ORIGINAL PAPER

The Journal of Venomous Animals and Toxins including Tropical Diseases
ISSN 1678-9199 | 2010 | volume 16 | issue 4 | pages 592-598

Bacillus subtilis induces morphological changes in *Fonsecaea pedrosoi in vitro* resulting in more resistant fungal forms *in vivo*

Machado AP (1, 2) Anzai M (1), Fischman O (2)

(1) Department of Basic Health Sciences, Federal University of Mato Grosso, Cuiabá, Mato Grosso State, Brazil; (2) Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo, UNIFESP, São Paulo, São Paulo State, Brazil.

Abstract: Interactions among microorganisms may be the cause of morphological modifications, particularly in fungal cells. The aim of this work was to examine the changes that occur in cells of the fungus *Fonsecaea pedrosoi* after *in vitro* co-culturing with *Bacillus subtilis* and to explore the results of this interaction *in vivo* in an experimental murine infection. *B. subtilis* strain was inoculated into a 15-day pure culture of *F. pedrosoi*. *In vitro*, after 48 hours of co-culturing, the fungal cells were roundish. The secretion of fungal dark pigments and production of terminal chlamydoconidia were observed in hyphae after one week. In the *in vivo* study, two animal groups of 30 BALB/c mice each were employed. One group was inoculated intraperitoneally with hyphal fragments from the co-culture of bacteria and fungi; the other group was infected only with *F. pedrosoi* hyphae. After seven days of infection, both animal groups developed neutrophilic abscesses. Phagocytosis of bacilli by macrophages occurred at three days. At later periods, generally after 25 days, only roundish cells similar to sclerotic bodies remained in the tissues while hyphae were eliminated by 15 to 20 days. These fungal forms originated mainly from terminal chlamydoconidia. The co-culturing between bacteria and fungi may constitute a mechanism to rapidly obtain resistant fungal forms for host defenses, especially for chromoblastomycosis (CBM) experimental infections.

Key words: *Bacillus subtilis*, *Fonsecaea pedrosoi*, microbial interaction, co-culture, fungal antagonism.

INTRODUCTION

Gram-positive bacilli are widespread in the environment, particularly in soils. These organisms have been used for several biotechnological applications, for example, as biocontrollers, probiotics, bioregulators, and as producers of antibiotics, insecticides and enzymes (1-6). Gram-positive bacteria such as the ones from *Bacillus* genera can secrete a variety of substances that are inhibitory to the growth of environmental and phytopathogenic fungi (7-10). This organism has also been reported to inhibit a fungal chromoblastomycosis (CBM) agent *in vitro* (11). It has also been reported that *Bacillus* spp. can induce morphological changes in dematiaceous fungi (12-14).

Dematiaceous fungi are saprobes found mainly in soils and organic matter functioning as decomposition agents (15). A natural characteristic of these microorganisms is their dark cellular pigment, which is the result of a melanization process. Melanin has been linked to high virulence, resistance in adverse environmental conditions and low susceptibility to antifungal drugs (16, 17). Many dematiaceous fungi are harmful to their hosts, in particular the *Herpotrichiellaceae* family, which includes a large number of CBM agents (18). Worldwide, *Fonsecaea pedrosoi* is the species most frequently

isolated in CBM disease, especially in humid areas (19). Generally, infection occurs after a traumatic inoculation with vegetable fragments that are contaminated with the fungus (20-22). A typical sign of the disease is the presence of parasitic forms that are commonly known as sclerotic cells (bodies) in the tissue (20).

In a preliminary study, we isolated an environmental gram-positive bacterium from Brazilian soil that antagonizes several fungi, including some dematiaceous species (23). Herein, we studied the interactions between this bacterium and a clinical strain of *F. pedrosoi* in two manners: firstly, we examined the *in vitro* changes in fungal cells after co-culturing with bacillus, and secondly, we explored the *in vivo* significance of this interaction.

MATERIALS AND METHODS

Animals

Two groups of 30 male BALB/c mice each weighing about 23 g (6 to 8-week-old), were used throughout this study. The specific-pathogen-free animals were purchased from CEDEME/UNIFESP (Brazil). The protocol used was approved by the UNIFESP Ethics Committee under project number 0808/05.

Microorganisms

F. pedrosoi strain (EPM – 380/03) was isolated from a patient with CBM examined in the Dermatology Outpatient Department, UNIFESP, in 2003. It was cultivated on Sabouraud dextrose agar (SDA – Difco Laboratories, USA) supplemented with 80 mg/L gentamycin at a temperature of 28°C, with periodic transfers at 15-day intervals into a new SDA medium containing antibiotics. An environmental bacillus isolated from random soil samples collected from the campus of the Federal University of Mato Grosso (Cuiabá, Mato Grosso state, Brazil) was used in this study; as in previous co-culture assays, it was able to inhibit the growth of some phytopathogenic fungi and to induce the release of dark pigment by dematiaceous molds. This bacterium was identified as *B. subtilis* by classical and molecular methods (23).

F. pedrosoi Hyphae

Cultures of *F. pedrosoi* were incubated for 15 days on Sabouraud dextrose broth (SDB) with and without gentamycin, pH 5.7, while shaking at 150 rpm at 28°C.

Co-culture of *F. pedrosoi* and *B. subtilis*

The *B. subtilis* strain was inoculated with a sterilized loop into 15-day *F. pedrosoi* cultures on SDA or SDB without antibiotic and maintained at a temperature of 28°C. SDB cultures were agitated at a rate of 150 rpm for one week. These co-cultures of cells were analyzed daily by optical microscopy.

Inocula

Broth cultures were vortexed with 4 mm glass beads for 20 minutes and filtered on sterilized gauze to retain macroscopic globoid mycelia. With a 50-mL needle, the filtrates were drained and dispensed several times in a 100 mL glass-beaker to break up small mycelia-clusters and obtain solitary hyphal fragments. Viability of inocula was assessed with LIVE/DEAD® Cell Vitality Assay Kit (L34951, Invitrogen, USA) by fluorescence microscopy. The viability of inoculum suspensions generally was defined by the presence of more than 97% of cells being alive. The suspensions containing fungal forms were adjusted to a final concentration of 1 x 10^6 cells in a Neubauer chamber. The separation between septa was used to distinguish individual hyphal cells, which were then counted.

Infection

Ten minutes prior to infection, animals were anesthetized by intraperitoneal route with 0.4 µL of Anasedan® and Dopalen® 0.35 mL/kg (Vetbrands, Brazil). Approximately 100 µL of the suspension containing 1 x 10^6 hyphal cells (about 1 x 10^5 short hyphal fragments) was inoculated intraperitoneally (IP) with a 25x8/21G1 needle. One group of mice was infected IP with hyphal fragments from the co-culture of fungi and bacteria, while the other group was inoculated only with *F. pedrosoi* hyphae. The kinetics of the infection was followed for one month. Three animals were sacrificed every three days, and the abscesses were immediately removed. Part of the infected tissue specimens was cultivated on SDA. This experimental infection was performed in duplicate.

Histopathology

Fragments of fresh tissue samples from abscesses were left in 10% formalin for 12

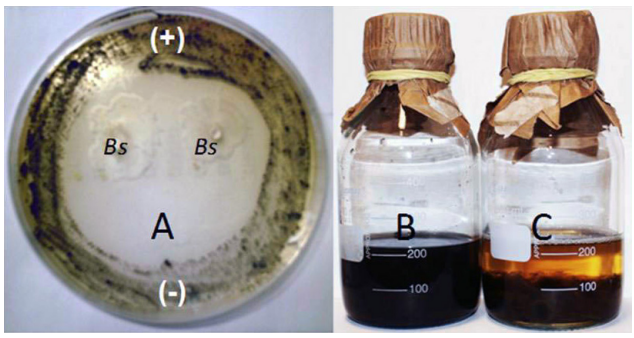

Figure 1. Chestnut-brown pigment secretion after one week of co-culturing *F. pedrosoi* with *B. subtilis*. (**A**) Plate co-culture on SDA medium, (+) positive reaction of pigment secretion proximal to bacterial colonies and (–) negative region. (**B**) Flask co-culture on SDB medium without antibiotic. (**C**) Pure culture of *F. pedrosoi* cultivated for 15 days on SDB medium with antibiotic. *Bs*: *Bacillus subtilis* colony.

hours. The specimens were embedded in paraffin, and serial sections of 3 to 5-µm from the blocks were stained with hematoxylin-eosin (HE) and by the Brown-Brenn Method (tissue gram stain).

RESULTS

Dark-Pigment Secretion on Media Culture

Viable *F. pedrosoi* cells from the SDB co-cultures with *B. subtilis* were observed daily over the course of the week. The secretion of a chestnut-brown pigment, probably melanin, was observed in co-cultures on SDA (Figure 1 – A) and SDB (Figure 1 – B) media. Cultures with antibiotic did not show dark pigments (Figure 1 – C).

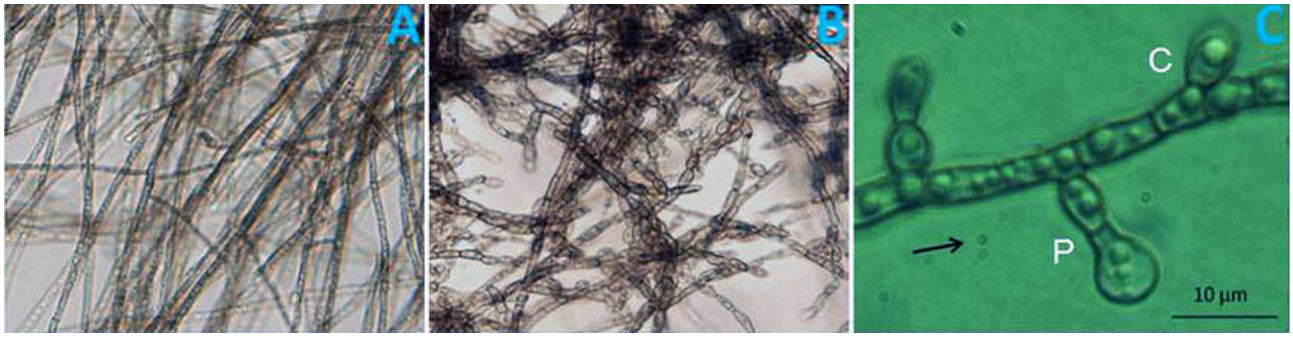

Figure 2. (**A**) *Fonsecaea pedrosoi* cells from a 15-day culture on SDB with antibiotic, 200x. (**B**) Cellular rounding (arthroconidium-like) and increase of conidiogenesis after two days of co-culturing with the *B. subtilis* strain, 200x. (**C**) Hyphal induction of lateral conidiophore (P, phialid-type) growth and conidiogenesis (C), 1000x (bacteria, arrow).

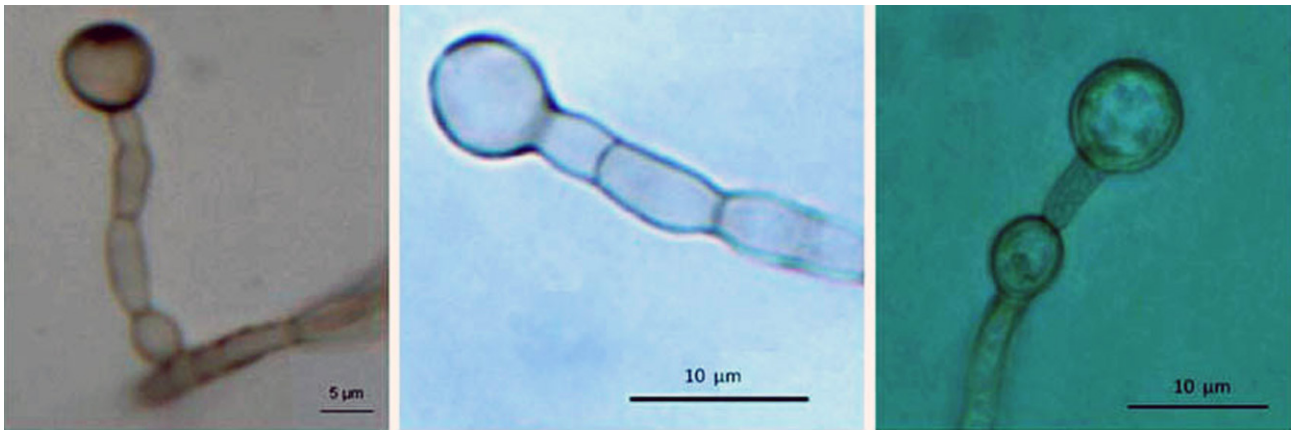

Figure 3. *Fonsecaea pedrosoi* terminal roundish-cell observed *in vitro* after a week of co-culturing with *B. subtilis* strain, 1000x.

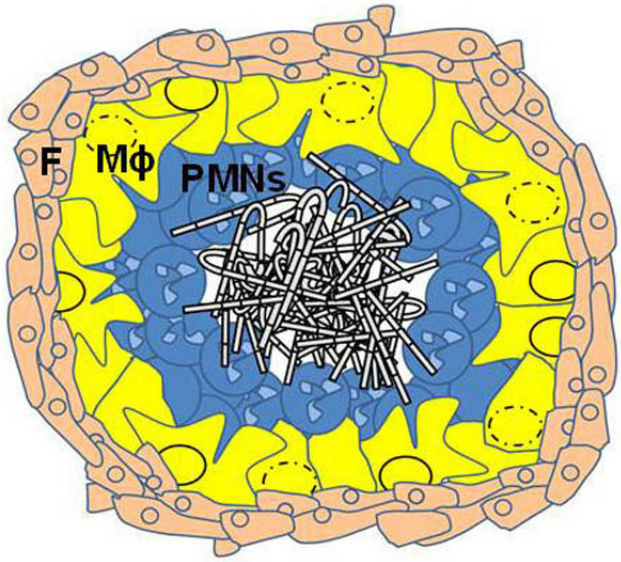

Figure 4. Scheme of peritoneal abscess observed in animals after 15 days post-infection with hyphae of *F. pedrosoi*. Abscess layers: fibroblasts (F, orange), macrophages (MΦ, yellow), polymorphonuclear leucocytes (PMNs, blue) and hyphae in abscess center.

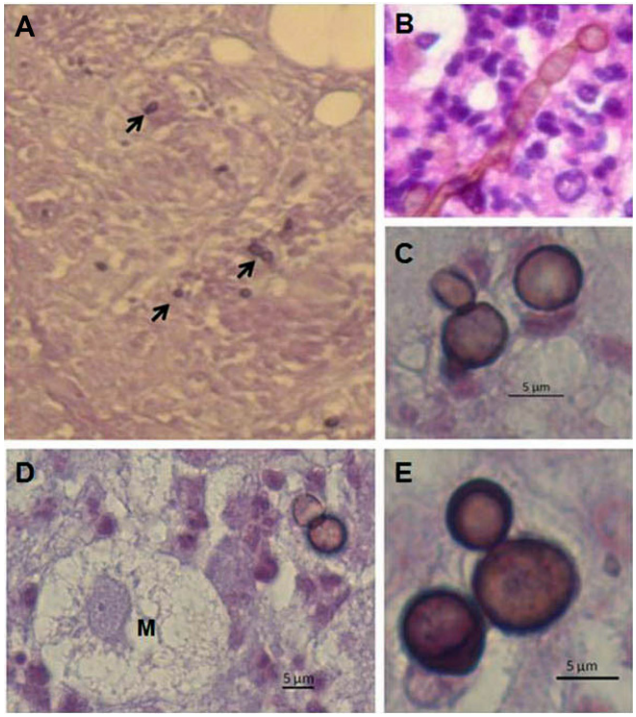

Figure 5. (**A**) Roundish cells observed 20 days after infection of mice with co-culture of *F. pedrosoi* and *B. subtilis*; Brown-Brenn (BB) stain, 100x. (**B**) Terminal chlamydoconidia on hyphae; HE, 400x. (**C**) Brown-roundish cells; BB stain, 1000x. (**D**) Foamy macrophage (M) and brown-roundish cells, BB stain, 400x. (**E**) Proliferation of brown-roundish cells, BB stain, 1000x.

Modifications of Fungal Morphogenesis

Usually, the morphology of *F. pedrosoi* hyphal cells on broth media with antibiotic is linear (Figure 2 – A), with lower conidium production by conidiophores. After 48 hours of co-culturing with *B. subtilis*, however, the *F. pedrosoi* hyphae were roundish (Figure 2 – B). The hyphal conidiogenesis and conidiophore (phialid-type) production were also enhanced (Figure 2 – C). Terminal globoid cells (chlamydoconidia-like) of 5 to 10 µm in diameter, usually with a thick wall, were found in hyphae after a week of co-culturing (Figure 3).

Murine Infection with *F. pedrosoi* Hyphae and Co-culture of Fungal Cells and *B. subtilis*

In both animal groups, we observed an intense inflammatory response and palpable lesions of approximately 8 mm in diameter a week post-infection. A capsulated neutrophilic abscess with a necrotic center was found at the inoculation site. Cellular layers were composed of distinct sets of specific cells, such as neutrophils in the central region, foamy macrophages in the periphery, and fibroblasts surrounding the abscess. A scheme showing abscess layers is presented in Figure 4. Lymphocytes were rarely detected. Dematiaceous hyphae were found in a necrotic center. Gram-positive bacilli had been phagocytized by macrophages by the third day post-infection with the co-culture. After one week, however, only *F. pedrosoi* cells remained and were recovered from the infected tissue. Fungal forms similar to sclerotic bodies, in particular those proceeding from the terminal chlamydoconidia, were observed in histological sections of the abscess produced by the infection with the co-cultured cells (Figure 5). These brown-rounded cells were *in vivo* less susceptible than hyphae to the microbicidal action of phagocytes. In general, most of the animals that were infected with the co-cultured cells healed after 25 to 30 days, while the clinical and mycological healing of mice infected with only *F. pedrosoi* hyphae were frequently achieved by 15 to 20 days of infection.

DISCUSSION

Gram-positive bacilli and fungi are important bioregulators widespread in soil (2, 24, 25). The coexistence of microorganisms in the environment during evolution has promoted several

interactions including predation, symbiosis, mutualism, competition and antibiosis (3, 26). Microbial antagonisms have been studied due to their numerous biotechnological applications, particularly those related to development of new biocontrol forms (1, 3, 25). Many *Bacillus* species present natural biocidal activities against fungi and other bacteria, and are frequently described as biocontrollers of phytopathogens (7, 13, 25, 27, 28). In the present study, an environmental bacterium (*B. subtilis* strain) with a good antagonic activity against environmental fungi and phytopathogens (23) was co-cultured with *F. pedrosoi* (clinical isolate).

The melanin secretion by the dematiaceous fungus increased after co-culturing with *Bacillus* and was continued later in broth and agar media. The ability of microbes to synthesize melanins is related to virulence and pathogenicity (17). Fungal melanogenesis was found to be stimulated after co-culturing *Cryptococcus neoformans* with *Klebsiella aerogenes* (29). Homogentisic acid, derived from the bacterial metabolism, seems to be capable of inducing fungal melanization (30). Several pathogenic species can be transformed into melanized forms or secrete melanin in order to increase antifungal resistance or evade host defense mechanisms (16, 17). It is possible that the melanin secretion by *F. pedrosoi* is associated with bacterial antibiosis or that metabolic substances are capable of inducing the melanogenesis pathway; however, more studies are necessary to confirm these hypotheses. In the present work, even though we observed the microbial growth and survival of two microorganisms in laboratory conditions, the results observed are likely to occur in nature.

Chlamydoconidia are roundish cellular forms, with a thick wall, found in filamentous fungi at intercalary or terminal positions in the hyphae. Generally, these cells are formed under environmental stress and improve fungus survival under adverse conditions. The formation of chlamydoconidia-shaped cells was observed after the interaction of dematiaceous fungi with *B. subtilis* or with bacterial metabolic products (13, 14). The introduction of bacillus into 15-day cultures of *F. pedrosoi* caused morphological changes in the fungal hyphae, including the presence of terminal chlamydoconidia-shaped cells. Previously, it has been noted that *Pseudomonas cepacia* mutants that are defective in the production of antifungal compounds fail to induce cellular abnormalities in phytopathogenic fungi, suggesting that antibiosis may be responsible for fungal morphological changes (31). Hence, our experiments raise concerns about the possibility that resistant fungal forms may be spread in nature as a result of several factors, including microbial interactions.

Curiously, we observed in histological sections that the host inflammatory response was not fully capable of eliminating all of the *F. pedrosoi* forms in the tissue. Some chlamydoconidia-shaped cells persisted a few days longer than the hyphae in host tissue. For this, morphological changes may be an important factor in fungal resistance to host defenses. Although these cells were more resistant to host immunity, all mice had healed by 25 days post-infection.

In the history of CBM research, several works have focused on establishing an experimental infection that is similar to the human form of the disease (32-36). Currently, however, there is no suitable animal model for this purpose. Nonetheless, chlamydoconidia-shaped cells have been shown to be worthy of future studies on the development of experimental infections, in which *B. subtilis* or other antagonistic strains can be used in co-cultures with CBM agents, especially because these forms may be precursors of sclerotic bodies. Recently, we developed a murine chronic infection after inoculation of round cells and chlamydoconidia from *F. pedrosoi* cells cultivated for long periods (23).

In conclusion, our results show that the interaction *in vitro* between *F. pedrosoi* and *B. subtilis* stimulated melanin secretion and induced morphological changes in a clinical isolate of *F. pedrosoi*. Chlamydoconidia-shaped cells were more resistant to the host immune response than hyphae. Thus, co-culturing antagonistic bacteria and CBM pathogens may be an interesting method for obtaining fungal forms that are more resistant to host defense.

ACKNOWLEDGMENTS

We thank Prof. Dr. Maria Regina Regis Silva, Department of Pathology (UNIFESP) for her help with histopathological analyses. Financial support was provided by CNPq and FAPEMAT.

COPYRIGHT

SUBMISSION STATUS

Received: April 8, 2010.
Accepted: May 26, 2010.
Abstract published online: June 7, 2010.
Full paper published online: November 30, 2010.

CONFLICTS OF INTEREST

There is no conflict.

FINANCIAL SOURCE

CNPq and FAPEMAT (project number 002.138/2007).

ETHICS COMMITTEE APPROVAL

The present study was approved by the UNIFESP Ethics Committee (project number 0808/05).

CORRESPONDENCE TO

ALEXANDRE PAULO MACHADO, Laboratório de Microbiologia, Departamento de Ciências Básicas em Saúde, Faculdade de Ciências Médicas, Universidade Federal de Mato Grosso, Av. Fernando Corrêa, s/n, Coxipó, Cuiabá, MT, 78060-900, Brasil. Phone: +55 65 3615 8835. Email: alexandre.paulo@unifesp.br or alepaulo@hotmail.com.

REFERENCES

1. Handelsman J, Stabb EV. Biocontrol of soilborne plant pathogens. Plant Cell. 1996;8(10):1855-69.
2. Keel C, Défago G. Interactions between beneficial soil bacteria and root pathogens: mechanisms and ecological impact. In: Gange AC, Brown VK, editors. Multitrophic interactions in terrestrial systems. London: Blackwell Science; 1997. p. 27-46.
3. Whipps JM. Microbial interactions and biocontrol in the rhizosphere. J Exp Bot. 2001;52(Spec Issue):487-511.
4. Guo X, Li D, Lu W, Piao X, Chen X. Screening of *Bacillus* strains as potential probiotics and subsequent confirmation of the *in vivo* effectiveness of *Bacillus subtilis* MA139 in pigs. Antonie Van Leeuwenhoek. 2006;90(2):139-46.
5. Niehaus F, Bertoldo C, Kahler M, Antranikian G. Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biotechnol. 1999;51(6):711-29.
6. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev. 1998;62(3): 597-635.
7. Stein T. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol. 2005;56(4):845-57.
8. Dass C, Teyegaga A. Growth suppression of some wood-decay and other fungi by *Bacillus subtilis*. Aust J Bot. 1996;44(6):705-12.
9. Cazorla FM, Romero D, Perez-Garcia A, Lugtenberg BJ, Vicente A, Bloemberg G. Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. J Appl Microbiol. 2007;103(5): 1950-9.
10. Pryor SW, Siebert KJ, Gibson DM, Gossett JM, Walker LP. Modeling production of antifungal compounds and their role in biocontrol product inhibitory activity. J Agric Food Chem. 2007;55(23):9530-6.
11. Romero H, Viscaya L, Ferrara G. *Bacillus subtilis* inhibits growth of *Cladophialophora carrionii in vitro*. J Mycol Med. 2006;16(2):92-4.
12. Romero D, de Vicente A, Olmos J, Davila JC, Perez-Garcia A. Effect of lipopeptides of antagonistic strains of *Bacillus subtilis* on the morphology and ultrastructure of the cucurbit fungal pathogen *Podosphaera fusca*. J Appl Microbiol. 2007;103(4):969-76.
13. Shirokov AV, Loginov ON, Melent'ev AI, Aktuganov GE. Protein and peptide factors from *Bacillus* sp.739 inhibit the growth of phytopathogenic fungi. Prikl Biokhim Mikrobiol. 2002;38(2):161-5.
14. Sharma N, Sharma S. Control of foliar diseases of mustard by *Bacillus* from reclaimed soil. Microbiol Res. 2008;163(4):408-13.
15. Vicente VA, Angelis DA, Queiroz-Telles F, Pizzirani-Kleiner AA. Isolation of *Herpotrichiellaceous* fungi from the environment. Braz J Microbiol. 2001;32(1):47-51.
16. Jacobson ES. Pathogenic roles for fungal melanins. Clin Microbiol Rev. 2000; 13(4):708-17.
17. Nosanchuck JD, Casadevall A. The contribution of melanin to microbial pathogenesis. Cell Microbiol. 2003;5(4):203-23.
18. de Hoog GS. Significance of fungal evolution for the understanding of their pathogenicity, illustrated with agents of phaeohyphomycosis. Mycoses. 1997;40 Suppl 2:5-8.
19. Santos AL, Palmeira VF, Rozental S, Kneipp LF, Nimrichter L, Alviano DS, et al. Biology and pathogenesis of *Fonsecaea pedrosoi*, the major etiologic agent of chromoblastomycosis. FEMS Microbiol Rev. 2007;31(5):570-91.
20. Esterre P, Richard-Blum S. Chromoblastomycosis: new concepts in physiopathology and treatment. J Mycol Med. 2002;12(1):21-4.

21. Queiroz-Telles F, McGinnis MR, Salkin I, Graybill JR. Subcutaneous mycoses. Infect Dis Clin North Am. 2003;17(1):59-85.
22. Lopez-Martinez R, Mendez Tovar LJ. Chromoblastomycosis. Clin Dermatol. 2007;25(2):188-94.
23. Machado AP, Vivi VK, Tavares JR, Gueiros FJ, Fischman O. Antibiosis and dark-pigments secretion by the phytopathogenic and environmental fungal species after interaction *in vitro* with a *Bacillus subtilis* isolate. Braz Arch Biol Technol. 2010;53(5):997-1004.
24. Gall LS. Significance of microbial interactions in control of microbial ecosystems. Biotechnol Bioengineer. 1970;12(3):333-40.
25. Boer W, Folman LB, Summerbell RC, Boddy L. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiol Rev. 2005;29(4):795-811.
26. Little AE, Robinson CJ, Peterson SB, Raffa KF, Handelsman J. Rules of engagement: interspecies interactions that regulate microbial communities. Ann Rev Microbiol. 2008;62(1):375-401.
27. Walker R, Powell AA, Seddon B. *Bacillus* isolates from the spermosphere of peas and dwarf French beans with antifungal activity against *Botrytis cinerea* and *Pythium* species. J Appl Microbiol. 1998;84(5):791-801.
28. Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology. 2001;91(2):181-7.
29. Frases S, Chaskes S, Dadachova E, Casadevall A. Induction by *Klebsiella aerogenes* of a melanin-like pigment in *Cryptococcus neoformans*. Appl Environ Microbiol. 2006;72(2):1542-50.
30. Frases S, Salazar A, Dadachova E, Casadevall A. *Cryptococcus neoformans* can utilize the bacterial melanin precursor homogentisic acid for fungal melanogenesis. Appl Environ Microbiol. 2007;73(2):615-21.
31. Upadhyay R, Jayaswal R. *Pseudomonas cepacia* causes mycelial deformities and inhibition of conidiation in phytopathogenic fungi. Curr Microbiol. 1992;24(4): 181-7.
32. Polak A. Experimental infection of mice by *Fonsecaea pedrosoi* and *Wangiella dermatitidis*. Sabouraudia. 1984;22(2):167-9.
33. Walsh TJ, Dixon DM, Polak A, Salkin IF. Comparative histopathology of *Dactylaria constricta*, *Fonsecaea pedrosoi*, *Wangiella dermatitidis*, and *Xylohypha bantiana* in experimental phaeohyphomycosis of the central nervous system. Mykosen. 1987;30(5):215-25.
34. Ahrens J, Graybill JR, Abishawl A, Tio FO, Rinaldi MG. Experimental murine chromomycosis mimicking chronic progressive human disease. Am J Trop Med Hyg. 1989;40(6):651-8.
35. Cardona-Castro N, Agudelo-Florez P. Development of a chronic chromoblastomycosis model in immunocompetent mice. Med Mycol. 1999;37(2):81-3.
36. Kurup PV. Pathogenicity of *Phialophora pedrosoi*. Mykosen. 1971;14(1):41-4.