EFFECT OF DIFFERENT CARBON AND NITROGEN SOURCES ON SPORULATION OF *BEAUVERIA BASSIANA* ROMANIAN STRAINS

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Abstract: A requirement for industrial-scale production of mycoinsecticides is the capacity of fungal strains to produce infective and stable propagules on inexpensive artificial substrates, with either solid-state or submerged liquid fermentation methods. The ability of entomopathogenic fungi to use different nutritive substrates is one of the factors influencing their effectiveness.Vegetative growth and sporulation yield depend on the composition of the culture medium and are specific to each fungal isolate. Our study has focused on fungal inoculum produced in artificial media. Native *Beauveria bassiana* strains was cultivated in liquid medium involving variations in carbon and nitrogen sources and the production of spores was evaluated. The results revealed that among the carbon and nitrogen sources tested, zaharose and ammonium nitrate were most efficiently used for the production of *B.bassiana* spores in submerged liquid fermentation.

Key words: *Beauveria bassiana, liquid medium, carbon/nitrogen sources*

INTRODUCTION

Entomopathogenic fungi can provide safe and effective control of many important insect pests. The current interest for the biological plant protection determined the direction of the research towards finding efficient solutions for biotechnological processing of entomopathogenic microorganisms in order to obtain biopesticides. Development of an efficient method for mass production of a mycopesticide requires detailed knowledge of the nutritional requirements for the growth and sporulation of the fungus (Gao, 2011). Biological control using entomopathogenic fungi will only become feasible if economic methods of mass production are available (Jenkins & Goettel, 1997).

Entomopathogenic fungus *Beauveria* sp. is considered promising candidate, given its cosmopolitan nature, its potential to control a wide range of harmful insects and its safety to most non-target organisms (Tuan et al., 2009). Other important parameters should be considered for commercial development of mycoinsecticides. For example, the production of infective and stable propagules on inexpensive artificial substrates is a requirement for industrial-scale production with either solid-state or submerged liquid fermentation methods (Jackson et al., 2010). The ability of entomopathogenic fungi to use different nutritive substrates is one of the factors influencing their effectiveness; the conidia germination on insect and in the culture medium is dependent on certain nutritional requirements. The virulence of fungal strains is influenced by the composition of the culture medium used, in vitro cultivation may cause a decrease in virulence, as the growth of fungi on special media ensures a virulence similar to that induced by insect passages (McCoy et al., 1988). On the other hand, the ability of *Beauveria bassiana* strains to degrade nutrients is a measure of their potential to adapt to varying environmental conditions, an important aspect in the activity of selecting fungal strains to obtain mycoinsecticides. The selection of fungal strains with high potential to adapt to different nutritional substrates guarantees satisfactory yields of fungal biomass both quantitatively and qualitatively. The capacity to use carbon sources is presented as a method to identify and differentiate the fungal isolates of *Beauveria* and *Tolypocladium*

(Todorova et al., 1998), also as a method to phenotypically characterize them (Draganova et al., 2011). The ability of fungal strains to use carbon sources has also been shown to influence their virulence (Pernfus et al., 2003).

Studies on the optimal liquid culture conditions for maximal sporulation of *B. bassiana* have been the subject of numerous papers (Bartlett & Jaronski, 1988; Brown, 1988; Feng et al., 2000; Pham et al., 2009; Yadav et al., 2013; Andrei, 2004; Dinu, 2013; Fatu, 2016). Bidochka et al. (1990) studied the ability of *B. bass*iana strains to store carbohydrates in the biomass. Hallswortht and Magan (1994) analized the effect of different carbohydrate concentrations on the growth and accumulation of polyols and trehalose in the conidia of *B. bassiana, Metarhizium anisopliae, Paratoxotus farinosus*. Effect of nitrogen sources on formation of the toxic protease in *B. bassiana* submerged culture was also studied (Kucera, 1971).

Considering the importance of host-specific isolates for the development of target-specific mycoinsecticides, the objective of this study was to evaluate the tolerance of some native *B.bassiana* strains to the different carbon and nitrogen sources, for maximal sporulation in submerged fermentation cultures.

MATERIALS AND METHODS

Fungal strains *B. bassiana* strains studied in terms of the capacity to use different sources of carbon and nitrogen belong to the Collection of entomopathogenic microorganisms from Research Development Institute for Plant Protection (RDIPP), Bucharest. These are native strains isolated from insects in natural outbreaks of infestation (Figure 1).

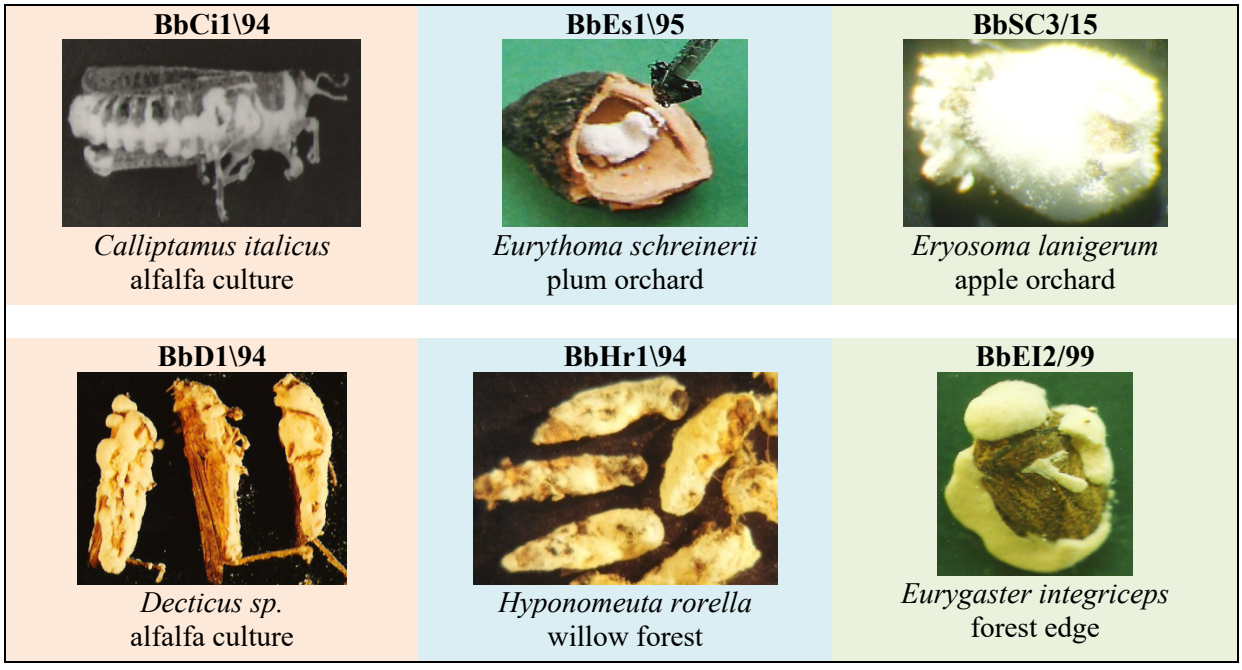

Figure 1. *Beauveria bassiana* strains studied, abbreviations and sources (host insect/habitat)

Preparation of fungal inoculum The production of the fungal inoculum was done in two stages: (i) *laboratory inoculum* was obtained by multiplying *B. bassiana* strains from the stock culture on potato-dextrose-agar medium; (ii) *batch inoculum* was obtained in submerged culture, using liquid basal medium (pH 5.0) based on glucose, KNO_3, KH_2PO_4, $MgSO_4$ and

trace salts (Thomas et al., 1987), after 5 days incubation period (26^0C). Considering the fact that different fungal isolates vary in their requirements for nutrition, in the basal medium, various carbon sources were incorporated at a final concentration of 6 g/l. Carbon sources tested were monosaccharides (arabinose, ribose, fructose) disaccharides (zaharose, maltose, lactose) and polisaccharides (celulose, starch); it was also tested molasses (about 40% zaharose). The nitrogen sources were incorporated in the basal medium at a final concentration of 4 g/l. Nitrogen sources tested were peptone, yeast extract and some by-products: wheat bran (by-product of wheat grain milling and grinding), soybean meal (by-product of the extraction of soybean oil, corn steep liquor (by-product of the corn wet-milling industry).

RESULTS AND DISCUSSION

The results regarding the capacity of some native *B.bassiana* strains to use different carbon and nitrogen sources when they are cultivated in liquid medium, are presented graphically in figures 2 and 3.

The average spores yealds had values between 6.13 and 10.28 ($x10^6$)/ml, with minimum values in the medium containing molasses ($4.91x10^6$ spores/ml - BbEI2/99 strain), cellulose ($2.00x10^6$spores/ml - BbEI2/99 strain), respectively starch ($1.53x10^6$ spores/ml - BbHr1/94 strain). The maximum yealds were registered in the media containing zaharose ($13.76x10^6$ spores/ml - BbD1/94 strain), respectively fructose ($12.89 x10^6$ spores/ml - BbSC strain). Arabinose and ribose were effectively used for sporulation by BbHr1/94 ($22.70x10^6$ spores/ml) and BbSC3/15 ($12.09 x10^6$ spores/ml) strains. Similar yealds were recorded in the medium with maltose ($12.88x10^6$ spores/ml - BbD1/94 strain), respectively lactose ($11.68x10^6$ spores/ml - BbSC3/15 strain). Among the nitrogen sources tested, only ammonium nitrate and yeast extract were effectively used in the sporulation process by all strains studied ($12.36x10^6$ spores/ml - BbD1/94 strain, $12.12x10^6$ spores/ml - BbSC3/15 strain, $22.0x10^6$ spores/ml - BbHr1/94 strain). In the case of the other nitrogen sources tested, although small amounts of spores were recorded, as in the case of carbon sources, the BbHr1/94, BbD1/94 and BbSC3/15 strains showed a superior ability to degrade the nutrient substrate. The BbEI2/99 strain proved to have the weakest capacity to use the carbon and nitrogen sources tested; there were maximum yealds of spore in the medium with zaharose ($9.01x10^6$ spores/ml), followed by fructose ($8.20x10^6$ spores/ml), lactose ($8.02x10^6$ spores/ml). In the case of BbCi1/94 and BbHr1/94 strains, the average yeald was of 1.30 respectively 1.27 lower, compared to BbEI2/99 strain. Zaharose was efficiently used, registering yealds of $13.07x10^6$ spores/ml (BbHr1/94 strain), respectively $11.34x10^6$ spores/ml (BbCi1/94 strain). Among the studied strains, the BbCi1/94 strain used the most efficiently molasses for the production of spores - $13.07x10^6$, an amount about 2 times higher than the other strains. The BbSC3/15 strain (average yeald $10.13x10^6$ spores/ml) produced large amounts of spores ($> 12x10^6$) in the mediua containing 6 of the carbon sources tested, with highest spores yeald maximum in the medium with zaharose ($13.3x10^6$ spores/ml), fructose ($12.89x10^6$ spores/ml), ribose ($12.35x10^6$ spores/ml), arabinosis ($12.09x10^6$ spores/ml).

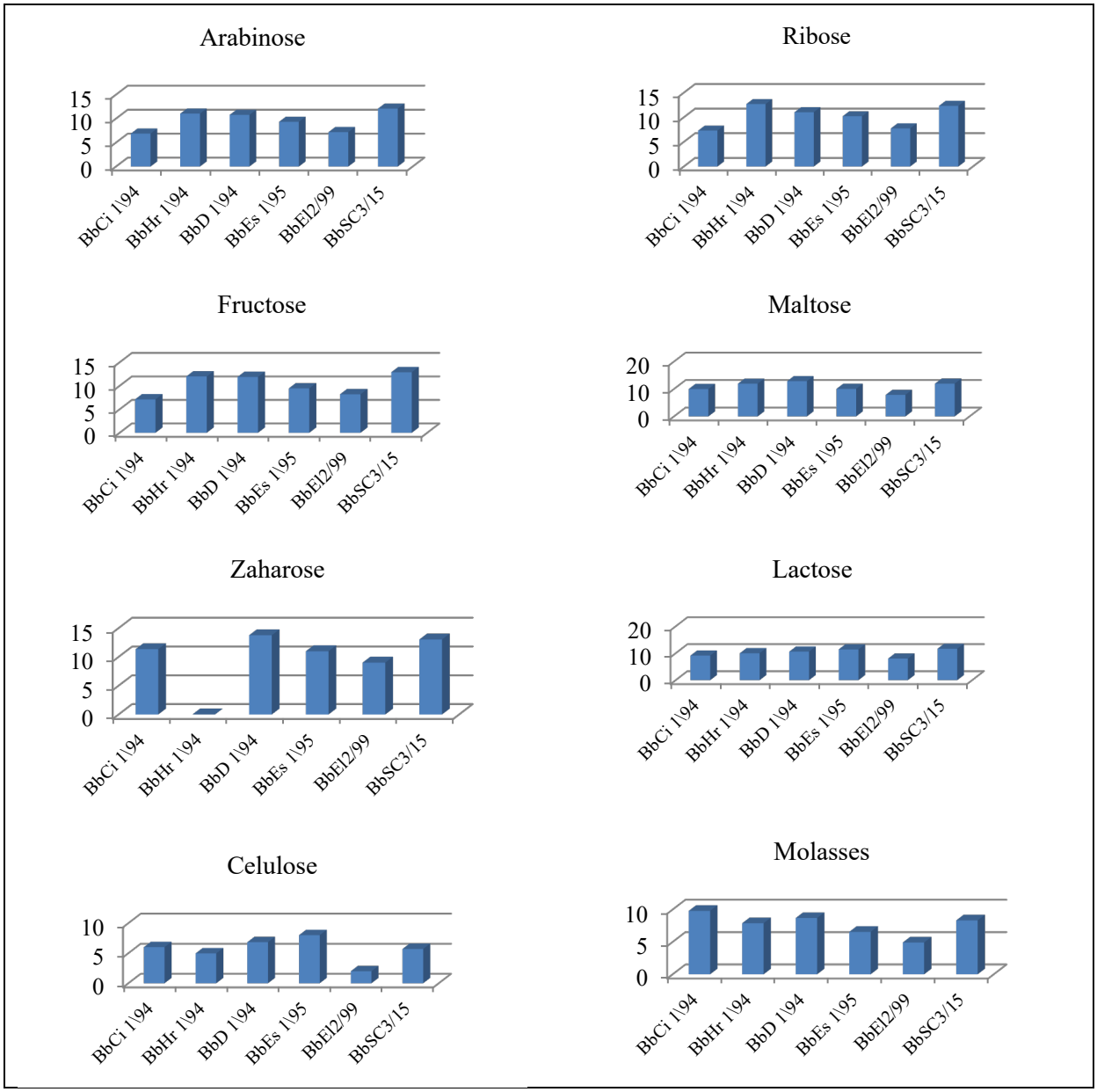

Figure 2. Spore production of *B. basiana* native strains in liquid culture medium containing various carbon sources

The results of our experiment prove that at the same *B.bassiana* strain, sporulation, expressed by the number of spores/ml, is different depending on the composition of the culture medium, mainly on the source of carbon and nitrogen. In similar experiments Leite et al. (2003) examined the effects of carbon and nitrogen sources on the growth of three genera of *Entomophthorales*: *Batkoa, Furia*, and *Neozygites* and found that the fungi had similar responses to various carbon sources but different responses to various nitrogen sources. The impact of nutrition on spore yields for various strains of *B. bassiana, Paecilomyces fumosoroseus* and *M. anisopliae* was studied by Vega et al. (2003) in six nutritionally different liquid media. The results proved that spore production was variable from one isolate to another. We find the same observation in the studies performed by Kassa et al. (2008); they found that when *B. bassiana* and *M. anisopliae* were mass produced on whey, spore yield and viability were significantly influenced by fungal isolate.

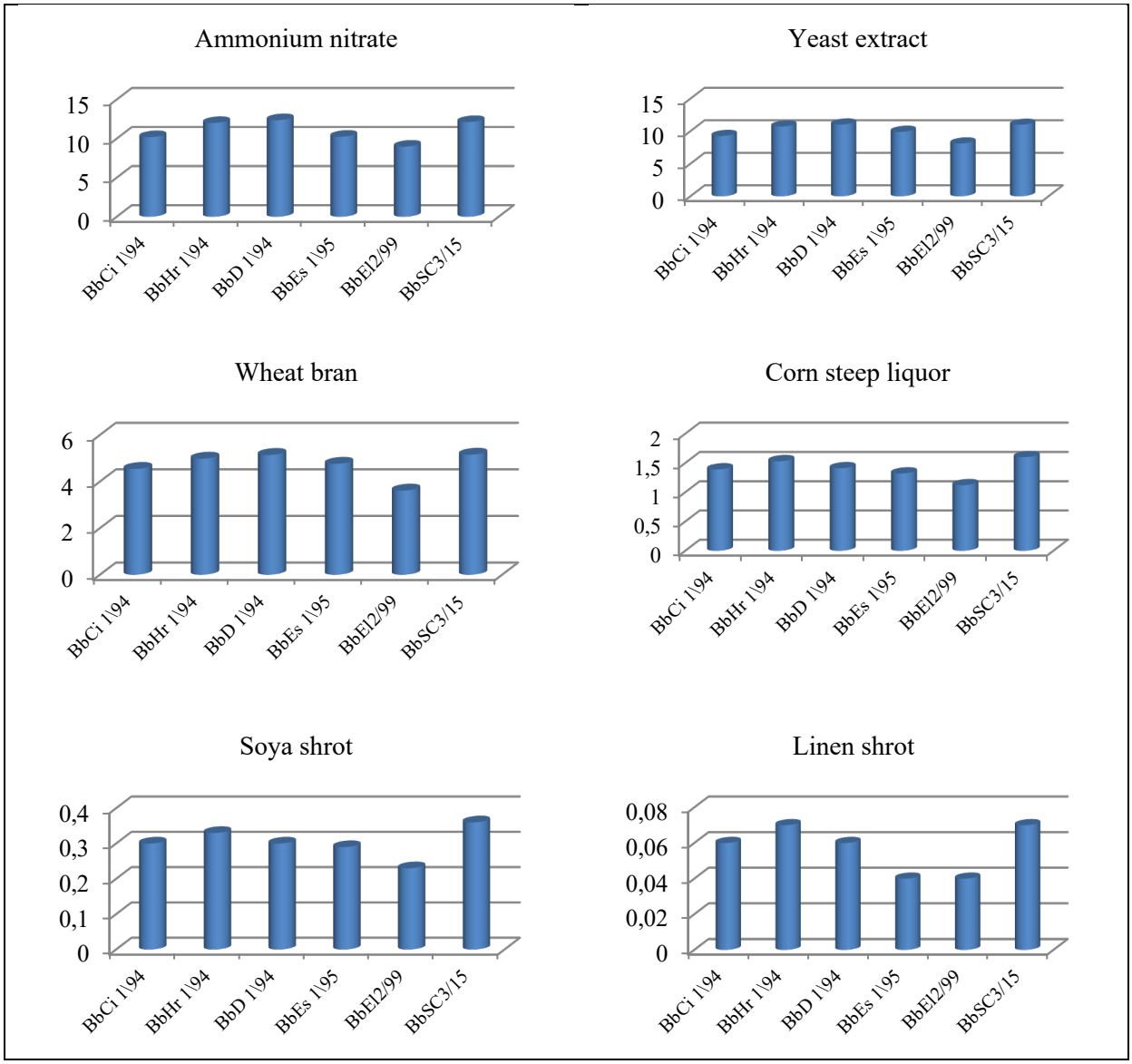

Figure 3. Spore production of *B. basiana* native strains in liquid culture medium containing various nitrogen sources

Barnes et al. (1975) studied the growth and the sporulation of *M. anisopliae* and *B. bassiana* on media containing various peptone sources and they have been reported that *B. bassiana* grew best on melizitose but sporulated best on zaharose, trehalose, and glucose, grew least on rhamnose, sporulated least on sorbose. Bidochka et al. (1987) experimented four liquid media (peptone, peptone-glucose, glucose and glucose-peptone-yeast extract) to define the developmental stages of *B.bassiana*; they found that in peptone-glucose, the yield of blastospores was four-fold higher than in glucose-peptone-yeast extract and were produced in all liquid media except glucose.

The effect of carbohydrate and nitrogen sources is presented by Rombach et al. (1989) which obtained maximum of 4.62×10^6 conidia/mg dry mycelium produced in a maltose (2%) / yeast extract (0.75%) medium. Using the data on mycelium yield (in liquid culture) and conidial production (by dry mycelium) it is calculated that the sucrose (3.5%)/yeast extract (3.5%) and the maltose (2%)/yeast extract (0.75%) media produce most

conidia per media volume/an equivalent of 3.52–3.72×10^7 conidia/ml (Rombach et al., 1988). Hegedus et al. (1990) studied growth, development and sporulation of *B. bassiana* in microcultures using growth media containing chitin monomers. For the production of submerged conidia growth media containing N-acetyl-D-glucosamine proved to be better than yeast extract-peptone-glucose, glucose plus ammonium salts or N-acetyl-D-galactosamine. In experiments aimed at selecting a medium for maximal sporulation of *B. bassiana*, these authors reported spore productions of 5.65×10^7 when the carbon source in liquid medium was represented by 3% sucrose and 1% casamino acid, after 5 days after inoculation, respectively 8.54×10^8 in medium containing 3% corn flour, 2% steep corn powder and 2% rice bran, after 8 days of inoculation. Thomas et al. (2011) studied the production and properties of *B. bassiana* conidia cultivated in submerged culture and maximum yield of conidia (5×10^8 conidia/mL) was obtained when glucose was the carbon source.

All studies targeting the cultivation of *B. bassiana* focused on the importance of various carbon and nitrogen sources, on submerged spore formation.

CONCLUSIONS

The results of our experiment prove that at the same *B.bassiana* strain, sporulation, expressed by the number of spores/ml, is different depending on the source of carbon and nitrogen. Of the strains tested in this study, the BbD1/94 strain recorded the highest average yeald of spores - 10,28x10^6 in media containing various sources of carbon, respectively 12,36x10^7 media containing various sources of nitrogen.

Cultivation on media containing different carbon and nitrogen sources proves the ability of *B. bassiana* to degrade different nutrient substrates, the yield in active biomass being different from one strain to another. Therefore, the ability of each fungal strain to use different nutrient substrates is a measure of its potential to adapt to highly variable environmental conditions. This is the reason why we recommend that the release of any *B.bassiana* strain in different habitats to be preceded by testing its ability to use a wide a range of nutrients, mainly carbon and nitrogen sources.

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