends in sporulation (6).

Light is essential for the formation and maturation of reproductive structures. Piskorz-Binczycka (11) detected an endogenous rhythm characterized by a long period in the sporulation process (72 h).

The expression of the sporulation rhythm is sensitive to many factors such as the composition of the nutrient medium, pH, lighting conditions, and also, to the properties of the strain itself (14). Sargent and Kaltenborn (12) reported that, among the substrate components, amino acids play an important role in the regulation of the sporulation rhythm.

The nutrient medium and its composition, in particular, are among the major factors determining the expression of the conidiation rhythm (3, 12, 13). According to Feldman and Dunlap (8), some sugars used in the nutrient medium (ribose, sucrose) have a stimulatory effect on the conidiation rhythm in *Neurospora*. Esser (7) and West (16) described the influence of the acidity of the nutrient medium as a regulatory factor which may occasionally determine the conidiation rhythm. In a study on *Neurospora crassa*, West (16) reported that the occurrence of the conidiation rhythm was observed in the pH range of 6.5 to 7.0.

Adjustment of the pH to 7.2 with K_2HPO_4 favored sporulation, suggesting that phosphate ion may have some influence on the production of spores by *Humicola* sp. (Fig. 1C). Sporulation was lower when the pH of PDA was adjusted with KOH.

ACKNOWLEDGMENTS

The authors thank CNPq for the financial support.

RESUMO

Condições ambientais que afetam a formação de conídios em *Humicola* sp

O fungo *Humicola* sp. foi cultivado em PDA com o pH do meio ajustado para 7.2 utilizando-se K_2HPO_4 ou KOH, e não ajustado (pH 5.76). As culturas foram comparadas quanto à capacidade de esporular no escuro ou na presença da luz, utilizando-se lâmpadas de tungstênio (6 volts/5w) ou lâmpadas fluorescentes (15 volts/54w), às temperaturas de 40°C (crescimento do fungo) e 20°C.

A melhor esporulação foi obtida quando o pH do meio foi ajustado com K_2HPO_4 e a cultura incubada a 20°C sob luz fluorescente.

Palavras-chave: *Humicola* sp., esporulação, pH, luz, temperatura

REFERENCES

1. Araújo, E. F.; Barros, E.G.; Caldas, R.A.; Silva, D.O. β-glucosidase activity of a thermophilic cellulolytic fungus, *Humicola* sp. *Biotechnol. Lett.* 5: 781784, 1983.
2. Barnett, A.L.; Hunter, B.H. *Illustrated genera of imperfect fungi*. 3rd ed. Burgess, Minneapolis, 1972, 241p.
3. Brody, S.; Harris, S. Circadian rhythms in *Neurospora*. *Science* 180, 498500, 1973.
4. Chaves, V.M.G. *Caracteristicas fisiológicas de um fungo termófilo isolado de compostagem e propriedades de seu complexo celulase*. Viçosa,1982, 60 p.(M.S. Thesis. Universidade Federal de Viçosa - Brasil).
5. Chaves, V.M.G.; Silva, D.O.; Brune, W.; Moreira, M.A. Cellulolytic activities of *Humicola* sp. *Rev. Microbiol.* 20: 460465, 1989.
6. Cochrane, V.W. *Physiology of fungi*. John Wiley and Sons, New York, 1963, 524p.
7. Esser, K. The influence of pH on rhythmic mycelial growth in *Poolospora anserina*. *Mycologia* 61: 10081011, 1969.
8. Feldman, J.F.; Dunlap, C.J. *Neurospora crassa*: A unique system for studying circadian rhythms. *Photochem. Photobiol. Rev.* 7: 319368, 1983.
9. Monti, R.; Terenzi, H.F.; Jorge, J.A. Purification and properties of an extracellular xylanase from the thermophilic fungus *Humicola grisea* var. *thermoidea*. *Can. J. Microbiol.* 37: 675686, 1991.
10. Peralta, R.M.; Terenzi, H. F.; Jorge, J.A. β-D-glycosidase activities of *Humicola grisea*: biochemical and kinetic characterization of a multifunctional enzyme. *Biochim Biophys. Acta*, 1033: 243249, 1990.
11. Piskorz-Binczycka, B. Rhythm of sporulation within the section *Penicillium clavigerum* I. Induction of endogenous rhythms in *Penicillium clavigerum* Demelius 310.48. I. *Acta Biologica Cracoviensia* series: *Botanica* 33:1323, 1991.
12. Sargent, M. L.; Kaltenborn, S. H. Effects of medium composition and carbon dioxide on circadian conidiation in Neurospora. *Plant Physiol.* 50, 171175, 1972.
13. Sargent, M.L.; Broggs, W.R.; Woodward, D.O. The circadian nature of rhythm expressed by an invertaseless strain of *Neurospora crassa*. *Plant Physiol.* 41, 13431349, 1966.
14. Sweiger, H.G. Circadian rhythms in unicellular organisms: An endeavor to explain the molecular mechanism. *Int. Rev. Cytol.* 51:171175, 1978.
15. Tosi, L.R.O.; Terenzi, H.F.; Jorge, J.A. Purification and characterization of an extracellular glucoamylase from the thermophilic fungus *Humicola grisea* var. *thermoidea*. *Can. J. Microbiol.* 39: 846 852, 1993.
16. West, D.J. Effect of pH and biotin on a circadian rhythm of conidiation in *Neurospora crassa*. *J. Bacteriol* 123, 387389, 1975.
17. Zimmermann, A. L. S.; Terenzi, H. F.; Jorge, J. A. Purification and properties of an extracellular conidial trehalase from *Humicola grisea* var. *thermoidea*. *Biochim. Brophys. Acta*, 1036: 4146, 1990.

Revista de Microbiologia (1996) 27:255-262
ISSN 0001-3714

EFFECT OF LECITHIN AND SOY OIL ON THE FERMENTATIVE PERFORMANCE OF *SACCHAROMYCES UVARUM* I Z 1904

Marta Cristina Teixeira Duarte[1]*, Alcides Serzedello[2], Gil Eduardo Serra[1], Maria Cecília de Faveri Leite de Oliveira[2], Alexandre Nunes Ponezi[1], Adilson Sartoratto[1]

[1]Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, CPQBA/UNICAMP, Paulínia, SP, Brasil; [2]Departamento de Bioquímica e Microbiologia - Instituto de Biociências - UNESP/Campus de Rio Claro, Rio Claro, SP, Brasil;

ABSTRACT

The proposal of this work was to study the effects of lecithin and soy oil on the fermentative performance of *Saccharomyces uvarum* I Z 1904, a yeast used in the industrial production of ethanol. High Test Molasses (HTM) was chosen as the fermentation media because it is a substratum that is poor in nutrients, and because it permits one to distinguish the action of lipids from other nutritional factors. The study of the optimization of the concentration of lipids by surface response analysis showed that the lipids favor the performance of the yeast principally when applied separately. Maximum concentrations of the two sources of lipids in the media stimulated the budding rate but did not constitute a protection against cell death. Considering the action of lipids on the cellular parameters studied, the supplementation of the media with 3.0 g/l of soy oil permitted the obtention of maximum responses of cellular viability, budding rate and viability of the buds after 6 successive cycles. In relation to the fermentative parameters, the use of 1.5 g/l of soy oil provided high yields and an equilibrium between the mass of ethanol produced (EM) and the alcoholic yield ($Y_{p/s}$) , whereas the cellular viability after 6 cycles did not differ statistically from that observed with 3g/l of oil.

Key words: *Saccharomyces*, ethanol, lipids metabolism, soy oil, soy lecithin.

INTRODUCTION

During the microbial production of ethanol, the performance of the fermentative yeast can be hindered by three main factors: nutrient impoverishment, unfavorable physical conditions of the media, or by the so called "staling" effect, which occurs due to the intracellular or extracellular accumulation of products of the metabolism itself, which limits the concentration of alcohol that can be reached (9). Ethanol itself is known to be the main product responsible for inhibition of fermentation, to which the diminishment of cellular viability of yeast is associated (14).

Hayashida *et al.* (10, 11, 12) verified that in the production of sake, the cells of *Saccharomyces sake* acquired greater resistance to ethanol because they grow in the presence of an extract of *Aspergillus oryzae* mycelium, rich in unsaturated fatty acids. In this way while in the majority of fermentative processes the maximum concentration of ethanol obtained is 10-12 % (v/v), the sake production can reach concentrations of up to 20 %. Other authors confirm the same action of lipids (15, 16).

* Corresponding author. Mailing address: Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, CPQBA/UNICAMP, Estrada PLN 393 nº. 999, CEP 13140-000, Paulínia, SP, Brasil.

The tolerance that yeast has to ethanol is related to the composition of the lipids in the cytoplasmatic membrane (5,18, 19, 21, 22), where the fatty acids perform important roles, not only from the structural point of view but also from a physiological one. In the absence of oxygen, a condition that establishes itself during fermentation, the yeast cells are incapable of growing because they do not synthesize unsaturated fatty acids and sterols (2, 3); Tyagi (23) relates similar observations in which, because of the absence of oxygen and an exogenous sources of lipids, the cells stops growth after 4 or 5 generations. The fermentation and the cellular viability can be prolonged by supplementation of the media with specific lipidic compounds, as well as some vitamins and proteins, capable of giving good results if applied industrially (17, 24, 1).

The main objective of this work was to study the use of soy oil and lecithin as lipidic supplements for the fermentation media of *Saccharomyces uvarum* I Z 1904, establishing an optimal concentration of lipids according to their effects upon the cellular viability, budding and fermentative activity. The soy oil was chosen for this study because it is a relatively low cost source of lipids, and because of its effects on fermentation which were verified before (1). The phospholipid lecithin is a natural addictive widely used in the chemical industry and food industry. Its functionality is great capable of acting as an emulsifier, stabilizer, dispersant, among others. The concomitant use of these lipids is attractive because lecithin can facilitate the dispersion of oil in the media.

MATERIALS AND METHODS

Microorganism. *Saccharomyces uvarum* I Z 1904 proceeding from Departamento de Ciências e Tecnologia Agroindustrial da ESALQ-USP. The yeast was preserved in tubes with solid YEPD media, refrigerated at 5°C. The renovation of the cells was done by inoculation of cells in the media, and incubation at 30°C for 48 hours.

Activation of the culture. The culture was activated before the fermentations, in a media containing (g/L): glucose, 40.0; KH_2PO_4, 5.0; NH_4Cl, 1.5; $MgSO_4.7H_2O$, 1.0; KCl, 1.0; yeast extract, 2.0; distilled water up to 1000 ml; pH 6.0. After growth in the YEPD media, cell samples were transferred to a 500 ml Erlenmeyer flask, containing 100ml of activation media and incubated at 30°C in rotary shaker (280 rpm), for 24 hours.

Dissemination of cells for inoculum. The media used for growth was HTM (High Test Molasses) at a concentration of 10°Brix, supplemented with 10 g/L of yeast extract. For the production of inoculum in an adequate quantity for fermentation, the dissemination was performed in two cycles. Aliquots of the activated culture were transferred to an 500 ml Erlenmeyer flask containing the media of HTM, keeping the proportion 12:100 (v/v). The cultures were incubated at 30°C under an agitation of 280 rpm for 24 hours, and the cellular mass obtained was aseptically separated from the media by centrifugation (5600 x g, 10°C, 10 min). The cells originated were reinoculated in the growth media, and subject to growth as before. After 24 hours the cultures were centrifuged, and the biomasses combined and suspended in the total volume of medium necessary for the first fermentative cycle, to guarantee homogeneous inoculum. Identical volumes were then distributed in the fermentation flasks. The number of cells at the beginning of the fermentation was of the order of 10^8/ml, and the cellular viability superior to 97%.

Experimental Planning - Optimization of the soy oil and lecithin concentration. To determine the ideal concentration of lipids in the fermentation media of *S. uvarum* I Z 1904, a complete factorial delineation 3^2 was used, according to Box *et al.* (6), with equidistant levels of two independent variables, in three variation levels. The factors or independent variables studied were: lecithin concentration (x_1) and soy oil concentration (x_2).The levels of the variables were determined from preliminary tests, and according to the statistical delineation 9 experiments were performed at random, in duplicate (Table 1). The answers or dependent variables studied were: cellular viability (Y_1), budding rate (Y_2) and ethanol yield (Y_3).

Table 1 - Experiments performed by factorial combination between the levels of x_1 e x_2.

Experiment	Level	Lecithin g/l	Soy Oil g/l
1	-1 -1	0,0	0,0
2	-1 0	0,0	1,5
3	-1 +1	0,0	3,0
4	0 -1	1,0	0,0
5	0 0	1,0	1,5
6	0 +1	1,0	3,0
7	+1 -1	2,0	0,0
8	+1 0	2,0	1,5
9	+1 +1	2,0	3,0

Fermentation with cell recycle. The experiments were conducted during 60 hours, divided in 6 cycles of 10 hours. HTM at a concentration of 18° Brix was used as a fermentation media. Aliquots of 50 ml of the inoculated fermentation media, were distributed in 125 ml Erlenmeyer flasks .With the exception of the control flasks, the oil and/or lecithin were added. The flasks were incubated under an agitation of 80 rpm and a temperature of 32°C for 10 hours. After this, a volume equivalent to 10 % of each culture was removed for analysis. The wine was separated by centrifugation (5600 x g, 10°C, 10 min) and the biomass originating from each flask was suspended again in 125 ml of fermentation media, in a corresponding flask, initiating the second fermentative cycle; this was repeated successively for 6 cycles. Samples of the corresponding cultures were analyzed at the end of the sixth cycle for the following parameters.

Cell counting and cellular viability. The counting of the cells was performed in a Neubauer camera and microscope. The evaluation of the number of cells and buds which were dead in the culture, using a coloring technique with erythrosine (20). The budding rate of the yeast was calculated from the data here referred to.

Determination of ethanol. Samples of the fermented wine were analyzed for the ethanol production in a gaseous chromatograph (HP 5890). Ethanol solutions of 5 and 10 % were used as external standard. Acetone (10% in water) was used as an internal standard. The operating conditions of the chromatograph were the following: HP FFAP column (capillarity 50 m; ID 0.2 mm; film 0.3 μm); temperature: injection 80°C, column 70°C, detector 150°C; injected volume 1 μl; flow of He 1ml/min.

Determination of TRS (Total Reducing Sugars). The proportion of TRS in the initial media and of the fermented wine was determined by the DNS method (13).

Ethanol yield ($Y_{p/s}$). It was calculated from the data of mass balance of produced ethanol (EM) and of the mass of sugar consumed (SM) in fermentation, in grams (4), by the formula:

$Y_{p/s}$ = EM/SM in which
EM = (EFF-TEF) x ETOHF - [(EFI - EFE) x 0.7 x ETOHP]
SM = (EFE - TEF) x TRSMF + (EFI - EFE) x 0.7 x TRSP - (EFF - TEF) xTRSF where:
EFF = weight of the Erlenmeyer at the end of fermentation (g)
TEF = weight of the empty Erlenmeyer (g)
ETOHF = ethanol produced at the end of the cycle (g/)
EFI = weight of the Erlenmeyer with inoculum (beginning of fermentation)(g)
EFE = weight of Erlenmeyer with only half (g)
0.7 = interstitial wine (g/g of biomass)
ETOHP = ethanol produced at the end of the cycle before (g/g)
TRSMF = sugar concentration in the must (g/g)
TRSP = sugar concentration at the end of the cycle before (g/g)
TRSF = sugar concentration at the end of fermentation (g/g)

Statistical analysis. The results obtained at the end of the 6th cycle for the answers studied in the tests performed by experimental planing, were submitted to an analysis of variance. The comparison of the mean values was done by the statistical test of Tukey (8), with confidence intervals to the level of 5 %. The program SANEST 2 (25) was used for this purpose.

Analysis by the response surface. The results related to the answers obtained at the end of the 6th fermentative cycle were modeled by the following quadratic polynomial equation: $Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1^2 + \beta_4 x_2^2 + \beta_5 x_1 x_2$ were:
Y = answer or dependent variable
β_0 = coefficient of the zeroth order effect
β_{1-2} = coefficient of the first order effects
β_{3-4} = coefficient of the second order effects
β_5 = coefficients of the interaction effects

The analysis of the data was performed according to Bruns *et al.* (7), and by the Statistical Analysis System (SAS).

RESULTS AND DISCUSSION

The supplementation of the fermentation media with lipids at different combinations of concentration, had a positive cumulative effect on the cellular viability of the yeast, confirmed by the higher mean values in relation to the non-supplemented test (Table 2). Comparing the results obtained in the media without lipids with those of the media supplemented with only soy oil, the effect of the oil on the viability of cells was found out to be proportional to the concentration used. In this way, higher mean values of cellular viability were obtained in the presence of 1.5

Table 2 - Celllular viability, budding and ethanol production for *S. uvarum* I Z 1904 cultivated in HTM supplemented with lecithin and/or soy oil. Mean values obtained at the end of the 6th fermentative cycle.

Test	Soy Lecithin g/l	Soy Oil g/l	Cellular Viability %	Budding rate %	Bud Viability %	SM g	EM g	$Y_{p/s}$
1	-	-	65,03	65,43	81,13	5,17	1,93	0,37
2	-	1,5	73,55	68,67	82,45	5,68	2,69	0,47
3	-	3,0	77,02	74,63	89,18	5,69	2,52	0,44
4	1,0	-	67,34	63,95	77,47	5,88	2,54	0,43
5	1,0	1,5	70,04	65,00	87,50	6,03	2,52	0,42
6	1,0	3,0	67,09	78,61	86,88	6,04	2,09	0,35
7	2,0	-	72,00	67,10	81,31	6,15	2,53	0,41
8	2,0	1,5	66,30	70,64	81,25	5,15	2,48	0,48
9	2,0	3,0	68,70	83,47	83,98	5,99	2,57	0,43

Initial data: Cellular viability = 96,12%; Budding rate = 32,95%; Bud viability = 96,26%; Total cells = 6,13 x 10^8/ ml; Total bud = 2,02 x 10^8/ ml; TSR initial = 18,10 g/ 100 ml. Culture temperature = 32°C; Agitation = 80 rpm.

Table 3 - Cellular viability (%), budding rate (%) and ethanol mass produced (g) for *S. uvarum* I Z 1904 cultivated in HTM supplemented with lecithin and soy oil. Comparison between mean values obtained at the end of the 6th fermentative cycle by the Tukey test.

Lecithin	Cellular viability Soy			Budding rate Soy			ME Soy		
g/l	0,0	1,5	3,0	0,0	1,5	3,0	0,0	1,5	3,0
0,0	65,03bB	73,55aA	77,02aA	65,43aB	68,67aAB	74,63bA	1,93bB	2,69aA	2,52aA
1,0	67,34bA	70,04aA	67,09bA	63,95aB	65,00aB	78,61abA	2,54aA	2,52aA	2,09bB
2,0	72,00aA	66,30bB	68,70bAB	67,10aB	70,64aB	83,47aA	2,53aA	2,48aA	2,57aA

The means followed by different letters differ between thenselves at a level of significance of 5% by the Tukey test. DMS = 3,69.
Small letter = vertical comparison = test for lecithin means in the cycles related to the soy factor.
Capital letter= horizontal comparison= test for soy means in the cycles related to the lecithin factor.

and 3.0 g/l of soy oil, were the cells showed a viability of 73.5 and 77.0%, respectively, contrasting to 65% of the non-supplemented media. This difference is statistically significant (Table 3). Similar effects were observed with lecithin. However, the effect of lecithin related to the non-supplemented media was significant only at concentrations of 2.0 g/l.

Cultivated cells in a media supplemented at the same time with lecithin and oil showed viability close to the one observed in a media containing the lipids apart only when intermediate concentrations of each one were used (test 5). Maximum concentrations of lecithin + oil resulted in viabilities close to the ones of non-supplemented media.

The budding of the cells in the presence of soy oil was proportional to its concentration (Table 2), which corresponded to the largest number of viable cells found in these conditions. In test 3, greater budding rates went together with a greater number of viable buds in the cultures and a larger population. There was no difference of budding between the test without supplementation and those in which the media received only lecithin, but there was an increase in budding when lecithin was used together with oil. The effects observed in the presence of 3.0 g/l of oil, increased gradually when 1.0g/l and 2.0 g/l of lecithin were added (Table 2). The statistical analysis shows that after 6 fermentative cycles, the budding mean values of tests 6 and 9 differ statistically from the rest (Table 3). Since the cellular viability in these tests did not differ from the one found in the media without supplementation, one can state that the lipids, when used in these concentrations, stimulate the budding rate but are not a protection against cellular death.

The effect on budding of the oil together with the lecithin could be a reflection of the dispersant function of the phospholipid. A greater dispersion of the oil in the media is believed to have facilitated the incorporation of the lipids by the cells, increasing the budding rate. However, the reason why the viability of the cells and buds did not agree is not yet clear. A possible explanation for this fact, is that in tests 6 and 9, the total concentration of lipids in the media in each cycle reached respectively 4.0 and 5.0 g/l, which may indicate an intoxicating effect on the cells. Lafon-Lafourcade *et al.* (15), mention that the addition of lipids in concentrations above those necessary to assure a satisfactory cellular structure, ends up

restricting fermentation, diminishing the permeability of the membrane and restraining the exchange of substances between cells and the media.

The use of 3.0 g/l of soy oil stimulates budding and does not affect the viability of the buds, and this is what must have been reflected in higher proportions of viable cells. A decrease of the yeast's mortality in the presence of soy oil was verified before (1), when the soy oil showed a protecting effect two times greater than oleic acid .

Apart from the action on cellular viability, the synergistic effect that the oil and lecithin exerted on the budding rate was evident, indicating that a greater lipidic enrichment of the plasma membrane may have stimulated the budding.

Taking into consideration the action of the lipids on the cellular parameters studied, the supplementation of the media with 1.5 and 3.0 g/l of soy oil showed a positive effect on cellular viability, consistent with the viability of the buds. The other treatments did not show consistent effects or showed only isolated effects related to the parameters.

Considering the ethanol mass produced (EM) at the end of 6 fermentative cycles, the yeast produced greater quantities in the presence of 1.5 g/l of soy oil and 2.0 g/l of lecithin + 3.0 g/l of soy oil (Table 2, tests 2 and 9). However the cells were more efficient in the conversion of sugars ($Y_{p/s}$) when cultivated in HTM containing 1.5 g/l of oil and 2.0 g/l of lecithin + 1.5 g/l of soy oil, respectively (Table 2, tests 2 and 8).

The results of Table 2 express that a greater sugar mass (SM) consumed did not implicate necessarily in greater ethanol yields. In the case of test 6, a high quantity of TRS was consumed in 10 hours, but corresponded to a low $Y_{p/s}$ value. Since this consumption of TRS did not result either in an increase of biomass, the consumption of media may be associated to metabolic activities in response to a cellular intoxication by lipids.

The fact that the yeast showed greater cellular viability after 6 cycles in the media with 3.0 g/l of soy oil (test 3), did not incur the obtention of higher ethanol yields ($Y_{p/s}$).

The comparison of the mean values of the ethanol produced (EM) performed by the Tukey test (Table 3), indicates that the ethanol production by the yeast was significantly lower than in test 1 and 6. Although there were no striking differences between the other supplemented tests, the use of 1.5 g/l of soy oil showed high and equilibrated yields between EM and $Y_{p/s}$.

Evaluation of the behavior of the responses after 6 fermentative cycles through the analysis of the response surface. The results obtained at the end of the 6th fermentative cycle for the answers or dependent variables studied, that is, cellular viability (Y_1), budding rate (Y_2) and ethanol yield (Y_3), were molded by the quadratic polynomial equation indicated before, and submitted to the analysis of surface response. The analysis of variance for the models is shown in Table 4.

According to the results, the analysis of variance applied to the model produced for cellular viability (Y_1) showed that it is significant at a confidence level of 95%, which indicates that the variable responded to the treatments in the interval studied of lipidic concentration. The lack of fit was not significant. The proportion of variance explained by model (R^2) is considered good, which indicates a good correlation between model and experimental data. Besides, the variance coefficient lower than 10% is satisfactory, which means a reinforcement to the fit of the model with the data. Considering that the linear and interaction effects (Crossproduct) are significant, the quadratic effects can be eliminated from the model making it simpler. The analysis also showed that the two variables studied are significant. The analysis shows that the model can be used for predicting purposes.

Through the surface response graphic (Fig. 1), it is observed that the yeast exhibited lower viability both when cultivated without lipid supplementation (level -1 -1) and in a media containing the maximum concentration for each lipid (level +1 +1). The Figure indicates that the tendency of cellular viability was to increase in the presence of the maximum levels of lecithin or soy oil.

Table 4 - Analysis of the variance and significance of the variables studied, executed by the Statistical Analysis System (SAS).

Independent variables	Cellular viability (%)	Budding rate (%)	Ethanol yield (%)
Linear	0,0090*	0,0000*	0,8698
Quadratic	0,2411	0,0677	0,0179*
Crossproduct	0,0009*	0,0837	0,2579
Total Regression	0,0035*	0,0002*	0,0780
x_1 (g/l)	0,0029*	0,0710	0,2365
x_2 (g/l)	0,0024*	0,0001*	0,0677
R^2	0,8846	0,8595	0,5521
Variation Coef.	2,77	4,69	9,48
Lack of Fit	0,0939	0,7470	0,0058*

* F significant test. Significance level ($p < 0,05$)

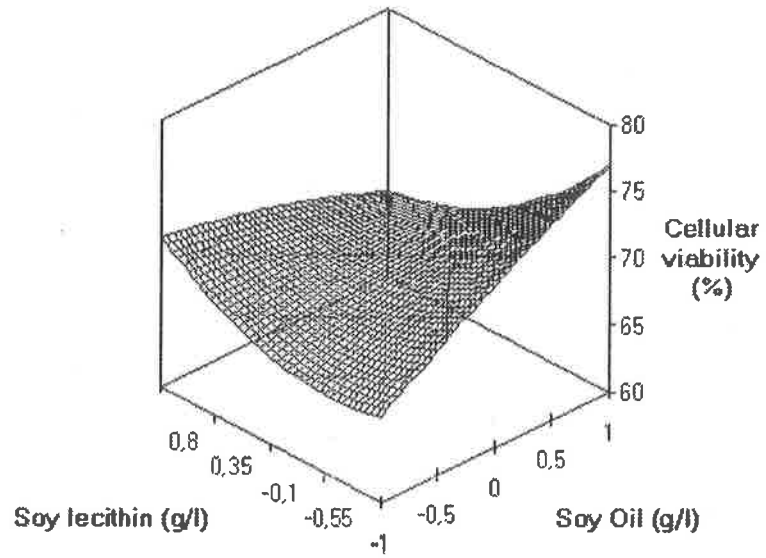

Figure 1 - Response surface for the variable cellular viability (%) of *S. uvarum* I Z 1904 cultivated in HTM supplemented with lecithin and soy oil, after 6 fermentative cycles.

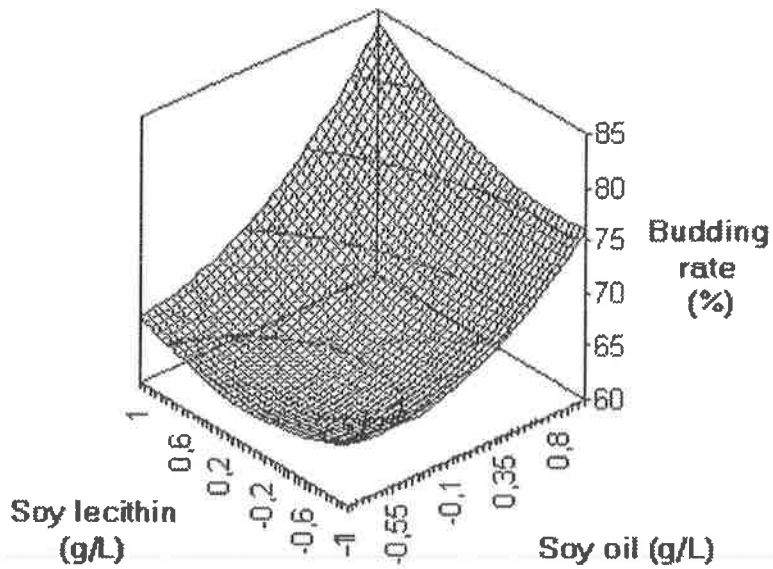

Figure 2 - Response surface of the variable budding rate (%) of *S. uvarum* I Z 1904 cultivated in HTM supplemented with lecithin and soy oil, after 6 fermentative cycles.
Regression Equation: $Y_2 = 66{,}37 + 2{,}07\ x_1 + 6{,}87\ x_2 + 2{,}74\ x_1^2 + 3{,}85\ x_2^2 + 1{,}85\ x_1.x_2$

The model obtained for budding rate (Y_2) is highly significant at the confidence level of 95%, and the lack of fit is not significant (Table 4). The analysis indicated that the only significant variable for the budding rate is x_2, that is, soy oil. In this way, it would be possible to make predictions for the budding rate using the model; if the model were valid for different values of lecithin and soy oil than those studied in this present work, extrapolations could be made to obtain budding values. Fig. 2 shows that the budding rate was especially favored when the media was supplemented with the maximum concentrations of lipids (level +1 +1), but that budding was also favored by the concentration increase of soy oil. The lecithin had no

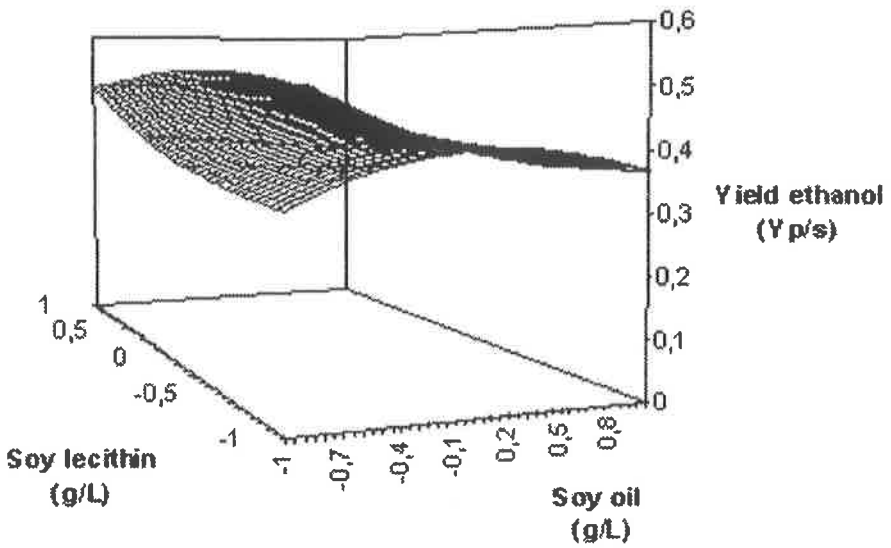

Figure 3 - Response surface for the variable ethanol yield ($Y_{p/s}$) produced by *S. uvarum* I Z 1904 cultivated in HTM supplemented with lecithin and soy oil, after 6 fermentative cycles.
Regression Equation: $Y_3 = 0,43 + 0,06\ x_1 - 0,0008\ x_2 + 0,038\ x_1^2 - 0,06\ x_2^2 - 0,02\ x_1.x_2$

effect on the yeast budding in the studied concentration intervals.

The variance analysis obtained for the model referring to the ethanol yield (Y_3) is not significant at a confidence level of 95 %, a result which indicates that the alcoholic yield did not depend on the changes of the lecithin and soy oil levels in the concentration intervals used in these tests. Furthermore, the significant lack of fit and low proportion of variance explained (R^2- Table 4), show that the quadratic model did not adequate itself to the experimental data. This happened because the addition of the squares of the experimental error is a very small value (0.003950), and for this reason was not taken into consideration. Neverthheless, it is possible that some other variable should have been controlled for the achievement of a more satisfactory fit of the data, or that the quadratic model is to simple to represent the data. The variance coefficient is satisfactory, but the model is not recommended for the prediction of results, mainly because of the value of R^2, so that it can be used only for a study of the tendency of the response. In relation to the significance's, only the quadratic effects (x_2x_2) are significant.

The fact that the lack of fit was significant to the response of the ethanol yield and not significant to other responses, supports the assumption that this lack of fit depends more on the complexity of the data than on the estimation of error, which had lower values for most of the terms of the model (data not shown). Therefore, it would be possible to try to mold the results with a cubic model. However, this model would be far too complex for the study, and in the attempt to explain the results, it could lead to graver errors than the lack of fit observed. Since there was not an adequate fit between the model and the data obtained for Y_3, Fig. 3 shows only a tendency of this response. According to the graphic, higher yields of ethanol are obtained with concentrations of 1.5 g/l of soy oil, or with its combination with the maximum concentration of lecithin employed.

RESUMO

Efeitos da lecitina e do óleo de soja sobre o desempenho fermentativo de *Saccharomyces uvarum* IZ 1904

A proposta deste trabalho foi estudar os efeitos da lecitina e do óleo de soja sobre o desempenho fermentativo de *Saccharomyces uvarum* I Z 1904, levedura utilizada para produção industrial de etanol. High Test Molasses (HTM) foi escolhido como meio de fermentação por ser um substrato pobre em nutrientes, e permitir distinguir a ação dos lipídios da de outros fatores nutricionais. O estudo de otimização da concentração dos lipídios por análise de superficie de resposta mostrou que os lipídios favoreceram o

desempenho da levedura principalmente quando aplicados separadamente. Máximas concentrações das duas fontes de lipídios no meio estimularam o brotamento mas não se constituíram em proteção contra a morte celular.

Considerando a ação dos lipídios sobre os parâmetros celulares estudados, a suplementação do meio com 3,0 g/l de óleo de soja permitiu obter máximas respostas de viabilidade celular, taxa de brotamento e viabilidade dos brotos após 6 ciclos sucessivos. Em relação aos parâmetros fermentativos, o emprego de 1,5 g/l de óleo de soja propiciou rendimentos elevados e um equilíbrio entre a massa de etanol produzida (ME) e o rendimento alcoólico ($Y_{p/s}$), sendo que a viabilidade celular após 6 ciclos não diferiu estatisticamente da observada com 3 g/l de óleo.

Palavras-chave: *Saccharomyces*, etanol, metabolismo lipídico, óleo de soja, lecitina de soja.

REFERENCES

1. Alterthum, F.; Mello Cruz, M. R. - Aumento do rendimento da fermentação alcoólica pela adição de óleo de soja. *Rev. Microbiol.* 18: 52-57, 1987.
2. Andreasen, A. A.; Stier, T. J. B. - Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. *J. Cell. Comp. Physiol.* 41:23-36, 1953.
3. Andreasen, A. A.; Stier, T. J. B. - Anaerobic nutrition of *Saccharomyces cerevisiae* . II. Unsaturated fatty acid requirement for growth in a defined medium. *J. Cell. Comp. Physiol.* 43: 271-281, 1954.
4. Andrietta, S. R; Andrietta, M. G. S.; Rodrigues, M. I. - Método de caracterização de leveduras de processo utilizando parâmetros cinéticos e produção especifica. *STAB* 13: 22-25, 1995.
5. Beaven, M. J.; Charpentier, C.; Rose, A. H. - Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. *J. Gen. Microbiol.* 128:1447-1455, 1982.
6. Box, G. E. P.; Hunter, W. G.; Hunter, J. S. - *Statistics for experiments, and introduction to design and model building.* John Wiley; Son Ed., New York, 1988.
7. Bruns, R. E.; Barros Neto, B.; Scarminio, I. S.- Otimização e Planejamento de Experimentos. *Uma Introdução à Quimiometria.* Editora da Unicamp. In the press.
8. Gomes, F. P. - O Teste de Tukey. *In: Curso Experimental de Estatistica*, Ed. Nobel, SP, 1982, p. 38-41.
9. Gray, W. D. - Studies on the alcohol tolerance of yeasts. *J. of Bacteriol.* 42: 561-574, 1941.
10. Hayashida, S.; Feng, D. D.; Hongo, M. - Function of the high concentration alcohol producing factor. *Agric. Biol. Chem.* 38: 2001-2006, 1974.
11. Hayashida, S.; Feng, D. D.; Hongo, M. - Physiological Properties of yeast cells grown in the proteolipid- supplemented media. *Agric. Biol. Chem.* 39: 1025-1031, 1975.
12. Hayashida, S.; Ohta, K. - Cell structure of yeasts grown anaerobically in Aspergillus oryazae-protolipid-supplemented media. *Agric. Biol. Chem.* 42:1139-1145, 1978.
13. Hostettler, F.; Borel, E.; Devel, H. - Helv. Chim. Acta, 34: 2132, 1951. *In* "H. V. Bergmeyer and K. Gawehn (eds), *Methods of Enzymatic Analysis*", Academic Press, N. Y., Vol. 2, 1972, p.885.
14. Jones, R. P. - Intracellular ethanol- accumulation and exit from yeast and other cells. *FEMS Microbiol. Rev.* 54: 239-258, 1988.
15. Lafon-Lafourcade, S. F. ; Larue, F.; Brechot, P.; Ribereau-Gayon, P. - Les steroids "facteurs de survie" des levures au cours de la fermentation alcoolique du moit de raisin. *C. R. Acad. Sci. Ser. D.* 284: 1939-1942, 1977.
16. Larue, F.; Lafon-Lafourcade, S. ; Ribereau-Gayon, P. - Relationship between the sterol content of yeast cells and their fermentation activity in grape must. *Appl. Environ. Microbiol.* 39; 808-811, 1980.
17. Mello Cruz, M. R.; Alterthum, F.; Santos, A. L. - *Aumento do rendimento da fermentação alcoólica com a adição de ácido oléico.* Anais do VI Simp. Nac. Ferm., 1982, p. 5.
18. Rose, A. H. - *In: "Biology and Activities of Yeasts"*. F A. Skinner. S. M. Passmore and R. R. Davenport, eds. Academic Press, London, 1980, p. 103.
19. Rose, A. H.; Beavan, M. J. - End-product tolerance and ethanol. *In:* Holland, A., ed. - *Trends in biology of fermentation for fuels and chemicals.* New York, Academic Press, 1980, p. 513-531.
20. Sharf, J. M. - *Exame Microbiológico de Alimento.* Editora Polígono, 2a. ed., 1972, 257pp.
21. Thomas, D. S.; Hossack, J. A.; Rose, A. H. - Plasma-membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*. *Arch. Microbiol.* 117: 239-245, 1978.
22. Thomas, D. S.; Rose, A. H. - Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affect by plasma-membrane lipid composition. *Arch. Microbiol.* 122: 49-55, 1979.
23. Tyagi, R. D. - Participation of oxygen in ethanol fermentation. *Process Biochem.* 19:136-141, 1984.
24. Viegas, C. A.; Sá-Correia, I.; Novais, J. M. - Nutrient-enhanced production of remarkably high concentrations of ethanol by *Saccharomyces bayanus:* mechanisms of action of soy flour supplementation. *Biotechnol. Lett.* 7: 515-520, 1985.
25. Zonta, E. P.; Machado, A. A. - Sanest 2, *Sistema de análise Estatistica para microcomputadores*, SEI 066060, 1992.

Revista de Microbiologia (1996) 27:263-267
ISSN 0001-3714

ETHANOL PRODUCTION FROM LACTOSE AND WHEY BY *ESCHERICHIA COLI* EXPRESSING GENES FROM *ZYMOMONAS MOBILIS*

Maria Lígia Carvalhal, Gerson Henrique Wuthstrack, Luciana Peixoto Montoro, Débora Fumie Takahashi and Flávio Alterthum*

Departamento de Microbiologia - Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brasil

ABSTRACT

The reduction of biochemical oxygen demand of whey with concomitant fuel production is one of the many challenges in biotechnology. *Escherichia coli ATCC 11775* containing plasmid-borne genes (pLOI297) encoding the enzymes for ethanol production from *Zymomonas mobilis* was used to convert lactose and whey to ethanol in batch fermentation. The best conditions were adjusted for: inoculum, lactose concentration and additional nutrients to whey fermentation. The inoculum was grown for six hours with agitation in Luria broth, under aerobic conditions. Inoculum was 10% of the fermentation's volume. With lactose concentrations higher than 60g/l, there is a detectable residue of galactose in the fermentation medium. The amount of ethanol produced from whey, supplemented with tryptone, was 38g/l with 85% efficiency of conversion in 60 hours. Yeast autolysate prepared either by addition of chloroform or heat treatment can replace tryptone as source of organic nitrogen. Further studies are needed to increase ethanol yield from whey as to convert this feedstock to potential for commercial expansion of fuel ethanol production.

Key words: *Escherichia coli*, whey, ethanol, lactose

INTRODUCTION

Whey is a by-product generated in large amounts during the making of cheese and represents an industrial waste disposal problem due to a high biochemical oxygen demand. In 1994, according to the Brazilian Association of Cheese Industries, cheese production was 290,000 ton/year and for each 100 liters of milk utilized, 90 liters of whey were generated. Although it is to some extent utilized by the food industry that extracts part of its protein content (1), lactose still remains as the main organic polutant. The fermentation of this sugar to produce ethanol employing *Kluyveromyces sp.* has been one of the alternative uses of whey (8, 12) yet with little success from an industrial standpoint, since ethanol concentrations above 30g per liter inhibit the yeast's activity (3,13). Other ways of producing ethanol from whey have been tried out such as the utilization of mixed cultures of *Kluyveromyces sp* and *Zymomonas mobilis* (7) or the insertion of the beta-galactosidase gene into *Saccharomyces cerevisiae* (11). The results obtained, however, are still incipient as the ethanol concentrations obtained are very low concerning to an industrial goal.

An alternative form of utilizing lactose from whey was recently proposed by Guimarães *et al.* (4), who employed *Escherichia coli* containing both the pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh) genes of *Z. mobilis* integrated to the chromossome (9). In the present work, a different genetically modified strain of *E. coli* harbouring the

* Corresponding author. Mailing address: Instituto de Ciências Biomédicas da Universidade de São Paulo. Av. Prof. Lineu Prestes, 1374. CEP05508-900 - São Paulo - SP. -Brazil. Fax:55-11-818-7354. E-mail: mlcarval@biomed.ICB2.USP.BR

same plasmidial genes and expressing them in large amounts (2) was employed. We tried to optimize the utilization of lactose from whey studying different ways of preparing the inoculum, various concentrations of whey and the addition of nutrients for better use of the fermentable substrate.

MATERIALS AND METHODS

Microorganism and culture medium: *Escherichia coli ATCC 11775* harbouring plasmid pLOI297 which contains the pdc and adh B genes of *Z. mobilis* (5) was used. Cultures were kept in Luria medium (LB) supplemented with lactose (20g/l) and tetracycline (12.5mg/l) (2)

Whey: According to the maker's analysis, the powdered whey used contained 72% lactose, 14% protein, 8% mineral salts, 1.5% fat and 4.5% humidity. Whey was dissolved in 0.2M phosphate buffer (pH 7.0) and pasteurized at 60°C for two hours before starting the fermentations.

For nutritional studies, whey was further supplemented with tryptone, yeast extracts and yeast hydrolysate. Yeast hydrolysate was obtained from 13g of dry yeast suspended in 100 ml of water containing 2g of $(NH_4)_3PO_4$ and treated in two different ways: a) 60°C for 18 hours and b) treatment with 1ml of chloroform at 60°C for 1 hour. In both cases, cell debris were removed by centrifugation and the supernatant used as yeast autolysate. Concentrations of tryptone and yeast extract are listed on Table 2. Yeast autolysate was added in the proportions of 1 to 19 or 1 to 7 of whey (v/v).

Inoculum: six isolated colonies for each 10ml of medium were inoculated in LB supplemented with 20g/l of lactose or in whey diluted so as to contain the same concentration of this sugar. The inoculum was incubated at 30°C for six hours with shaking, and the added volume was of 10% of the volume to be fermented. Other conditions are described in Results and Discussion.

Fermentations: Carried out in 200 ml round-bottom flasks containing 180ml of medium or whey at the concentrations specified for each test. The flasks were stoppered with rubber cap, which were perforated with a needle for sampling and outflow of gases. Fermentations were conducted at 30°C with shaking until all the sugar was consumed or ethanol was no longer produced.

Chemical Analyses: Ethanol and sugars were measured by HPLC as previously described (12). Some ethanol measurement were concomitantly made by the potassium dichromate oxidation method specified by Joslyn (6).

The results represent the mean of three or more tests and, for computations, the theoretical yield is of 0.54g ethanol/ per gram of lactose.

RESULTS AND DISCUSSION

Ethanol production from lactose and whey was previously compared between two American Type Culture Collection strains: *E.coli* ATCC 11303 (pLOI297) and ATCC 11775 (pLOI297). These strains were selected for their caracteristics in environment tolerance, plasmid stability, substrate range and ethanol production (2). When ethanol production was measured with whey as substrate E.coli ATCC 11775 presented better results (data not shown). On the basis of these comparative studies, strain ATCC 11775 appeared to be the best constructs for ethanol production from whey.

The inoculum was prepared in LB medium supplemented with lactose (20g/l) and tested taking into consideration the following parameters: time of growth (6 and 18 hours), shaking and stationary culture, presence or absence of oxygen and addition to the fermentation medium of either 10 or 20% of the volume. The conditions that gave the best results were: six hours of growth with shaking, presence of oxygen and inoculum of 10% of the volume. These parameters were taken as standard for all subsequent tests. The inoculum was made in LB medium even for the fermentation experiments carried out in whey, because this LB preparation proved to be physiologically better than the one made with whey.

Table 1 shows the results of fermentation using *E.coli* ATCC 11775 (pLOI297) in the presence of increasing concentrations of lactose. The conversion

Table 1 - Ethanol production by *Escherichia coli* ATCC 11775(pLO1297) in Luria broth containing different concentrations of lactose (96 hours).

Lactose (g/l)	Ethanol (g/l)	Yield (g et/g sug.)[a]	Efficiency[ab] (%)
60	32,2	0,53	99
80	40,6	0,50	94
100	40,8	0,40	75
120	44,4	0,37	68

[a] Calculations are based on total sugar added initially
[b] Calculations are based on theoretical maximum conversion of lactose (54g of ethanol per 100 grams of sugar)

is total and the efficiency is close to the maximum at up to 80g/l of the sugar. From this concentration onwards, a residue of lactose and galactose was observed in the medium, galactose being generated during the hydrolysis of lactose. The incorporation into *E.coli* of the *Z. mobilis* genes responsible for the production of ethanol from lactose, proved to be more successful (Table 1) than the use of mixed cultures of *Z. mobilis* and *K. fragilis* where the best ethanol yield was 0,43 (7). On the other hand, the ATCC 11775 strain was as efficient as the ATCC 11303 previoulsy employed (2) and the KO11 that had its plasmid incorporated into the chromosomal DNA (4).

To evaluate the influence of each of the components of the LB medium, fermentation experiments were carried out with added lactose (80g/l) followed by the separately addition of the following supplements: tryptone (10g/l), yeast extract (5g/l) and NaCl (5g/l). The results are presented in Table 2. Tryptone is a rich source of protein, peptides and amino acids. Although whey already contains proteins, the use of an additional source of nitrogenous compounds has been reported to cause a significant increase in the growth and in the production of alcohol (4). We observed that none of the components introduced led to a production of ethanol similar to that obtained with the standard Luria medium, yet both tryptone and yeast extract are two sources that may be employed whenever available and economically feasible.

Fermentation using concentrated whey containing 60g/l of lactose yielded a discrete production of ethanol (data not shown). For this reason, whey was supplemented with tryptone (10g/l) and tryptone plus lactose at the additional concentrations of 20, 40 and 60g/l. The results presented in Table 3 show that whey containing tryptone and lactose at up to 80g/l can be fermented with an efficiency of about 85%. Guimarães *et al.* (4) employed another recombinant strain of *E. coli* B (ATCC 11303, strain KO11) and cultured it in whey containing salts, vitamins and proteinases; they obtained, under the best conditions, 30g/l of ethanol starting from 60g/l of lactose. Higher concentrations led to yields and times of fermentation similar to the ones presently reported were whey was supplemented only with either tryptone or yeast extract.

Considering the performance of modified microorganims, however, this genetic makeup (5) proved to be superior to that described by Porro *et al.*(10), who transformed *Saccharomyces cerevisiae* and studied the fermentation of whey by it, obtaining yields not greater than 18g/l of ethanol in medium containing 60g/l lactose.

Tryptone at 10, 5 and 2.5 g/l was added to double concentrated whey (lactose=80g/l). The results, expressed in Figures 1A and 1B, show that lactose

Table 2 - Ethanol production by *Escherichia coli* ATCC 11775(pLO1297) in batch fermentation, in media containing lactose supplemented with tryptone, yeast extract and NaCl added together (LB) or separately

Lactose (8%) supplemented with	Ethanol (g/l)	Yield (g.et/g.sug[a])	VP(48hrs)[d] (g.et/l/h)	Efficiency[ab] (%)	Conversion efficiency[c] (%)
LB	40,6	0,50	0,70	94	94
Trypt.(10g/l)	31,9	0,39	0,62	74	89
Y.extr (5g/l)	32,3	0,40	0,61	75	80
NaCl (5g/l)	14,3	0,17	0,23	33	57

[a] Calculations are based on total sugar added initially.
[b] Calculations are based on theoretical maximum conversion of lactose (54g of ethanol per 100g sugar)
[c] Calculations are based on theoretical maximum conversion of lactose consumed.
[d] VP= Volumetric productivity - grams of ethanol/l/hour.

Table 3 - Ethanol production by *Escherichia coli* ATCC 11775(pLO1297) in batch fermentation, in whey (60g lactose/l) supplemented with tryptone and different lactose concentrations.

Whey plus:: Tryptone (10g/l)	Ethanol (g/l)	Yield[a] (g.et/g sug)	VP (72hrs)[d] (g.et/l/h)	Efficiency [ab] (%)	Conversion efficiency(%)[c]
Lactose 20g/l	35,4	0,44	0,49	82	82
Lactose 40g/l	38,4	0,38	0,53	70	85
Lactose 80g/l	39,7	0,28	0,55	51	82

[a] Calculations are based on total sugar added initially.
[b] Calculations are based on the theoretical maximum conversion of lactose (54grams of ethanol per 100 grams of sugar)
[c] Calculations are based on theoretical maximum conversion of lactose consumed.
[d] VP - Volumetric productivity - grams of ethanol/l/hour.

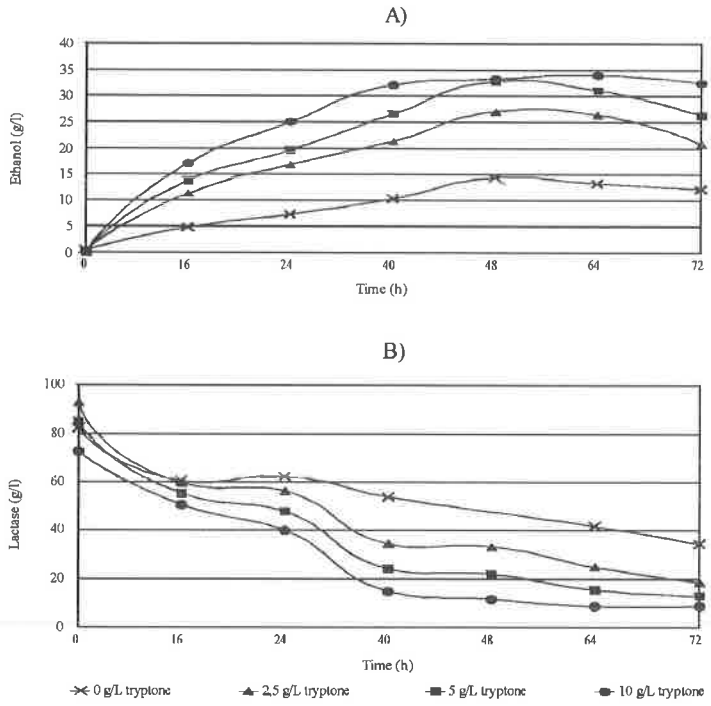

Fig. 1 - Ethanol production (A) and lactose utilization (B) by *Escherichia coli* ATCC 11775(pLOI297), in batch fermentation in whey (80 g/l lactose), supplemented with different concentrations of tryptone (0 g/l; 2,5 g/l; 5 g/l; 10 g/l).

consumption is linear and proportional to the amount of tryptone added, at least for the first 48 hours. After this period, the rate of consumption changes and the production of ethanol falls, leaving residual sugar in the medium. The maximal volumetric productivity measured at 10 and 20 hours was of 1.06g/l/h, 0.86g/l/h, 0.71g/l/h and 0.23g/l/h when adding 10, 5 and 2.5g/l and no tryptone, respectively. When tryptone was supplemented at a concentration of 10 g/l, the production of ethanol, besides being faster, reached higher levels than those reported by Guimarães *et al.* (4) after 48 hours of fermentation. Experiment revealed that yeast autolysate can be substituted by yeast extract provided it is added at a proportion of 1 ml of autolysate to 7 ml of whey, independently of the way it is prepared. Preparation of autolysate by yeast treatment with chloroform is faster than heating at 60°C and allows the release of nutrients from within the cell, thereby enriching the culture medium. The preparation of the yeast suspension in $(NH_4)_3PO_4$ worked better than that in water (data not shown) since it supplied additional sources of phosphate and nitrogen.

Although further investigations are needed to optimize ethanol production by recombinant *E.coli* from whey the convertion rate of lactose exceeded previous rates by yeasts and other *E.coli* tested with a more expensive system.

Further studies to increase ethanol yield offer potential for commercial expansion of fuel ethanol production by an alternative feedstock as whey which do not have competing value as food besides being a polutant.

ACKNOWLEDGMENTS

This work was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo).

RESUMO

Produção de etanol a partir de lactose e soro de leite por *Escherichia coli* expressando genes de *Zymomonas mobilis*

Um dos maiores desafios da biotecnologia é a redução da demanda bioquímica de oxigênio do soro de leite com concomitante produção de um

combustível líquido. Utilizamos, para este fim, *Escherichia coli ATCC 11775* contendo o plasmídio pLOI 297 que codifica as enzimas etanologênicas de *Zymomonas mobilis* capazes de converter lactose em etanol. No processo fermentativo, os seguintes parâmetros foram estudados: preparo do inóculo, concentração de lactose e adição de nutrientes ao soro de leite. As melhores condições em relação ao inóculo foram: crescimento em seis horas com agitação em meio Luria. O inóculo foi de 10% do volume de fermentação. Em relação a concentração de lactose, detectamos que acima de 80g/l, há um resíduo de galactose no meio de fermentação. A quantidade de etanol produzida a partir de soro de leite suplementado com triptona foi de 38g/l com uma eficiência de 85% de conversão, em 60 horas. Autolisado de levedura preparado pela adição de clorofórmio ou tratamento térmico pode substituir a triptona como fonte de nitrogênio orgânico. São necessários estudos adicionais para aumentar a produção de etanol a partir de soro de leite de forma a tornar a utilização deste resíduo economicamente viável.

Palavras-chave: etanol, *Escherichia coli*, soro de leite, lactose

REFERENCES

1. Ahmed, I.; Morris, D.(editors) - Alcohol fuels from whey: novel commercial uses for a waste product - Institute for Local Self - Reliance. Whashington D.C., 1991.
2. Alterthum, F.; Ingram, L.O.-Efficient ethanol production from glucose, lactose and xylose by recombinant *Escherichia coli*. *App. Environ. Microbiol.* 55:1943-1948, 1989.
3. Bothast, R. J., Kurtzman, C. P., Satarelli, M. D.; Slinger, P. J.- Ethanol production by 107 strains of yeasts on 5, 10 and 20% lactose. *Biotechnol. Lett.*, 8:593-596, 1986.
4. Guimarães, W. V., Dudey, G. L.; Ingram, L. O. -Fermentation of sweet whey by ethanologenic *Escherichia coli*. *Biotechnol. Bioengng.*, 40:41-45, 1992.
5. Ingram, L. O., Conway, T.; Alterthum, F. - Ethanol production by *Escherichia coli* strains co-expressing *Zymomonas* **pdc** and **adh** genes. United States 5.000.000 patent, March, 1991.
6. Joslyn, M. A. *Methods in food analysis*. 2nd. ed. Academic Press, 1970.
7. Kamini, N. R.; Gunasekaran, P. - Simultaneous ethanol production from lactose by *Kluyveromyces fragilis* and *Zymomonas mobilis*. *Curr. Microbiol.* 16:153-157, 1987.
8. Marwaha, S. S.; Kennedy, J. F.- Ethanol production from whey permeate by immobilized yeast cells. *Enz. Microbial Technol.*, 6:18-22, 1984.
9. Ohta, K.; Beall, D. S.; Mejia, J. P., Shaanmugam, K. T.; Ingram, L. O. - Genetic improvement of *Escherichia coli* for ethanol production:chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate descarboxylase and alcohol dehydrogenase II *App. Environ. Microbiol.*, 57:893-900, 1991.
10. Porro, D., Martegani, E., Ranzi, B. M.; Alberghina, L.- Lactose/whey utilization and ethanol production by transformed *Saccharomyces cerevisiae* cells. *Biotechnol. Bioengng.* 39: 799 - 805, 1992.
11. Ramakrishnan, S.; Hartley, B. S.- Fermentation of lactose by yeast cells secreting recombinant fungal lactase. *App. Environ. Microbiol.*, 59:4230-4235, 1993.
12. Takahashi, D. F., Carvalhal, M. L. C.; Alterthum, F.- Ethanol production from pentoses and hexoses by recombinant *Escherichia coli*. *Biotechnol. Lett.*, 16:747-750, 1994.
13. Vienne, P.; von Stockar, V.- An investigation of ethanol inhibition and other limitations occurring during the fermentation of concentrated whey permeate by *Kluyveromyces fragilis*. *Biotechnol. Lett.*, 7:521-526, 1985.

Revista de Microbiologia (1996) 27:268-272
ISSN 0001-3714

VARIABILITY OF STRAINS OF *METARHIZIUM ANISOPLIAE* VAR. *ANISOPLIAE* (METSCH.) SOROKIN IN TERMS OF PROTEIN CONTENT AND SPORE GERMINATION

Neiva Tinti de Oliveira[1], Claudio Luiz Messias[2]*

[1]Departamento de Micologia, CCB, Universidade Federal de Pernambuco, Cidade Universitária, Recife, PE, Brasil. [2]Departamento de Genética e Evolução, IB, Universidade Estadual de Campinas - UNICAMP, Campinas, SP, Brasil.

ABSTRACT

The variation of total protein of spore during germination of strains E9, Pegro and BSA of *Metarhizium anisopliae* var. *anisopliae* was investigated. Minimal germination time (MGT, defined as the time during which 85% of the conidia germinated) for the three strains was 12 hours of incubation in minimal medium. When the total protein content of the three strains was determined, variations related to increased germination time were detected and the beginning of protein synthesis was found to differ among strains. Strain E9 started protein synthesis between 0 and 7 hours of germination time, whereas strains Pegro and BSA started germination between 7 and 13 hours.

Key words: *Metarhizium anisopliae*, spore germination, conidial protein content.

INTRODUCTION

The fungus *Metarhizium anisopliae* has been used to control insect pests and has been shown to have a great potential as a biological control agent (12). More than 200 insect species belonging to 43 families and 7 orders (14) are hosts to this entomopathogen.

Entomopathogenicity, defined as the ability to produce disease in insects, depends on sequential events of mechanical and biochemical order that are triggered synchronously by the deposition of the spore on the insect cuticle, followed by germination, penetration, colonization, exteriorization of fungal structures and spore production on the insect's carcass (13,15).

For a pathogenic fungus, spore germination with or without the production of specialized structures such as the appressoria is the first step in the penetration and initiation of infection (15). The germination of *M. anisopliae* spores is asynchronous and in some strains, such as E9, a germ tube is present within 12 hours at 28°C in 90% of the spores under conditions of culture in a medium of semi-defined composition (1).

Water absorption by *M. anisopliae* spores increases the germination rate as well as the mortality rate of the insect *Manducca sexta*, a fact that appears to be related to synchronism that favors the formation of appressoria, important structures for pathogenesis (7).

Germination is preceded by a rapid increase in protein synthesis rate (3,17) and this synthesis is essential for the formation of the germ tube in all fungi (5,8). In most fungi DNA synthesis starts after the initiation of RNA and protein synthesis but before the appearance of the germ tube. Cycloheximide, a substance that inhibits protein synthesis, is known also to inhibit the formation of the germ tube in *M. anisopliae* (2).

* Corresponding author. Mailing address: Departamento de Genética e Evolução, IB, Universidade Estadual de Campinas - UNICAMP, Cidade Universitária Zeferino Vaz, Caixa Postal 6109, CEP 13081-970, Campinas, SP, Brasil.

In view of the importance of germination for an entomopathogenic fungus, in the present study we evaluated the protein content of germinating and non-germinating spores of three *M. anisopliae* strains.

MATERIALS AND METHODS

Strains E9, Pegro and BSA of *M. anisopliae* var. *anisopliae* were isolated from insects known in Brazil as "spittleburgs" (Homoptera, Cercopidae), i.e, *Deois flavopicta* from the state of Espírito Santo, *Deois flavopicta* from the state of São Paulo, which occurs in pastures and *Mahanarva posticata*, from leaf of the sugar cane plant, from the state of Alagoas. The strains are stored in liquid nitrogen and maintained in the germplasm bank of entomopathogenic fungi of the Department of Genetics and Evolution, State University of Campinas, Campinas, SP.

Preparation of synchronized spores - The method of Law and Timberlake (8), modified, was used to obtain spores of similar physiological level in all experiments. Spores produced on solid minimal medium (MM) (11) were gently collected from the surface of the culture medium and transferred to saline solution. Aliquots (10 ml) of the spore suspension were transferred to 1000 ml Erlenmeyer flasks containing 300 ml liquid MM (LMM). Eight Erlenmeyer flasks per strain were incubated at room temperature (± 28°C) for 24 hours with shaking. The pellets formed after this period of time were separated by vacuum filtration through n°.1 Whatman filter paper measuring 30 x 20 cm. The pellets were washed in 1000 ml sterilized distilled water to remove culture medium residues. The filter paper with the pellets was placed on a layer of nylon beads used as support on Pyrex trays containing 100 ml LMM so they would be slightly soaked. The Pyrex trays (19.5 x 30 x 4.5 cm) thus prepared were covered with plastic film and incubated at 28°C for 7 days in order to obtain synchronized spores. After development, the spores were scraped from the filter paper, suspended in sterilized distilled water and filtered through three layers of sterilized gauze for the retention of possible mycelial fragments. The filtrate was centrifuged at 400 g for 10 minutes, yielding a humid spore mass that was used for the analysis of total protein content.

Evaluation of protein amount in germinated and non-germinated spores - Humid portions of spores of known mass of the strains E9, Pegro and BSA were left to germinate in LMM and collected at different times (0, 7, 13 and 24 hours) by centrifugation. The portions were ground separately in liquid nitrogen in a mortar in 6 ml 0.1 M phosphate buffer, pH 7.0, the ground mass was collected in 6 additional ml of the same buffer and immediately centrifuged at 2000 g for 25 minutes at 3°C. The supernatant was collected, filtered through a 0.45 µm Millipore filter and dialyzed for 12 hours in 500 ml phosphate buffer, pH 7.0. The amount of protein present in the extracts was measured by the method of Bradford (4), modified. Forty µl samples of the dialyzed supernatant were placed in a test tube containing 1.5 ml phosphate buffer and 1 ml of CBB reagent (0.06 g Coomassie Brilliant Blue G-250 and 1000 ml 3% perchloric acid) at room temperature. A reading was taken 2 minutes later in a Beckmann DU-70 spectrophotometer at 595 nm wavelength.

Electrophoresis for the determination of total spore protein - Aliquots of the extracts obtained as described above were submitted to polyacrylamide gel electrophoresis using a vertical run and a discontinuous system of buffers. The sample was prepared by mixing 100 µl of the sample with 10 µl bromophenol blue solution (0.025 g bromophenol blue + 10 ml Tris-HCl buffer, pH 6.7). The run buffer was Tris-glycine, pH 8.3. The run was started at 15 mA, corrected to 20 mA after the bromophenol blue front passed through the stacking gel. A bovine serum albumin solution was used as standard (0.001 g bovine serum albumin in 5 ml 0.15 M NaCl solution).

After the run the gel was fixed in 20% glacial acetic acid + methanol (1:1, v/v) for 30 minutes and stained with a freshly prepared solution of Coomassie Brilliant Blue R 250 + 10% glacial acetic acid. An aqueous mixture of 40% glacial acetic acid and 2.5% glycerol was added at a proportion of 1:20. Minimum staining time was 8 hours. The gel was destained in a 20% methanol + 5% acetic acid + 2.5% glycerol solution.

Germination was evaluated on the basis of the minimum time needed by 85% of the spores to germinate (MGT) (2) in 150 ml Erlenmeyer flasks containing 10 ml liquid MM inoculated with 10^6 synchronized spores/ml. Aliquots of the inoculated medium were removed at one hour intervals, 500 spores were counted under the light microscope for the presence or absence of a germ tube and the percentage of germinated spores was calculated.

RESULTS AND DISCUSSION

The percentages of germinated spores after 7, 8, 9, 10, 11 and 12 hours of incubation with shaking are

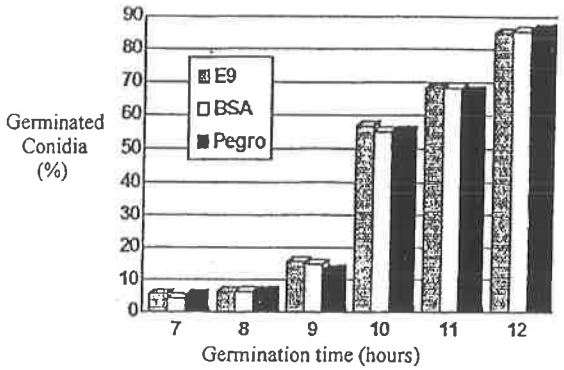

FIGURE 1 - Percent conidial germination in strains E9, BSA and Pegro of *M. anisopliae* in minimal medium at 28°C.

presented in Fig.1. The MGT obtained for strains E9, BSA and Pegro was approximately 12 hours of incubation in LMM, with a mean germination rate of 84.80%, 85.53% and 86.11%, respectively, in agreement with the data reported by Arrais de Matos (2) for strain E9.

The data for total protein analysis in the three strains with different germination times are listed in Table 1. There was a significant interaction between germination time and strains, indicating that the effect of germination time in terms of total protein content depends on the strain. The mean effects of the three strains differed from one another, with strain E9 presenting the highest mean total protein content, followed in decreasing order by strains BSA and Pegro. The mean effects of germination time also differed, with the 24 hour time presenting a higher total protein value, followed in decreasing order by 13, 7 and 0 hours.

Partitioning of the main effect "Time of germination" with the "Strain x Time of Germination" interaction revealed a significant difference between germination times for all strains at the 5% level of probability, with the effect being more intense for strain E9 (Table 2).

Comparison of the means by the Tukey test at the 5% level of probability revealed the following features: in strain E9 the mean values of total protein content differed significantly at all four germination times, with the 24 hour germination time presenting the highest total protein content, followed in decreasing order by 13, 7 and 0 hours. In the BSA strain there was a significant differences between 24, 13 and 7 hours of germination, whereas no difference was observed between 7 and 0 hours. In the Pegro strain, mean total protein content differed between 24 and 13 hours but did not differ between 13 and 7 hours or between 7 and 0 hours. It can be seen that E9 spores started protein synthesis between 0 and 7 hours of germination, the BSA strain started after 7 hours of incubation and the Pegro strain only after 13 hours of incubation. Fitting of the linear model $yi = A + Bxi$ to the data showed a correlation coefficient of 0.9449, indicating affinity among the variables through the adjusted model. The regression equation obtained was

Table 1. Total protein content of the spores (mg/mg dry spore weight) of three *M. anisopliae* strains with different germination times.

	Total protein content* germination time				
Strains	0	7	13	24	Means
E9	406.32Bd**	1116.04Ac	5696.27Ab	7116.47Aa	3583.77A
BSA	535.62Ac	423.61Bc	597.87Bb	758.05Ba	578.28B
Pegro	111.48Cc	256.88Cbc	321.05Cb	548.67Ca	309.52C
Mean CV=3.53%	350.45d	598.84c	2205.96b	2807.73a	

* - mean of three replications

** - means followed by the same letter did not differ at the 5% level of probability in the column (capital letters) or on the line (lower case letter)

Table 2. Partitioning of the major effect of "Germination time" with the "Strains x Germination time" interaction in terms of total protein in spores from three *M. anisopliae* strains with different germination times.

Sources of variation	d.f.	SS	MS	F
Germination time/Strain E9	3	99385593	3312831	11943.78*
Germination time/Pegro strain	3	298036.	99345.58	35.82*
Germination time/BSA strain	3	175867.57	58622.52	21.13*
Residue	24	66568.96	2773.71	

*Significant at the 5% level of probability

$y = 0.0026064 + 0.0011181x$, demonstrating that time zero of germination corresponds to an amount of protein of 0.0026064 g/g dry spore weight, and that at each hour of germination time there was an increase in protein amount of 0.001118 g/g dry spore weight.

Intraspecific variability was observed in total protein content of *M. anisopliae* spores. Spore total protein content increased significantly with time of incubation, with values depending on the strain involved. This indicates that an intense protein synthesis process occurs in germinating spores, as also observed in other fungi (16). Furthermore, protein synthesis started at different times in the three strains studied. It has been previously observed that protein synthesis occurs before the formation of the germ tube in *M. anisopliae* spores, since the germination of spores treated with the protein synthesis inhibitor cycloheximide is also inhibited (2). This need for synthesis before the appearance of the germ tube has been emphasized by others (5,9,10).

In conclusion, we may state that the method used in the present study, i.e., total protein analysis, showed that protein synthesis increases with incubation time until germination, although total protein electrophoresis showed that, qualitatively, these proteins are the same as those preexisting in non-germinated spores. This may also indicate that, in qualitative terms, the spore contains all the protein material it needs for this phase, requiring no synthesis of new protein type(s) for germination. This may be the factor, or may reinforce the factor related to the type of exogenous dormancy of *M. anisopliae*. Destefano (6), in a study of O_2 consumption in *M. anisopliae* spores during germination, noted that the spores do not germinate if the medium does not contain a source of carbon and nitrogen.

During germination, endogenous metabolism occurs through organelles and storage materials originating from conidiogenesis and also depends on factors such as the presence of a substrate on the spore surface, for example, lipids that might prevent water absorption.

The protein synthesis rate differed among the three strains used, being highest in E9 and lower in Pegro and BSA. This difference may indicate the initiation of synthesis needed for germination. Thus, even though all proteins are present, there must be control of the synthesis of a specific type that is synthesized sequentially at different rates in E9, Pegro and BSA, causing a difference in the time of appearance of the germ tube. Alternatively, other proteins may be present in lower amounts not detected by the methodology employed that may be related to this differentiation and may not be the most abundant ones. From a practical viewpoint, these differences may indicate variability in virulence, since the infectious process starts with spore germination on the host's surface.

In view of the importance of germination, further studies are needed to elucidate the mechanism of germination control in *M. anisopliae*, contributing to the understanding of the process of host infection that starts with spore germination.

ACKNOWLEDGMENTS

The authors are indebted to CNPq, FINEP/PADCT and CAPES/PICD for financial support.

RESUMO

Variabilidade de linhagens de *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin quanto ao conteúdo protéico e germinação de esporos

A variação de proteínas totais e a germinação de esporos das linhagens E9, Pegro e BSA de *M. anisopliae* var. *anisopliae* foram analisadas neste trabalho. Verificou-se que o tempo de germinação mínimo (TGM, no presente estudo empregado para expressar o período no qual 85% dos esporos estão germinados) para as três linhagens estudadas em MM foi de 12 horas. O conteúdo de proteína total foi analisado e mostrou que há variações quantitativas mas não qualitativas relacionadas com o tempo de germinação e início da síntese destas proteínas para as três linhagens. A linhagem E9 iniciou a síntese de proteínas entre os tempos de germinação de 0 e 7 horas e as linhagens Pegro e BSA entre 7 e 13 horas.

Palavras-chave: *Metarhizium anisopliae*, germinação de esporos, conteúdo protéico de esporos.

REFERENCES

1. Arrais de Matos, A.J. *Crescimento, germinação, conidiação e resistência a luz ultra-violeta de linhagens de* Metarhizium anisopliae. Campinas, SP, Brasil, 1983, 78p. (M.S. Thesis. Universidade Estadual de Campinas)
2. Arrais de Matos, A.J. *Germinação conidial em* Metarhizium anisopliae *(Metsch.) Sorokin.* Campinas, SP, Brasil, 1988, 175p. (Ph.D. Thesis. Universidade Estadual de Campinas).
3. Bartinicki-Garcia. Spore germination in fungi: Basic concepts - *In*: Roberts, D.W. & Aist, J.R. (eds). *Infection Process of Fungi.* The Rockefeller Foundation, USA, 1984. p. 111-117.

4. Bradford, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye bynding. *Anal.Biochem.* 72: 248-254, 1976.
5. Brambl, R; Dunkle, L.D.; Van Etten, J.L. Nucleic acids and protein synthesis during fungal spore germination - *In*: Smith, J.E. & Berry, D.R. (eds). *Filamentous Fungi: Developmental Mycology.* Edward Arnold, London,1978, v.3, p. 94-118.
6. Destefano, R.H.R. *Evolução de O_2 viabilidade e virulência de* Metarhizium anisopliae *(Metsch.) Sorokin, durante armazenamento.* Piracicaba/SP.1993, 85p.(M.S. Thesis. Universidade de São Paulo - USP).
7. Hassan, A.E.M.; Dillon, R.J.; Charnley, A.K. Influence of accelerated germination of conidia on the pathogenicity of *Metarhizium anisopliae* for *Manduca sexta. J. Inv. Pathol.* 54: 277-279, 1989.
8. Law, D.J.; Timberlake, W.E. Developmental regulation of laccase levels in Aspergillus midulans. *J. Bacteriol.* 144: 509-517, 1990.
9. Lovett, J.S. Regulation of Protein Metabolism during spore germination *In*: Weber, D.J. & Hess, V.W. (eds). *The Fungal spore: Form and Function.* John Wiley and Sons, New York, USA, 1976. p. 189-242.
10. Macko, V. Inhibitors and stimulants of spore germination and infection structure formation in fungi. *In*: Turian, G.; Hohl, H.R. (eds). *The Fungal Spore: Morphogenetic Controls.* Academic Press, New York, USA. 1981, p. 565-585 .
11. Pontecorvo, G.; Roper, J.A.; Hemons, L.M.; MaDonald, K.D.; Button, A.W.J. The Genetics of *Aspergillus nidulans. Adv. Gen.*, 5: 141-238, 1953.
12. Roberts, D.W. World Picture of Biological Control of Insects by Fungi. *Memórias do Instituto Oswaldo Cruz*, Supl.III, 84: 89-100, 1989.
13. Roberts, D.W.; Campbell, A.S. Stability of entomopathogenic fungus. *Miscellaneous Publications of Entomological Society of America,* 10: 19-76, 1977.
14. Roberts, D.W.; Humber, R.A. Entomopathogenic fungi *In*: Conference on Infection Processes of Fungi, Bellagio, Report Bellagio Conference Center, p. 1-6, 1983.
15. ST. Leger, R.J. Integument as a Barrier to Microbial Infection *In*: Retnakaran, A. & Binnington (eds). *Physiology of Insect Epidermis*, CSIRO, Australia, 1991, p.286-308.
16. Sussman, A.S. Dormancy and spore germination. *In*: Ainsworth, G.C. & Sussman, A.S., *The Fungi*. Academic Press, New York, USA, 1966, v. 2, p. 733-764.
17. Turian, G. Polarizing process for germ tube outgrowth from monilioid fungal spores (Neurospora conidia model). *Proceedings of the Indian Academy of Science Plant Sciences* 94: 165-173, 1985.

Revista de Microbiologia (1996) 27:273-276
ISSN 0001-3714

IN VITRO INTERACTIONS BETWEEN AMPHOTERICIN B AND HALOACETAMIDE DERIVATIVES AGAINST *CANDIDA* SP

Arlete Emily Cury*

Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, Brasil

ABSTRACT

Antifungal activities of amphotericin B (AmB) and haloacetamide derivatives were studied against 17 strains of *Candida albicans* and 6 of *Candida tropicalis*. Concentrations from 0.03 μg/ml to 8.0 μg/ml for AmB and from 0.25 μg/ml to 64.0 μg/ml for ethophamide or teclosan were prepared in enriched yeast nitrogen base agar to establish the minimum inhibitory concentration by the checkerboard technique. The minimum fungicidal concentration was determined by inoculum subculture on Sabouraud dextrose agar. In concentrations up to 64.0 μg/ml, the haloacetamide derivatives did not show antifungal activity against *Candida* strains. Combinations between ethophamide or teclosan with AmB showed synergistic interactions against most of the studied strains, standing out the fungicidal activity of the AmB-ethophamide combination against all *C.albicans* strains. These results show good perspectives for experimental *in vivo* studies with combined therapy.

Key words: antifungal drugs, amphotericin B, ethophamide, teclosan, *Candida*.

INTRODUCTION

Amphotericin B (AmB) is the only polyenic compound utilized in the systemic therapy of mycoses, even though it is one of the most toxic antimicrobial agents in clinical use (4). In an attempt to minimize the toxicity and to take advantages of the combined therapy, studies of interactions have been done between AmB and some other drugs with systemic activity, such as azoic antifungals, antibacterians or antineoplastic drugs against yeasts (3, 6, 9).

Combinations of AmB with antiamoebic drugs, such as haloacetamide derivatives, applied in topic therapy have not been yet evaluated, although the results of these studies could be interesting in specific clinical cases of candidoses (11, 13, 14).

The aim of this study was the determination of the *in vitro* antifungal activity of the haloacetamide derivatives ethophamide and teclosan by themselves and in combinations with amphotericin B against *Candida albicans* and *C.tropicalis*.

MATERIALS AND METHODS

1. Microrganisms and inoculum

C.albicans (17 strains) and *C.tropicalis* (6 strains) from patients with invasive candidosis were selected for the study. *Saccharomyces cerevisae* ATCC 9763 sensible to polienic agents was included as control.

The yeasts were cultivated on Sabouraud dextrose agar (ASD) at 30°C. The inoculum of 10^5 cells/ml of phosphate buffer (0.01 M, pH 7.0) was achieved after 24 h growth.

* Corresponding author. Mailing address: Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, CEP 05508-900 - São Paulo- SP - Brasil

2. YNBG broth

This medium contained Bacto-YNB, (0.67 g), L-asparagine (0,15 g) and glucose (1.0 g) in phosphate buffer (100 ml).

3. YNBG agar

The YNBG broth was prepared 10x concentrated and was diluted (1:10) with phosphate buffer containing 1.1% of Bacto-agar at 50°C.

4. Drugs and dilutions

Amphotericin B (Squibb), ethophamide (Farmitalia Carlo Erba) and teclosan (Winthrop), kindly supplied in powder form, were dissolved with dimethylsulphoxide. Double dilutions in series were performed in YNBG broth. AmB concentration ranged from 0.6 μg/ml to 160 μg/ml and the haloacetamide derivatives concentration ranged from 5.0 μg/ml to 1280 μg/ml.

5. Susceptibility tests

The tests were performed three times in duplicate, according to the following procedures:

5.1. Minimum inhibitory concentrations (MIC).

This test was conducted according to the checkerboard technique (9, 12).

For 20 ml of final medium, each dilution of the drug alone and each dilution of AmB mixed with each dilution of the haloacetamide derivatives were added to YNBG agar at 50°C. The final concentration of AmB ranged from 0.03 μg/ml to 8.0 μg/ml and the haloacetamide derivative from 0.25 μg/ml to 64.0 μg/ml. Growing controls were performed on agar medium added of formaldeyde (0.1 ml) and without drug.

Each medium was plated, and the yeast suspensions were inoculated with the Steers's replicator. After incubation for 24 h at 30°C the MICs were determined.

5.2. Minimum fungicidal concentration (MFC)

The inoculum that did not result in growth on the MIC test were subcultured on ASD, and after 48 h of incubation at 30°C the MFCs were determined.

6. Activity evaluation of single and combined drugs against yeasts (1, 2, 12).

The final results of MICs and MFCs of the drugs against each *Candida* strain were obtained by geometric mean of the values found in repeated tests. From these results, fractional inhibitory concentration (FIC) and fractional fungicidal concentration (FFC) were respectively determined. For each drug in the combination, the FIC was equal to the MIC divided by the MIC of the drug alone. The sum of the FICs of the two combined drugs resulted in FIC index. From the drugs MFCs, same calculation was done to obtain the FFC index. When FIC or FFC indexes were < 1 the drug interaction was considered synergistic; = 1, additive; > 1 and < 2, indifferent, and ≥ 2, antagonist.

MIC and MFC > 64.0 μg/ml were established in 128 μg/ml; values ≤ 0.25 μg/ml and ≤ 0.03 μg/ml were kept in 0.25 μg/ml and 0.03 μg/ml, respectively.

RESULTS

In the antifungal activity study of single drugs, the MICs of AmB ranged from 0.5 μg/ml to 4.0 μg/ml

Table 1. Interactions between amphotericin B and haloacetamide derivatives against *Candida spp*

Yeast	AmB + Et		AmB + Te	
	∑ FIC	∑ FFC	∑ FIC	∑ FFC
C.albicans				
1	1.00	0.63	0.50	0.31
2	1.00	0.63	1.00	0.50
3	1.00	0.53	0.75	0.75
4	0.75	0.75	1.00	0.19
5	0.63	0.63	0.53	0.28
6	0.75	0.56	0.63	0.50
7	1.00	0.75	0.50	0.38
8	0.56	0.75	1.00	0.53
9	0.75	0.75	1.00	0.56
10	1.00	0.75	0.56	1.00
11	1.00	0.52	1.00	0.51
12	1.00	0.63	1.00	0.25
13	1.00	0.53	1.00	0.52
14	0.53	0.75	1.00	1.00
15	0.52	0.75	0.53	0.31
16	0.63	0.56	0.56	0.52
17	0.63	0.75	1.00	0.75
C. tropicalis				
1	1.00	0.52	0.56	0.56
2	0.31	0.56	0.51	0.75
3	0.18	0.75	0.98	0.53
4	0.53	1.00	1.00	0.25
5	0.37	0.31	1.00	1.00
6	0.75	0.75	0.75	0.56

Et = ethophamide; Te= teclosan; ∑FIC or ∑FFC < 1 synergistic interacton; = 1 additive interaction.

against *C.albicans* strains and from 0.25 μg/ml to 4.0 μg/ml against *C.tropicalis* strains. For both *Candida* species, the MFCs of AmB ranged from 2.0 μg/ml to 8.0 μg/ml . The haloacetamide derivatives did not show either inhibitory or lethal activity by themselves in concentrations up to 64.0 μg/ml.

Compared with the drug alone, AmB combined with ethophamide showed lower MICs values, ranging from 0.25 μg/ml to 1.0 μg/ml , against 11 strains of *C.albicans*, and from 0.03 μg/ml to 1.0 μg/ml against 5 strains of *C.tropicalis*.The combined ethophamide showed inhibitory activity against all the strains in MICs from 0.25 μg/ml to 64.0 μg/ml. For the two combined drugs the lethal activity was potentialized against all strains, except the unchanged MFC of AmB against one strain of *C.tropicalis* .

The FIC indexes of AmB-ethophamide combination showed synergistic interactions against 9 strains of *C.albicans* and 4 of *C.tropicalis*. Based on FFC index, the synergy occured against all the strains of *C.albicans* and 5 of *C.tropicalis*. Both indices showed only additive interactions against the others *C.albicans* and *C.tropicalis* strains (Table 1).

The AmB-teclosan combination also showed potentialization of antifungal activity of both drugs against most of the strains. AmB showed MICs from 0.5 μg/ml to 2.0 μg/ml against 13 strains of *C.albicans* and from 0.03 μg/ml to 2.0 μg/ml against 5 strains of *C.tropicalis*. The MFCs ranged from 0.5 μg/ml to 4.0 μg/ml against 15 strains of *C.albicans* and 5 strains of *C.tropicalis*. For the combined teclosan, the MICs and MFCs ranged from 0.25 μg/ml to 64.0 μg/ml against the strains of both species (Table 1).

As in AmB-ethophamide combination, the AmB-teclosan combination showed a lower number of synergistic interactions based on inhibitory activity than on lethal activity against both *Candida* species. The FIC index showed these interactions against 8 strains of *C.albicans* and 4 strains of *C.tropicalis*, while the FFC index showed them against 15 strains of *C.albicans* and 5 strains of *C.tropicalis*. According to both indices, additive interactions occured against the others strains (Table 1).

DISCUSSION

Ethophamide and teclosan are called luminal or contact amebicides that seem to act inhibiting the protein synthesis of the parasite. They are administered orally, and may be used in association with systemic amebicides in the eradication of intestinal forms of amebiasis (5,7).

In this study, ethophamide and teclosan showed antifungic activity only when combined with AmB. Depending on the strain, synergistic or additive interactions were observed for both combinations. The number of synergistic interactions was much higher than the additive, and even more when related to lethal activity than to inhibitory activity of the drugs against *C.albicans* and *C.tropicalis* strains. In this context, AmB-ethophamide combination was more active, showing synergism related to lethal activity against all the strains of *C.albicans*.

The explanation for the resulted synergism of AmB-haloacetamide combination could be based on the mechanism of action of the drugs. The destabilization of the yeast membrane function caused by AmB (4) eased the haloacetamide cell penetration and its action against the protein synthesis. However the occurence of these events need confirmation, because the action mechanism described for the haloacetamides serve to explain the activity of these drugs against amebae, but it was not yet described against yeasts. At any rate, the results obtained, principally for the combination between amphotericin B and ethophamide, show good perspectives for effective and short-time therapy of cutaneous and mucosal candidoses, sometimes with poor response to the usual treatments (8, 11, 13, 14). The results of synergistic interactions reported in the present paper suggest that further experimental *in vivo* researchs are warranted.

RESUMO

Interações *in vitro* entre anfotericina B e derivado haloacetamídico contra *Candida* sp

Estudou-se a atividade antifúngica da anfotericina B (AnB) e de derivado haloacetamídico, contra 17 cepas de *Candida albicans* e 6 de *Candida tropicalis*. As concentrações inibitórias mínimas foram avaliadas a partir de cultivos em ágar "yeast nitrogen base", utilizando-se concentrações de AnB variáveis de 0,03 μg/ml a 8.0 μg/ml e de etofamida ou de teclosan de 0,25 μg/ml a 64.0 μg/ml. As concentrações fungicidas mínimas foram determinadas a partir de subcultivos em ágar Sabouraud dextrose. Nas concentrações estudadas, os derivados haloacetamídicos não mostraram atividade antifúngica contra as cepas de *Candida*. Combinações entre a etofamida ou o

teclosan com AnB resultaram em interações sinérgicas contra a maioria das cepas, destacando-se a atividade fungicida da combinação AnB-etofamida contra todas as cepas de *C.albicans*. Esses resultados mostram boas perspectivas para estudos do tratamento de candidose experimental com as drogas combinadas.

Palavras-chave: drogas antifúngicas, anfotericina B, etofamida, teclosan, *Candida.*

REFERENCES

1. Berenbaun, M.C. - A method for testing for synergy with any number of agents. *J. Infect. Dis.*, 137: 122-130, 1978.
2. Edwards, D. I. - *Antimicrobial drug action.* London, Macmillan, 1980.
3. Ghannoum., M.A.; Abu-Elteen, K.H.; Motawy, M.S.; Abu-Hatab, M.A.; Ibrahim, A.S.; Criddle, R.S. - Combinations of antifungal and antineoplastic drugs with interactive effects on inhibition of yeasts growth. *Chemotherapy,* 36: 308-320, 1990.
4. Hoeprich, P.D. - Clinical use of amphotericin B and derivatives: lore, mystique and fact. *Clin. Infect. Dis.*, 14 (Suppl.1): S114-S119, 1992.
5. Korolkovas, A. - *Essencials of medical chemistry.* 2 ed. New York: Wiley-Intercience, 1988, p. 658-661.
6. Meyer, R.D. - Current role of therapy with amphotericin B. *Clin. Infect. Dis.*, 14 (Suppl.1): S154-S160, 1992.
7. Neal, R.A. - Experimental amoebiasis and the development of anti-amoebic compounds. *Parasitology*, 86: 175-191, 1983.
8. Odds, F.C. - *Candida and candidosis,* 2 ed., London, Baillière Tindall, 1988. 468 p.
9. Petrou, M.A. ; Rogers, T.R. - Interactions *in vitro* between polyenes and imidazoles against yeasts. *J. Antimicrob. Chemother.*, 27: 491-506, 1991.
10. Shadomy, S.; Wagner, G.; Espinel-Ingroff, A.; Davis, B.A. - *In vitro* studies with combinations of 5-fluorocytosine and amphotericin B. *Antimicrob. Agents Chemother.*, 8: 117-121, 1975.
11. Sobel, J.D. - Pathogenesis and treatment of recurrent vulvovaginal candidiasis. *Clin. Infect. Dis.*, 14 (Suppl.1): S148 - S153, 1992.
12. Van Der Auwera, P. - Synergistic, additive, and antagonistic activity of antimicrobial agents: how to measure? What are the clinical consequences? Application to the new fluoroquinolones. *Infect. Dis. Newslett,* 11: 45-56, 1992.
13. Walsh, T.J.; Lee, J.W. - Prevention of invasive fungal infections in patients with neoplastic diseases. *Clin. Infect. Dis.*, 17 (Suppl.2): S468-S480, 1993.
14. White, A.; Goetz, M.B. - Azole-resistant *Candida albicans*: report of two cases of resistance to fluconazole and reviwe. *Clin. Infect. Dis., 19:687-692, 1994.*

Revista de Microbiologia (1996) 27:277-282
ISSN 0001-3714

CONSUMPTION OF BLOOD COAGULATION FACTORS ASSOCIATED WITH *ACTINOBACILLUS PLEUROPNEUMONIAE* INFECTION

Marcio M. Folly[1]*, Catherine Kolly [2]

[1]Laboratório de Sanidade Animal, Universidade Estadual do Norte Fluminense; Rio de Janeiro, Brasil. [2] Centro de Pesquisa Agrícola da Ciba Geigy, Suíça

ABSTRACT

A study of the effect of *Actinobacillus pleuropneumoniae* infection on the blood coagulation system of pigs was carried out. 25 Specific Pathogen Free (SPF) weaned piglets were randomly assorted into different groups. Ten piglets were infected with $5X10^6$ CFU of *Actinobacillus pleuropneumoniae* serotype 1 and the remaining fifteen were used as negative controls. Significant concentration reductions ($P<0.005$) of blood coagulation factors IX, VIII, VII, X and V were demonstrated. Activated partial thromboplastin time increased while prothrombin time, expressed as percentage, decreased. Antithrombin III concentration decreased significantly ($P<0.005$). These observations were consistent with consumption of blood coagulation factors in the intrinsic, extrinsic and common pathways of blood coagulation, therefore intrapulmonary haemorrhage and clot formation could be observed in the lungs of piglets infected with *Actinobacillus pleuropneumoniae.*

Key words: pleuropneumonia, pigs, blood coagulation

INTRODUCTION

Porcine pleuropneumonia is caused by *Actinobacillus pleuropneumoniae*, a Gram negative coccobacillary bacterium. The susceptibility to infection increases with stress and changes in climate. Each of these factors are present in modern pig farming. Porcine pleuropneumonia has been reported in the United States, Canada, Mexico, South America, Australia, Japan, Taiwan and most of Europe (16).

In the peracute form of the disease, pigs show high temperature (41.5°C - 42°C), anorexia, cough, respiratory distress, shock with blood-stained nasal froth and oral discharge and finally death. In the acute form, there is fever (40.5°C - 41°C), coughing and dyspnoea. A chronic form of the disease can result from the acute form, leading to decrease in constant rate and persistent cough.

The pathological lesions produced by *A. pleuropneumoniae* are characterized by local to generalised fibrinous pleuritis and a necrotizing fibrino-haemorrhagic pneumonia of the apical, cardiac, diaphragmatic and intermediary lobes (12). An increase of the phagocytic activity of alveolar macrophages in piglets infected with *A. pleuropneumoniae* was demonstrated (3). Smits *et al.*(13) suggested that cytolisins produced by serotypes of *Actinobacillus pleuropneumoniae* could be involved in the induction of pneumonic lesions. The primary lesion caused by this bacterium is not due to the haemolysin (15) but *A. pleuropneumoniae* endotoxin has been involved in the development of pulmonary lesions (5). Prolongation of prothrombin time (PT) and activated partial thromboplastin time (aPTT) during the acute course of APP with various doses of *Actinobacillus pleuropneumonia* given to piglets were demonstrated in preliminary studies,

* Corresponding author. Mailing address: Laboratório de Sanidade Animal, Universidade Estadual do Norte Fluminense, Av. Alberto Lamego 2000, CEP 28015-620, Campos dos Goytacazes - RJ - Brasil. E-mail folly@uenf.br

showing disorders in the blood coagulation mechanisms (6).

The aim of this study was to investigate *in vivo* the effect of *Actinobacillus pleuropneumoniae* on blood coagulation factors and proteins in the peracute and acute course of porcine pleuropneumonia. Clinical and bacteriological studies of the lungs of piglets infected were also done.

MATERIALS AND METHODS

Animals and housing

Twenty five Specific Pathogen Free (SPF) piglets of the Large White breed, both sexes, weighing 20-25 kg, were used. Fifteen piglets were left uninfected as negative controls and ten piglets were infected with *Actinobacillus pleuropneumoniae*. The piglets were placed in the isolation station on flatdecks and let to adapt to the new environment for one week before the experiment started.

Bacterium and Infection Model

Actinobacillus pleuropneumoniae, Serotype 1, was obtained from Veterinary Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon - Canada S7H OWO. The strain was cultivated in Brain Heart Infusion + polyvitamin (Polyvitex from bioMérieux, France) + factor V (Taxo V from Becton-Dickinson, Cockeysville, USA) and incubated for 24h at 37°C.

Groups of 5 piglets were placed in the aerosol chamber and infected with a nebulisor (Ultra Neb 99 De Villbis GMBH - West Germany) according to the methods of Osborne *et al.* (14). The inoculum was prepared as a bacterial suspension corresponding to $5x10^6$ CFU in 200 ml of PBS (0.1 M phosphate, 0.15 M NaCl, pH 7.2) with 10% fetal calf serum. Each group of animals was exposed for 10 min and then removed from the aerosol chamber.

Parameters analysed

Temperature was taken once a day and clinical signs of pleuropneumonia, i.e. anorexia, cough, dyspnoea, hyperthermia and epistaxis, were registered for the sick piglets. Necropsy was performed on all dead piglets.

Blood samples were taken before infection (day 0) and once a day until the end of the trial (day 4).

Samples were taken as follows:

Vacutainer with 0.5 ml sodium citrate (0.129M) for the coagulation tests. The blood was centrifuged for 15 min at 3000 rpm and 5°C. Vacutainer with 0.048 ml (0.34M) K_3 EDTA, for the white blood cells and platelets counts. All Vacutainer tubes were from Becton-Dickinson, Basle, Switzerland. White Blood Cells (WBC) and platelets counts were made for all animals, using a Sysmex E-5000 Digitana TOA counter, Lausanne, Switzerland.

Fibrinogen (FIB) was measured by the modified Clauss methods. Plasma was diluted 1:5 with Michaelis buffer "Roche". 0.2 ml of this dilution was pre-warmed at 37°C for 2 min in Cobas Fibro cuvettes. 0.2 ml of thrombin reagent (Roche-Cobas Fibro 60NIH U/ml) was added and the coagulation end-point measured following the instructions (Cobas fibro machine, Hoffmann la Roche, Basle, Switzerland).

For the thrombin time (TT), a standardized quantity of bovine thrombin (thrombin reagent "Roche") was diluted 1:7.5 in distilled water. 0.2 ml of piglet blood plasma was incubated in pre-warmed Cobas fibro cuvettes at 37°C for 2min. Then 0.2 ml of the thrombin reagent was added to the blood plasma and the coagulation end-point determined following the instructions for Cobas fibro.

Activated Partial Thromboplastin Time (PTT): 0.1 ml of piglet plasma was incubated with 0.1 ml of PTT reagent "Roche" at room temperature for 3 min in Cobas fibro cuvettes, then 0.1 ml of calcium chloride (0.025 mol/l) pre-warmed at 37°C was added. The coagulation time was recorded following the instructions for Cobas fibro.

The Prothrombin Time (PT) was measured with Calibrated Rabbit Brain (CRB) thromboplastin reagent "Roche" dissolved in 10 ml of distilled water. 0.1 ml of piglet plasma was put in Cobas fibro cuvettes at 37°C for 2 min, then 0.2 ml of CRB thromboplastin reagent was added and the clotting time recorded following the instructions for Cobas fibro.

The blood coagulation factors VIII, VII, X, IX and V were assayed with a standard test kit Bio Mérieux, France. The tests were carried out according to the instructions of the manufacters. Calibration curves corresponding to the clotting time found were made and the results expressed in percentage.

Antithrombin III(ATIII) was measured by a colorimetric method (ATIII chromoline kit, bioMérieux, France) and a spectrophotometer. 0.1 ml of piglet plasma was diluted 1:20 in physiological saline and incubated with 0.5 ml of human thrombin reagent in the presence of heparin for 1 min at 37°C, then 0.1 ml of a synthetic chromogenic substrate

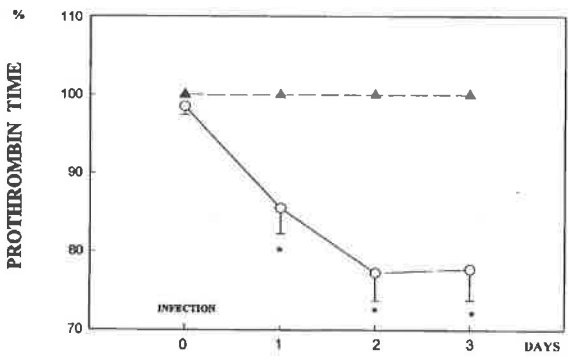

Figure 1. Decrease (as percentage) in the Prothrombin Time in the infected animals.
*A significant difference (P<0.005) was observed between uninfected (▲) and infected (O) groups.

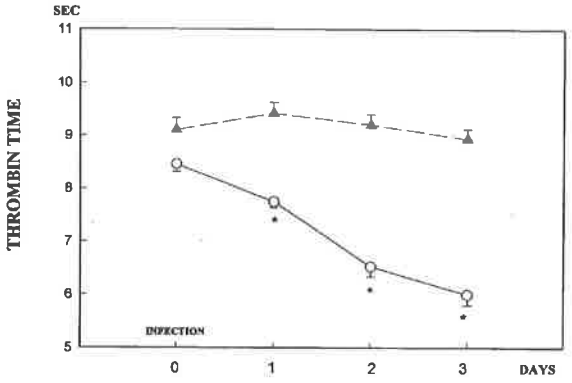

Figure 3. Decrease in the thrombin time in the infected animals.
*A significant difference (P<0.005) between the uninfected (▲) and infected (O) groups was demonstrated.

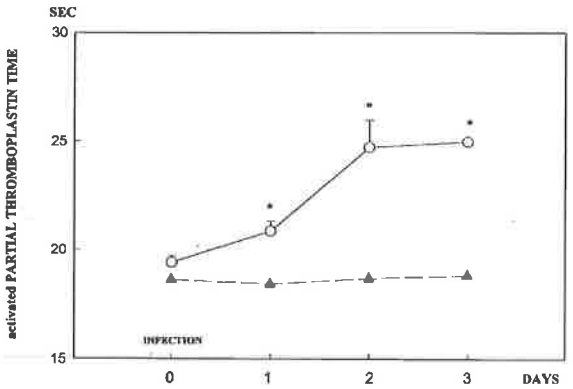

Figure 2. Increase in the activated partial thromboplastin time in the infected animals.
*A dignificant difference (P<0.005) between the uninfected (▲) and infected (O) groups was demonstrated.

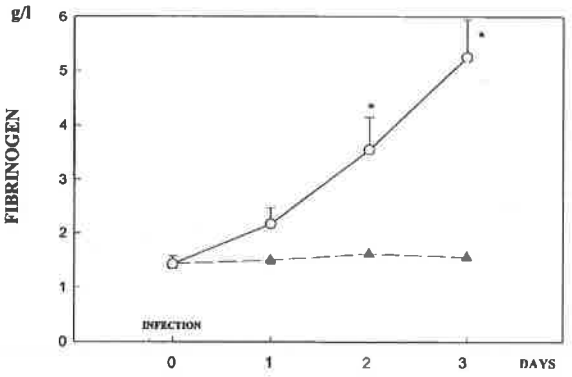

Figure 4. Increase in the levels of fibrinogen during the challenge.
*The uninfected (▲) and infected (O) groups showed significant difference (P<0.005).

(2 AcOH,H-D-CHA-NVal Arg-pNA) was added and incubated for 1 min at 37°C. The residual thrombin was evaluated for the rate of substrate hydrolysis. The concentration of ATIII is inversely proportional to the quantity of substrate hydrolysed. Calibration curves were made and the results expressed in percentage.

Means and standard errors of means (SEM) were calculated using Symphony program (Lotus Development Corporation, Cambridge, MA 024142 - USA). Student "t" test was also applied to results obtained with infected and uninfected groups.

RESULTS

All piglets in the infected group showed clinical signs of pleuropneumonia, i. e., anorexia, cough, dyspnoea, hyperthermia (40.5°C ± 0.70) and epistaxis. Leucocytosis (from 24.50 X 10^3 µl) was also observed. There was no significant change in platelets counts in the two groups.

The uninfected group had no mortality. The infected group presented 80% mortality during the trial, i.e., 8 out of 10 animals died.

In the infected group, the piglets were cyanotic with a Blood-stained froth at the nose and mouth. The trachea and larynx contained blood-stained froth. The lungs were swollen, firm with haemorrhagic lesions forming occasional nodules of clotted blood in the apical, cardiac, diaphragmatic and intermediary lobes. Fibrinous pleuritis was observed on the pleura. *Actinobacillus pleuropneumoniae* was isolated in bacterial culture of lung lesions from all dead piglets.

A progressively shortening in the prothrombin time (expressed as percentage) was observed in the

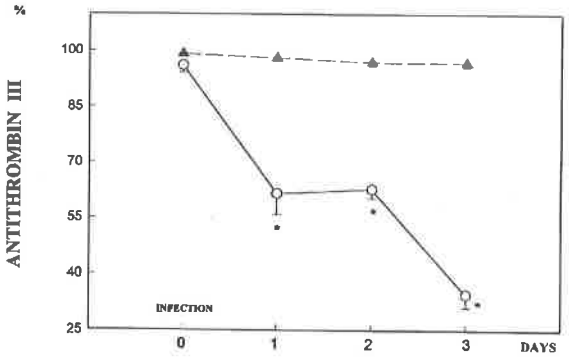

Figure 5. Decrease in the antithrombin III levels during the challenge.
*A significant difference (P<0.005) was demonstrated between the uninfected (▲) and infected (○) groups.

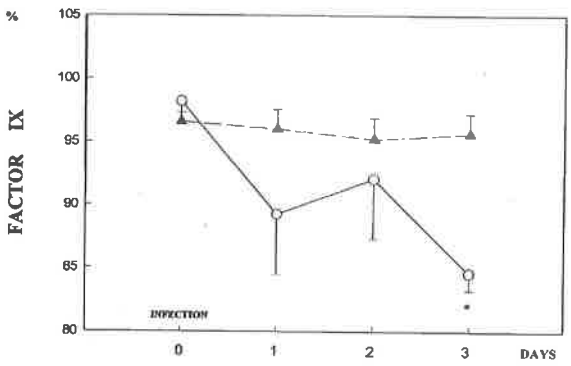

Figure 7. Decrease in the concentrations of the blood coagulation factor IX in the infected group.
*A significant difference (P<0.005) was observed between the uninfected (▲) and infected (○) groups.

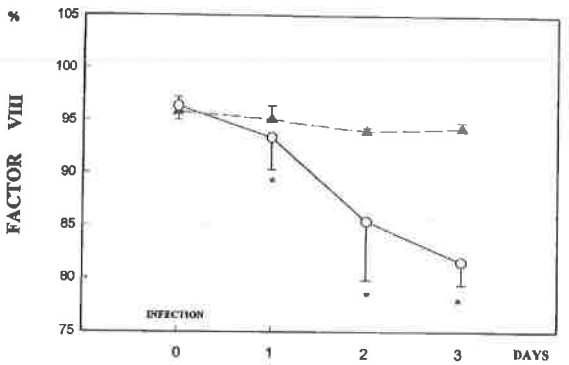

Figure 6. Decrease in the concentrations of the blood coagulation factor VIII in the infected group.
*A significant difference (P<0.005) was observed between the uninfected (▲) and infected (○) groups.

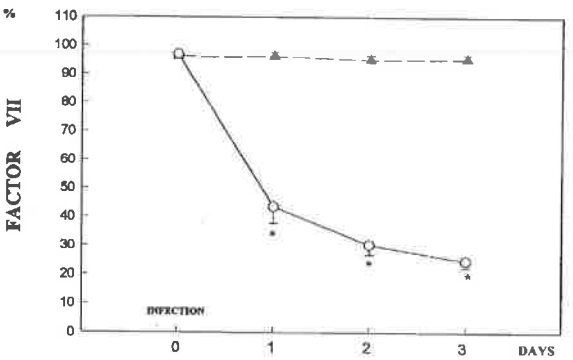

Figure 8. Decrease in the concentrations of the blood coagulation factor X in the infected group.
*A significant difference (P<0.005) was observed between the uninfected (▲) and infected (○) groups.

infected group during the trial period. The uninfected group did not change.(Fig. 1)

There was a progressive increase in the activated partial thromboplastin time (Fig. 2) a day after the infection has started.

The thrombin time (Fig. 3) and the levels of antithrombin III (Fig. 4) in the infected group decreased one day after infection up to the end of the trial.

The concentration of fibrinogen (Fig. 5) increased on the first day after infection until the end of the trial.

A decrease in the concentration of blood coagulation factors VIII (Fig. 6), IX (Fig. 7), VII (Fig. 8), X (Fig. 9) and V (Fig. 10) was observed on the second day after infection had started and persisted until the end of the trial period.

DISCUSSION

Actinobacillus pleuropneumoniae, serotype 1, produced clinical signs and mortality. Pleuritis and haemorrhagic pneumonia were observed at autopsy.

Bertram (1) demonstrated that the early effects of *Actinobacillus pleuropneumoniae* infection involve macrophage and platelet activation and a marked increase in interalveolar septal capillary permeability. Simon and Ward (17) related the importance of inflammatory mediators as endotoxin, the complement system, arachidonic acid metabolites and blood coagulation system to the genesis of pulmonary injury. Nakajima *et al.* (11) suggested that severity of the hemorrhagic and thrombotic lesions might relate to the amount of endogenous Tumor Necrosis Factor activity.

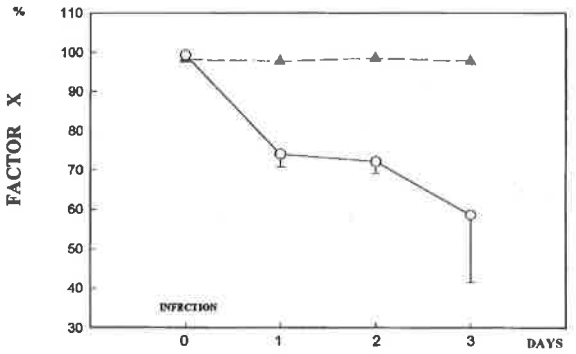

Figure 9. Decrease in the concentrations of the blood coagulation factor VII in the infected group.
*A significant difference (P<0.005) was observed between the uninfected (▲) and infected (O) groups.

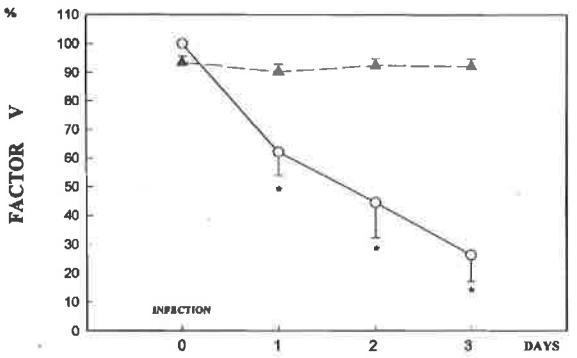

Figure 10. Decrease in the concentrations of the blood coagulation factor V in the infected group.
*A significant difference (P<0.005) was observed between the uninfected (▲) and infected (O) groups.

Movat and Burrowes (10) described the effects of endotoxin on the local inflammatory reaction, including stimulation of polymorphonuclears, macrophages and activation of blood coagulation factor XII. The latter intiates the activation of blood coagulation factors in the intrinsic and extrinsic pathways of blood coagulation. During the trial the decrease in the concentration of factors IX and VIII following the increase in the activated partial thromboplastin time reflected consumption of factors and cofactors in the intrinsic pathway of blood coagulation. Activated factor IX in the presence of activated factor VIII, phospholipides and Ca^{++} forms a complex which activates factor X in the common pathway of blood coagulation. An increase in the production of tissue factor by inflammatory cells, specially alveolar macrophages and endothelial cells stimulated by endotoxin and immune complexes, contributes to the generation of procoagulant activity leading to further consumption of factor VII of the extrinsic pathway of blood coagulation (2,18). In this study, a decrease in the concentration of factor VII and a decrease in the prothrombin time reflected alterations in the extrinsic pathway of blood coagulation. Tissue factor forms an active complex with factor VIIa and Ca^{++} which activates factor X in the common pathway of blood coagulation. A consumption of factor X was demonstrated during the trial in the infected animals.

Activated factor X in the presence of activated factor V, phospholipides and Ca^{++} results in the conversion of prothrombin to thrombin. Thrombin catalyses the conversion of fibrinogen into fibrin, activates blood coagulation factors VIII, V and XIII, and may form active complexes with thrombomodulin on the surface of endothelial cells. The thrombin-thrombomodulin complexes activate factors V and VIII. Activation of the extrinsic and intrinsic pathways of the clotting cascade as decreases in factors VII, IX and VIII were observed in this study. Endotoxin and cytokines as interleukin-1 and tumor necrosis factor act on endothelial cells to supress thrombomodulin activity (8,9). Consequently a decrease in thrombin-thrombomodulin complexes leads to increase in the concentration of thrombin. Decrease in the thrombin time reflects the hyperfibrinogenemia observed in the fibrinogen assay. Green (7) described that hepatic fibrinogen production may be increased in chronic disseminated intravascular coagulation.

Antithrombin III exerts its controlling effect on coagulation through rapid, heparin-facilitated inhibition of thrombin and activated factor X, although it may also inhibit factors IXa, XIa and XIIa. A decrease in antithrombin III concentration observed in this study may contribute to intravascular coagulation. Egbring *et al.* (4) reported that antithrombin III replacement therapy is necessary to stop disseminated intravascular coagulation.

In conclusion, inflammatory mediators produced by *Actinobacillus pleuropneumoniae* caused activation of blood coagulation factors in the extrinsic, intrinsic and common pathways of blood coagulation. These activations may contribute to generate thrombin which degrades fibrinogen into fibrin. Acute and chronic disseminated intravascular coagulation may be also induced leading to systemic hypercoagulability. The nodules of clotted blood

observed in the pulmonary lobes of the piglets infected with *Actinobacillus pleuropneumoniae* may have been produced through activation and consumption of factors in the blood coagulation system.

RESUMO

Consumo de fatores da coagulação do sangue associado a infecção causada por *Actinobacillus pleuropneumoniae*

O efeito da infecção causada por *Actinobacillus pleuropneumoniae* no sistema da coagulação do sangue de leitões foi estudado. 25 leitões desmamados isentos de organismos patogênicos específicos (IOPES) foram distribuídos de forma aleatória em 2 grupos. 10 leitões foram infectados com $5X10^6$ UFC de *Actinobacillus pleuropneumoniae* sorotipo 1, e 15 leitões usados como controles negativos. Reduções significativas nas concentrações ($P<0.005$) dos fatores de coagulação do sangue IX, VIII, VII, X e V foram demonstradas. O tempo parcial ativado da tromboplastina aumentou enquanto que o tempo da protrombina (em porcentagem) diminuiu. A concentração de antitrombina III diminui de forma significativa ($P<0.005$). As alterações observadas no tempo de trombina e na quantidade de fibrinogênio estão relacionadas com a formação de fibrina no processo de coagulação. Essas observações foram consistentes com o consumo de fatores de coagulação do sangue nas vias intrínsica, extrínsica e comum da coagulação sangüínea. Em conseqüência disso, a hemorragia pulmonar e a formação de coágulos podem ser observados em pulmões de leitões infectados com *Actinobacillus pleuropneumoniae*.

Palavras-chave: pleuropneumonia, porco, coagulação sanguínea

REFERENCES

1. Bertram, T. A. Quantitative morphology of peracute pulmonary lesions in swine induced by *Haemophylus pleuropneumoniae*. *Vet. Pathol.* 22, 598-609, 1985.
2. Car, B.D. The pathogenesis of fibrin deposition in pneumonic pasteurellosis .Thesis Cornell University, 1990.
3. Caruso, J. P.; Ross, R.F. Effects of *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* infections on alveolar macrophages functions in swine. *Am. J. Vet. Res.* 51, 227-231, 1990.
4. Egbring, R., Seitz, R., Blanke, H., Leititis, J., Kesper, H.J., Burghard, R., Fuchs, G., Lerch. L. The proteinase inhibitor complexes (antithrombin III-thrombin, α_2 antiplasmin-plasmin and α_2 antitrypsin-elastase) in septicemia, fulminant hepatic failure and cardiac shock: Value for diagnosis and therapy control in DIC/F syndrome. *Behring Inst. Mitt.*, 79, 87-103, 1986.
5. Fenwick, B.W., Osburn, B.I.; Olander, H.J. Isolation and biological characterization of two lipopolysaccharides and a capsular-enriched polysaccharide preparation from *Haemophilus pleuropneumoniae.*. *Am. J. Vet. Res.* 47, 1443, 1986
6. Folly, M., Kolly, C.; Martinod, S. Coagulation induced by challenge with various doses of *Actinobacillus pleuropneumoniae*. *Vet. Rec.* 128, 186-187, 1991.
7. Green, R A. *Veterinary Internal Medicine*, Stephen J.Ettinge, Editors: W.B. Sanders Company Philadelphiar, 3rd Edition, 2246-2264, 1989.
8. Jaffe, E.A. *Inflammation - Basic Principles and Clinical Correlates*, Eds: J. I. Gallin, I. M. Goldstein and R. Snyderman, Raven Press, Ltd., New York, 559-576, 1988.
9. Moore, K.L., Andreoli, S.P., Esmon, N.L., Esmon, C.T. and Bang, N.U. Endotoxin enhances tissue factor and supresses thrombomodulin expression of human vascular endothelium in vitro. *J. Clin. Invest.*, 79, 124-130, 1987.
10. Movat, H. Z.; Burrowes, C. E. The local Shwartzmann reaction endotoxin mediated inflamatory and thrombo-hemorrhagic lesion. Handbook of endotoxin 3, 260-302, 1985.
11. Nakajima Y., Momotani E., Takahashi H., Isshikawa Y., Ito T., Kanesaki M., Madarame H. Endogenous tumor necrosis factor (TNF) production and modification of pathological lesions in experimental *Escherichia coli* endotoxemia of piglets. *Vet. Immunol. Immunopathol.* 45(1-2), 45-54, 1995.
12. Nicolet, J. und Koenig, H. Zur *Haemophilus pleuropneumoniae* beim Schwein. Bakteriologische, pathologisch-anatomische und histologische *Befunde. Pathol. Microbiol.* 29,301-306, 1986.
13. Smits M., Kamp E., Briaire J., Jansen R., Leengoed L.V., Dijk J.V. Induction of pneumonic lesions by recombinant cytolysin of *Actinobacillus pleuropneumoniae*. Proceedings 12th IPVS Congress, 187, 1992.
14. Osborne, A.D., Saunders, J.R., Sebunya, T.K., Wilson, P. and Green, G. H. A simple aerosol chamber for experimental reproduction of respiratory disease in pigs and others species. *Can. J. Comp. Med.* 49, 434-435, 1985.
15. Rycroft, A.N., Williams, D., McCandlish, I.A.P., Taylor, D.J. Experimental reproduction of acute lesions of porcine pleuropneumonia with a haemolysin-deficiente mutant of *Actinobacillus pleuropneumoniae*. *Vet. Rec.*, 16, 441-443, 1991.
16. Sebunya, T.N.K., Saunders, J.R. *Haemophilus pleuropneumoniae* infection in swine: A review. *J. Am. Vet. Med. Assoc.*, 182, 1331-1337, 1983.
17. Simon, R.H; Ward, P.A. *Inflammation. Basic principles and clinical correlates* Eds: J. I. Gallin, I. M. Goldstein and R. Snydermann (Raven Press NY) 815-827, 1988
18. Wasi, S., Burrowes, C.E., Hay, J.B.,and Movat, H.Z. Plasminogen activator and thromboplastin activity from sheep alveolar macrophages. *Thromb. Res.* 30, 27-45, 1983.

REVISTA DE MICROBIOLOGIA

Volume 27 Number 1 January-March 1996

REVISTA DE MICROBIOLOGIA

Volume 27 Number 2 April-June 1996

REVISTA DE MICROBIOLOGIA

Volume 27 Number 3 July-September 1996

REVISTA DE MICROBIOLOGIA

Volume 27 Number 4 October-December 1996

ENVIRONMENTAL MICROBIOLOGY/SOIL MICROBIOLOGY

MICROBIAL PHISIOLOGY/GENETICS

INDUSTRIAL MICROBIOLOGY

REVISTA DE MICROBIOLOFIA

Volume 27 (1-4) January - December 1996

Author Index

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Guidelines to authors

Scope of the Journal

Revista de Microbiologia (Journal of the Brazilian Society for Microbiology), published by the Brazilian Society for Microbiology, is intended for publication of original research papers, research notes and, occasionally, reviews, covering all aspects of Microbiology.

Submitting manuscripts

Submission of a manuscript to Revista de Microbiologia (Journal of the Brazilian Society for Microbiology) is understood to imply that it has not previously been published (except in an abstract form) and that it is not being considered for publication elsewhere.
All manuscripts should be typewritten in English and submitted in triplicate to the Editors.

Publication of a manuscript

Manuscripts are accepted for publication only after they are critically reviewed. Papers are reviewed by referees indicated by the Section Editors. After review, the manuscript will be returned to the nominated author for revision according to suggestions made by the reviewers. The author should return the reviewed manuscript to the Editors. The author is notified when a manuscript is received and also when it is accepted or rejected for publication.
On acceptance of the paper, the nominated author will be requested to send the text on a computer diskette. Galley proofs will be sent to the author for correction. They should be checked carefully and handled promptly (5 days) according to instructions which are attached.
Membership in Brazilian Society for Microbiology is not a prerequisite for acceptance of a manuscript for publication. Nonmembers scientists from Brazil and other countries are invited to submit papers for consideration for publication.
Submission of a manuscript implies that all authors and their institutions have agreed to its publication.
Revista de Microbiologia assumes no responsability for errors made by the authors. Furthermore, Revista de Microbiologia assumes no responsability for conclusions reached by the authors.

Types of papers

The following types of papers are acceptable for publication in Revista de Microbiologia (Journal of the Brazilian Society for Microbiology):

Research paper: the research paper reports results of original research which have not been published elsewhere. It consists of 12 to 15 double-space typewritten or computer-written pages plus appropriate references, Tables and Figures. An abstract with title (Resumo) and three to five key-words (palavras-chave) in Portuguese must also be included.

Short Communication: a Short Communication is a concise account of new and significant findings. It should be written according to the guidelines given for research papers (see below) but without the heading divisions. It's abstract and resumo (in Portuguese) should not exceed 50 words. Figures and Tables should be restricted to a maximum of two Figures or two Tables, or one Table and one Figure. The designation "short communication" will appear above the title of this type of paper. The author should specify that his manuscript is a short communication so that it can be properly evaluated during the review process.

Mini-review: Review articles should deal with microbiological subjects of broad interest. Specialists will be called upon to write them. In addition to an abstract in English and in Portuguese (resumo), they may contain a list of contents.

Preparation of Manuscripts

General

1 - All manuscripts should be typed double-spaced with wide margins and the pages should be numbered sequentially. Research papers should be restricted to 15 printed pages, including Figures and Tables. Short Communications should be restricted to 6 printed pages.
2 - All manuscripts should be submitted written in English. The Editor recommends that a manuscript should be read critically by someone fluent in English before it is submitted. Manuscripts in poor English will not be accepted.
3 - The paper should be organized in topics, as described in the next paragraph. The name of the topics should be typed in capital letters (e.g. ABSTRACT, INTRODUCTION, etc.).
4 - Abbreviations of therms and symbols should follow the recommendations of the IUPAC-IUB Commission and the Metric System is to be used throughout.
5 - As a rule, the references in the text should be cited by their numbers. Exceptionally, when authors are mentioned in the text, the mention should be done according to the following examples: Bergdoll (number) reported that..., Bailey and Cox (number) observed that..., or Smith *et al.* (number) mentioned that... Do not use capital letters.
6 - Authors of accepted papers will be requested to send a 3 1/2" diskette containing the text prepared in a P.C. based word processor.

Organization

TITLE PAGE: A separate page should be used to give the title of the paper, complete name (including first name and middle initial) and affiliation of each author. An asterisk should be placed after the name of the author to whom correspondence about the paper should be sent. The telephone and fax numbers of this author should be given on the bottom of the page. No text of the manuscript should appear on the title page.
The title should be as brief as possible, contain no abbreviations and be truly indicative of the subject of the paper. Expressions such as "Effects of", "Influence of", "Studies on", etc., should be avoided. Care should be exercised in preparing the title since it is used in literature retrieval systems.

ABSTRACT: The abstract should be typed in a separate page and should not exceed 250 words. It should summarize the basic contents of the paper. The abstract should be meaningful without having to read the remainder of the paper. An abstract should not contain references, tables or unusual abbreviations. Abstracts are reprinted by abstracting journals and hence will be read by persons who do not have access to the entire paper. Hence the abstract must be prepared with great care. Three to five key words should also be included.

RESUMO: *Resumo* is the abstract written in Portuguese. Its preparation should follow the same recommendations for the abstract in English. The *resumo* should also contain a title in Portuguese. The rules for the title in Portuguese are the same for the title in English (see above). Three to five *palavras-chave* (key words) have also to be included. The *resumo* and the title in Portuguese should also be typed in a separate page.

INTRODUCTION: The introduction should begin on a new page and provide the reader with sufficient information so that results reported in the paper can be properly evaluated without refering to the literature. However, the introduction should not be an extensive review of the literature. The introduction should also give the rationale for and objectives of the study that is being reported.

MATERIALS AND METHODS: This section should provide enough information for other investigators to repeat the work. Repetition of details of procedures which have already been published elsewhere should be avoided. If a published method is modified, such modification(s) must be described in the paper. Sources of reagents, culture media and equipment (company, city, state, country) should be mentioned in the text. Names that are registered trade marks should be so indicated. Subheading often make this section easier to read and understand.

RESULTS: This section should, by means of text, tables and/or figures, give the results of the experiments. If a *Discussion* section is to be included, avoid extensive interpretation of results but do so in the *Discussion* section. If *Results* and *Discussion* are combined. then results should be discussed where, in the text, it is most appropriate. Tables should be numbered independently of the figures using Arabic numerals. All tables and figures must be mentioned in the text. The approximate location of tables and figures in the text should be indicated.

DISCUSSION: The discussion should provide an interpretation of the results in relation to known information.

ACKNOWLEDGMENTS: This section is optional and follows the *Discussion*. It aknowledges financial and personal assistance.

REFERENCES: Arrange the references in alphabetical order, by last name of the author. All authors must be cited. Number the references consecutively. Cite each reference in the text by its number. Journal names should be abbreviated according to the style of *Biological Abstracts* or *Chemical Abstracts*. All references given in the list should be cited in the text and all references mentioned in the text must be included in the list. List references according to the style shown in the following examples.

a. Paper in a journal
Campos, L.C.; Whittam, T.S.; Gomes, T. A.T.; Andrade, J.R.C.; Trabulsi, L.R. *Escherichia coli* serogroup O111 includes several clones of diarrheagenic strains with different virulence properties. *Infect. Immun.*, 62: 3282-3288, 1994.

b. Paper or chapter in a book
Nelson, E.B. Current limits to biological control of fungal phytopathogens. *In*: Arora, D.K.; Rai, B.; Mukerji, K.G.; Knudsen, G. (eds). *Handbook of applied mycology; soils and plants*. Marcel Dekker, New York, 1991, p.327-355.

c. Book by author(s)
Salyers, A.A.; Whitt, D.D. *Bacterial pathogenesis. A molecular approach*. ASM, Washington, 1994, 418p.

d . Patent
Hussong, R.V.; Marth, E.H.; Vakaleris, D.G. Manufacture of cottage cheese. *U.S. Pat. 3,117,870*. Jan. 14, 1964.

e. Thesis
Calzada, C.T. Campylobacter jejuni *e* Campylobacter coli - *caracterização em sorogrupos e biotipos das cepas isoladas no município de São Paulo, no período de 1983-1989*. São Paulo, 1991, 131p. (Ph.D. Thesis. Instituto de Ciências Biomédicas. USP).

f. Publication with no identifiable author or editor
Anonymous. The economy of by-products. *Álcool Alcoolquim.*, 2;33-40, 1985.

g. Communications in events (Symposia, conferences, etc)
Simão, G.S.; Silva, J.; Toledo, A.S.; Gontijo Filho, P.P. *Micobactérias não tuberculosas isoladas de pacientes com a síndrome de imunodeficiência adquirida.* XVII Congresso Brasileiro de Microbiologia, Santos, 1993, p.41.

References citing "personal communication" or "unpublished data" are discouraged, although it is recognized that sometimes they must be used. In these cases, they should be cited in the text and not in the list of references. References consisting of papers that are "accepted for publication" or "in press" are acceptable. However, references of papers that are "submitted" or "in preparation" are not acceptable.

Tables
Tables should not be included in the text. Each Table must be typed in a separate page and numbered sequentially with an Arabic number. The title of a Table should be placed in the top of it and should be brief but fully descriptive of the information in the Table. Headings and subheadings should be concise with columns and rows of data carefully centered below them.

Figures
Arabic numbers should be used for numbering the Figures. Data in Tables should not be repeated in Figures. The legend of the Figures should be placed in the bottom of them.

Photographs and line drawings
Only those photographs which are strictly necessary for the understanding of the paper should be submitted. Photoprints must be of sufficient quality to ensure good reproduction. They should be numbered on the back and identified with the nominated author's name. Legends of line drawings and photographs should not exceed the printing area. All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

Reprints

Fifteen reprints of each paper will be mailed to the nominated author, free of charge Additional reprints will be billed at cost price if requested upon returning the corrected galley proofs.