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BIOLOGICAL CHARACTERIZATION OF SOME MICROORGANISMS OF BIOTECHNOLOGICAL INTEREST

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Abstract

Successful use of biological insecticides depends on their "active substance" quality, which ensures the biological efficacy and performance of bioproducts in field conditions: the active substance have to be virulent and to have ecological competences (epizootiological qualities, multiplication capacity, spreading and persistence in habitats and target pests populations).These parameters are objective criteria for selection of microorganisms that can be successfully included in biological control programs of pests. In this paper are presented results of some laboratory tests aimed to estimate biological parameters specific for entomopathogenic fungi, including the spores germination and spores production on culture media and insects. The biological material used in this study consisted in five Beauveria bassiana entomopathogenic biotypes, isolated from insects belonging to the ord. Coleoptera, Hymenoptera and Diptera in natural epizootic outbreaks, during the years 2008-2010. The results led to the following conclusions: (i) B. bassiana strains having different origins in terms of habitat and host insect exhibit different biological qualities, (ii) the evaluation of test insects mortality induced by B. bassiana doesn't provides a complete pathological characterization of fungal isolates; to assess the biological control potential of B. bassiana isolates, the virulence have to be correlated with the biological cycle of the fungus and the spores production on the insect cuticle; (iii) the quantification of spore production on insect is also necessary to further evaluate the fungal strains potential to naturally increase of virulence.

Key words**:** *entomopathogenic fungi, biotypes, Beauveria bassiana*

INTRODUCTION

Agricultural production is still the main source of food for humankind. Population explosion has determined the increase of agricultural production in response to growing food requirements. The growth in human population around the world affects all people through its impact on the economy and environment. In many cases it was made an irrational exploitation of natural resources leading to ecological imbalance. One of the biggest ecological challenges facing plant protectionists is the development of environmentally friendly alternatives to the extensive use of chemical pesticides for crop diseases and pests control. Improvements in crop management systems can have an important influence on product quality as well.

Uses of synthetic pesticides led to undesirable effects on non-target organisms sharing the ecosystem and decrease the loss of efficacy due to adaptation of pest agents. Moreover, the toxicity of their residues affects the environment and people health too (Keller&Brenner, 2005; NRC, 1996; Robinson et. al, 1980).

Research on biological pest control methods can make an important contribution to reducing the use of chemicals for crop protection. (Butt&colab, 2001; Gerhardson, 2002; Lacely&colab., 2001).

Beauveria bassiana (Bals.) Vuill. is an enthomopathogenic fungus that grows naturally in soils (Steinhaus, 1956). It is being used as a biological insecticide all over the world in order to control pests such beetles, whiteflies, aphids, mealybugs, grasshoppers etc.

B.bassiana strains vary in their host ranges, having, generally, a wide host range. *B.bassiana* strains isolated from different hosts inhabiting different geographical regions differ in theirs ecological capacities.

Ecological competences of different strains (epizootiological qualities, multiplication capacity, spreading and persistence in habitats and target pests populations) are objective criteria for selection of those which can be successfully included in biological control programs of pests (Andrei, 1999).
Several strains of the entomopathogenic fungi (including 5 strains of *B. bassiana* presented in this experiment) have been isolated in order to use them for biological insecticides production at the Research-Development Institute for Plant Protection Bucharest.
This work aimed at selecting *B. bassiana* strains favourable growth, sporulation, viability and infectivity responses on different culture media.

MATERIAL AND METHOD

Five strains of *B. bassiana* were used as biological material in this study. All strains were isolated from natural outbreaks, purified and stored on sterilized potato dextrose agar (PDA) slants, at 4^0C (Table 1).

Table 1. *Beauveria bassiana* strains isolated from natural epizootic outbreaks during the period 2008-2010

Fungal strain	Insect		
	Name and order	Isolated from/at	Natural outbreaks
BbIt	The European spruce bark beetle (*Ips typographus*) Coleoptera	Iacobeni / Sept. 2010	Spruce trunk in a coniferous forest
BbAll	East Asian sawfly (*Aprocerus leucopoda*) Hymenoptera	Şoldăneşti, / June 2010	Elm leaf in a deciduous forest
BbBrodoc		O.S. Brodoc / July 2011	
BbCr	Leaf beetle (*Chryptocephalus* sp.) Coleoptera	Cap Kaliakra / Aug. 2009	Sunflower crop
BbSc	Fungus gnats (*Sciara* sp.) Diptera	Topoloveni, Aug. 2008	Pot with wet soil

The strains used in the experiment were grown on plates using three different culture media (Sabouraud, Czapek-Agar and PDA) as monosporal cultures, and incubated at 25^0C, under dark conditions. For each strain were prepared separately nine Petri dishes, three plates for each type of culture media (three replications).

The colonies were measured daily during 14 days. Macroscopic observations were made at 10 and 15 days after inoculation and were analyzed the average growth of colonies, their appearance, the sporulation, the emergence of sporulating structures and pigmentation for every strain and for each of the three media.
The experiment aimed also the virulence of these five strains. Insects from order Coleoptera and Lepidoptera (2^{nd} and 3^{rd} larval stage) were selected for this purpose: *Galleria mellonella* L., *Plodia interpunctella* (Hübner) and *Tenebrio molitor* L. The artificial inoculation was made by spraying on insects the *B.bassiana* conidial suspensions. The larval mortality was analyzed after 5 and 10 days. The sporulation rates of each strain on death test insects were analyzed after 30 days using serial dilution method for counting fungal spores.

RESULTS AND DISCUSSIONS

The influence of solid media on the development of the *B. bassiana* strains is presented in Table 2-7.
The growth rate was monitored daily by measuring the colony diameter.
The highest mycelial growth rate of *B. bassiana* colonies cultivated on Czapek medium was observed at BbAl strain (Fig. 1). The average fungal colonies diameter was 39.67 mm. The lowest growth rate was recorded for BbIt strain, with an average diameter of 33.33 mm colony growth after 14 days.

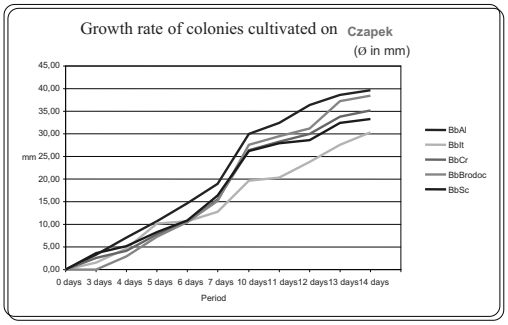

Fig. 1. Growth rate of colonies cultivated on Czapek medium

On Sabouraud medium, the highest mycelial growth rate of colonies was observed at BbCr strain. The average fungal colonies diameter

was 32.23 mm. The lowest growth rate was recorded for BbSc strain, with an average diameter of 19.11 mm colony growth after 14 days (Fig 2).

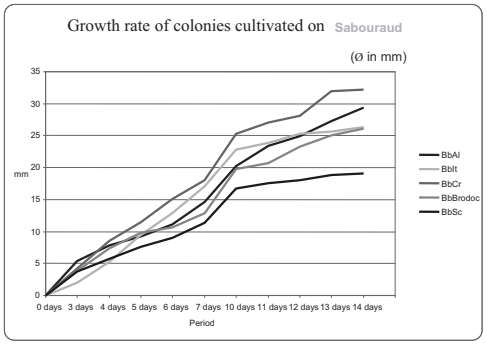

Fig. 2. Growth rate of colonies cultivated on Sabouraud medium

The highest mycelial growth rate of *B. bassiana* colonies on PDA medium was observed at BbCr strain. The average fungal colonies diameter was 45.50 mm. The lowest growth rate was recorded for BbBrodoc strain, with an average diameter of 29.78 mm colony growth after 14 days (Fig 3).

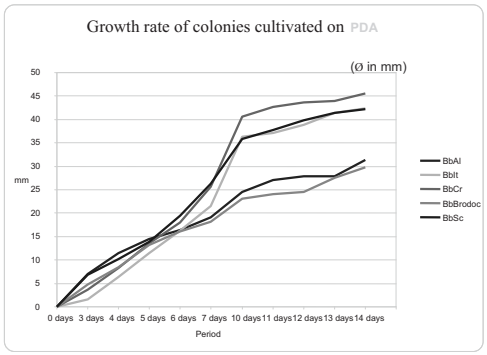

Fig. 3. Growth rate of colonies cultivated on PDA medium

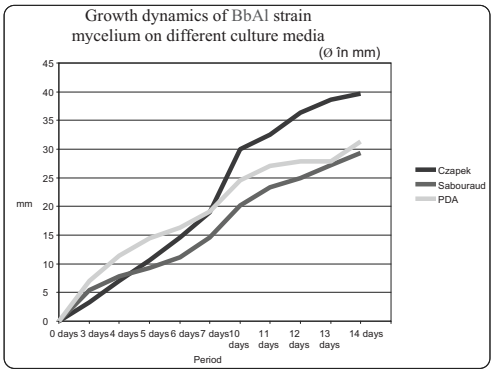

Fig. 4. Growth dynamics of BbAl strain mycelium on different culture media

The highest increase in BbAl mycelial colony diameter was recorded on those cultivated on Czapek medium (39.67 mm), followed by those on PDA (31.39 mm) and Sabouraud (29.33 mm) (Fig 4).

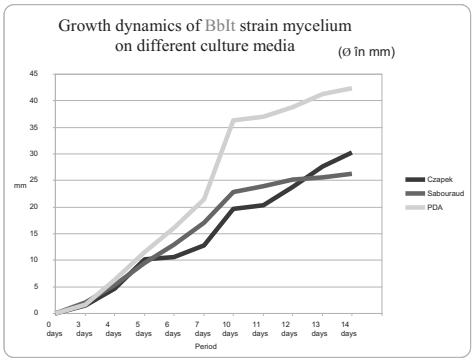

Fig 5. Growth dynamics of BbIt strain mycelium on different culture media

The highest increase in BbIt mycelial colony diameter was recorded on those cultivated on PDA medium (42.33 mm), followed by those on Czapek (30.33 mm) and Sabouraud (26.29 mm) (Fig. 5).

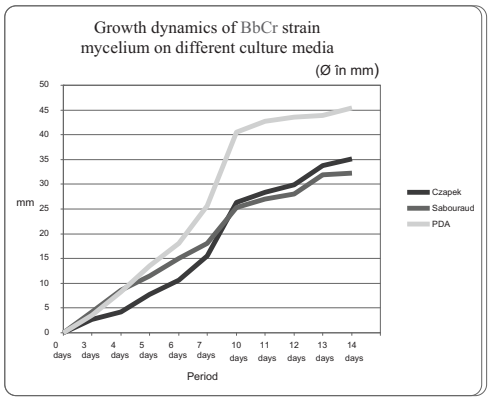

Fig. 6. Growth dynamics of BbCr strain mycelium on different culture media

The highest increase in BbCr mycelial colony diameter was recorded on those cultivated on PDA medium (45.50 mm), followed by those on Czapek (35.13 mm) and Sabouraud (32.23 mm) (Fig 6).

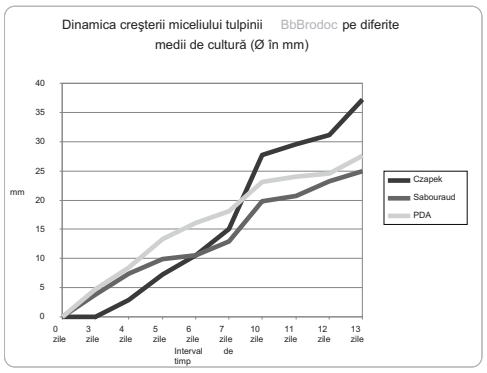

Fig. 7. Growth dynamics of BbBrodoc strain mycelium on different culture media

The highest increase in BbBrodoc mycelial colony diameter was recorded on those cultivated on Czapek medium (38.50 mm), followed by those on PDA (29.78 mm) and Sabouraud (26.11 mm) (Fig 7).

The highest increase in BbSc mycelial colony diameter was recorded on those cultivated on PDA medium (42.19 mm), followed by those on Czapek (33.33 mm) and Sabouraud (19.11 mm) (Fig 8).

The influence of culture media in viability test of conidia of entomopathogenic fungi was experimented on *Lecanicillium lecanii*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* isolates (Francisco et al., 2006).

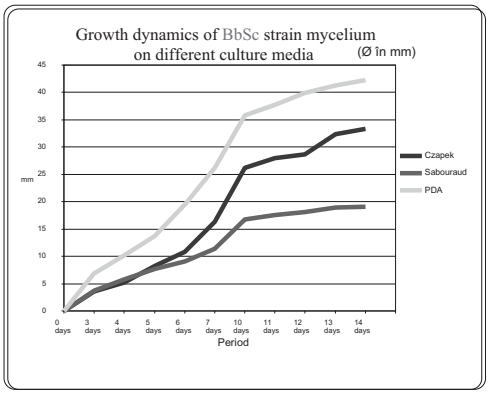

Fig. 8. Growth dynamics of BbSc strain mycelium on different culture media

The tests were performed on microscope slides containing one of the culture media: agar-water, minimal medium, potato-dextrose-agar, potato-dextrose-1% yeast extract agar, sabouraud-dextrose-yeast extract-agar and complete medium. The culture media influenced the germination of the species studied, verifying within and inter specific variations. *B. bassiana* development was also tested in different liquid cultures, resulting in different yield of blastospores. In peptone-glucose, the yeald of blastospores was four-fold higher than in glucose-peptone-yeast extract (Bidochka et al., 2004).

Rombach (2006) tested several simple liquid media for *B. basiana* submerged conidiation. The results proved maximum yields of conidia in medium consisting of sucrose-yeast extract and basal salts and maximum yields of hyphal bodies in a sucrose-yeast-extract medium. More than that, Kmitowa (1979) studied the effect of the quantitative gradient of nitrogenous compounds added to the culture medium has on the growth and pathogenicity of over 30 *B. bassiana* strains. The author reported that among the *B. bassiana* strains under study some formed poorly growing and weakly sporulating colonies while others grew rapidly and proliferously forming dense and sporulating colonies. Biomass of some strains exceeded many times that of the others. The pathogenicity of these strains was not correlated with their rates of growth.

Colonial morphology of *B.bassiana* biotypes is presented in Photo 1-8 and Table 2-6.

BbAl strain growth and sporulation

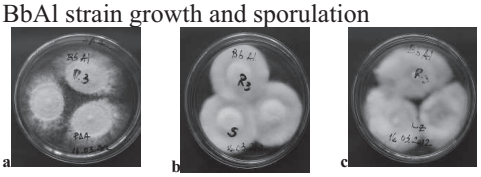

Photo 1. Mature colonies of BbAl strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 2. Colonial morphology of BbAl strain grown on different culture media

Colony morphology (10 days after inoculation)	BbAl strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular, raised	circular
Elevation	flat	hemispheric, fluffy mycelia	convex
Margin	regular edge	entire	entire
Opacity	translucent	opac	opac
Sporulation	unsporulated	unsporulated	sporulated

BbIt strain growth and sporulation

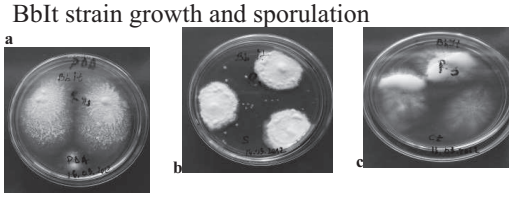

Photo 2. Mature colonies of BbIt strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 3. Colonial morphology of BbIt strain grown on different culture media

Colony morphology (10 days after inoculation)	BbIt strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular	circular
Elevation	flat	crateriform	flat
Margin	filiform	curled	regular edge
Opacity	translucent	opac	translucent and 1/3 opac
Sporulation	sporulated	sporulated (2/3 started from center)	sporulated (1/3 started from center)

BbCr strain growth and sporulation

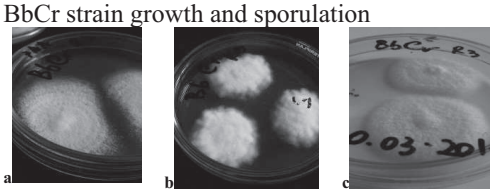

Photo 3. Mature colonies of BbCr strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 4. Colonial morphology of BbCr strain grown on different culture media

Colony morphology (10 days after inoculation)	BbCr strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular	circular
Elevation	flat	crateriform	flat
Margin	regular	curled	regular
Opacity	translucent	opac	translucent in growing area and opac in area with sporulation
Sporulation	sporulated	sporulated	sporulated

BbBrodoc strain growth and sporulation

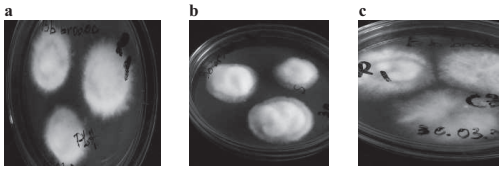

Photo 4. Mature colonies of BbBrodoc strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Table 5. Colonial morphology of BbBrodoc strain grown on different culture media

Colony morphology (10 days after inoculation)	BbBrodoc strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular, gibbous	circular, slightly bulging
Elevation	plat	hemispheric fluffy mycelia	flat
Margin	filiform	entire	regular
Opacity	translucent	opac	opac
Sporulation	unsporulated	unsporulated	unsporulated

BbSc strain growth and sporulation

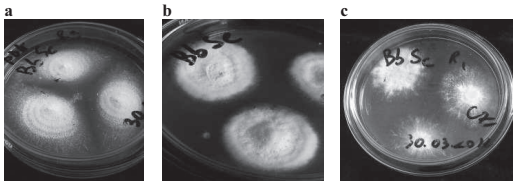

Photo 5. Mature colonies of BbSc strain grown on three different culture media: a. PDA; b. Sabouraud; c. Czapek

Culture media differentially influenced the growth, colony character and sporulation of *B.bassiana* tested strains. The results reported by Barnes G.L. (Barnes et al., 1975) proved

that *B. bassiana* growth and sporulation are influenced by various peptone sources in culture.

Table 6. Colonial morphology of BbSc strain grown on different culture media

Colony morphology (10 days after inoculation)	BbSc strain on culture media		
	Czapek	Sabouraud	PDA
Shape	circular	circular, gibbous	circular, slightly bulging
Elevation	plat	hemispheric, fluffy mycelia	flat
Margin	filiform	entire	regular
Opacity	translucent	opac	opac
Sporulation	sporulated	sporulated	sporulated in concentric circles

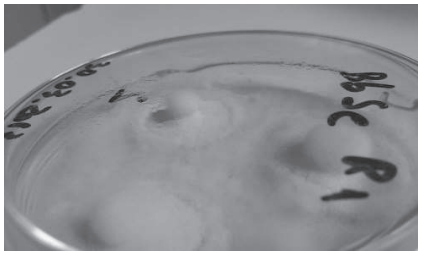

Photo 6. Fungal coremia of BbSc strain grown on Sabouraud

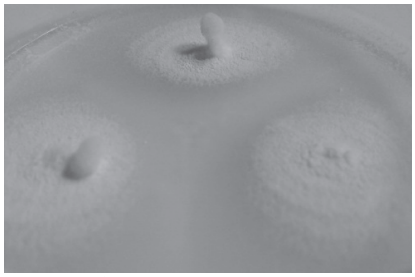

Photo 7. Fungal coremia of BbSc strain grown on PDA

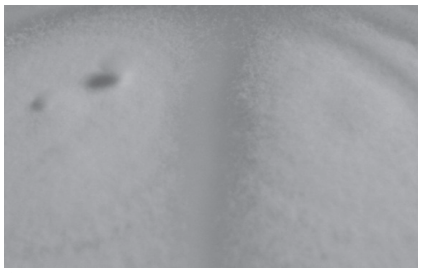

Photo 8. Fungal coremia of BbSc strain grown on Czapek

Pathogenicity tests performed in laboratory conditions and quantified in external sporulation on test-insects (Photo 9) proved that the saprophytic development of *B. bassiana* biotypes vary from one to another strain (Table 7).

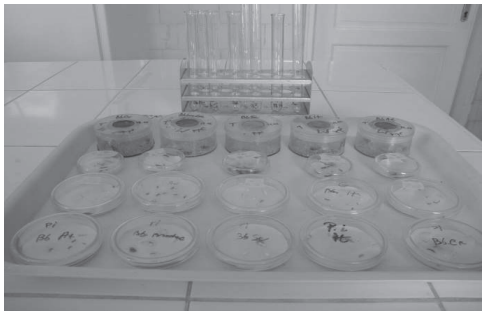

Photo 9. Pathogenicity test on insects

From all tested strains, BbSc strain recorded the largest amount of spores, both on *G. mellonella* and *P. interpunctella* larvae.

Table 7. *B. bassiana* strains spore production on test insects

B. bassiana strains	test insects	
	Galleria mellonella	*Plodia interpunctella*
	spore concentration ml^{-1}	spore concentration ml^{-1}
BbCr	0.525	1.175
BbSc	0.825	10.9
BbIt	0.425	0.975
BbBrodoc		0.675
BbAl		0.55

CONCLUSIONS

The results of this work show that BbAl and BbCr strains are recommended for further investigation in order to determine the most favourable substrate for mass production of bioformulated products.

Statistically, PDA environment has provided the best carbon source for vegetative development *B.bassiana* strains.

B. bassiana tested strains, regardless of their origin, has biotechnological potential, related to the ability to degrade various synthetic nutritive substrates and to keep the pathogenicity against insects belonging to different orders.

Spores production tests proved different capacities of *B. bassiana* biotypes to efficiently exploit the nutritive substratum.

B. bassiana strains with different origins in terms of habitat and host insect exhibits different biological qualities.

The evaluation of test insects mortality induced by *B. bassiana* doesn't provides a complete pathological characterization of fungal isolates; to assess the biological control potential of *B. bassiana* isolates, the virulence have to be correlated with the biological cycle of the fungus and the spores production on the insect cuticle.

The quantification of spore production on insect is necessary to further evaluate the fungal strains potential to naturally increase of virulence.

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