EXPLORING ENDOPHYTIC POTENTIAL OF AN AUTOCHTHONOUS STRAIN OF *BEAUVERIA BASSIANA* (Bals.) Vuill. (ASCOMYCOTA: HYPOCREALES)

Dinu Mihaela Monica, Fătu Ana-Cristina, Andrei Ana-Maria*

Research-Development Institute for Plant Protection Bucharest

*Correspondence address:
Research Development Institute for Plant Protection Bucarest
Bd. Ion Ionescu de la Brad no.8, CP 013813, Bucarest, Romania
Tel. 004-021-2693231 (32, 34)
E-mail:frommaca@gmail.com

Abstract: The overall objective of the method presented was to investigate the endophytic potential of an autochthonous strain of *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales), isolated from a natural outbreak and maintained in the RDIPP Bucharest Collection of entomopathogenic microorganisms, for evaluation of its biological control potential. For this experiment, young plants of *Capsicum anuum* L. (Solanaceae) were planted in pots with pre-sterilized soil. The inoculation was done by watering plants with a spore-based liquid suspension (soil drench method) formulated at a concentration of $6x10^9$ UFC / ml, using a *B. bassiana* strain isolated from *Lymantria dispar* L. (Lepidoptera: Lymantriidae). The assessment was made after one month from the application of the inoculum, by determining the colonization of the tested fungus in the roots, stem and leaves. Plant tissue colonization was assessed through re-isolation of *B. bassiana.* The tested *B. bassiana* strain had colonized plants at a rate of 68%, including foliage-level. The highest incidence of colonization was observed in absorbing roots, and the lowest was observed in the leaves. Other fungi have been identified in plants, predominantly *Fusarium* sp., *Alternaria* sp. and *Penicillium* sp.

Key words: biological control, endophytic fungi, B. bassiana, entomopathogenic

INTRODUCTION

Entomopathogenic fungi are very important microorganisms for the regulation of insect populations within agro-ecosystems and they have a great potential for biological control, especially when they are formulated as mycopesticides. These micromycetes have also the ability to colonize different substrates: they live saprophytically in the soil without a host, as spores, mycelium or resistance forms and they are able to colonize plants as endophytes, both naturally and by artificial inoculation.

The ecological function of endophytic entomopathogenic fungi remains largely unknown (Parsa et. al, 2013), but some studies have revealed diverse phenological characteristics of inoculated plants (better development, enhanced growth) as well as a better resistance to disease (including virosis) and pests.

Some fungal endophytes can also act as insect pathogenic agents by infecting arthropods. Endophytic *B. bassiana* reduces arthropod pest infestation in host plants (Arnold & Lewis, 2005; Reddy et al., 2009; Akello & Sikora, 2012; Biswas et al., 2013). Jaber & Araj (2017) reported the possibility of using endophytic fungal entomopathogens, *B. bassiana* and *Metarhizium brunneum* in combination with the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) for the management of the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae) in sweet pepper *Capsicum annum* L. (Solanaceae). (Bamisile et al., 2018).

The objective of experiment was to study the possibility of artificial inoculation of an entomopathogenic endophytic *B. bassiana* strain in pepper using soil drench method. This

experiment also aimed to identify possible factors which reduce the method's efficacy or help to improve the experimental design.

MATERIALS AND METHODS

Source of conidia. The fungus used in the experiment was isolated from *Lymantria dispar* (L.) Lepidoptera: Lymantriidae) and preserved in Culture Collection of Entomopathogenic Fungi of Useful Organisms Department - Research-Development Institute for Plant Protection, Bucharest. The fungus was identified as *Beauveria bassiana* (Bals.) Vuill.This strain was preserved on agar culture medium, as sporulated pure colonies, at 4^0C and periodically refreshed by successive passages on potato-dextrose-agar (PDA) medium (maximum 3 passages on artificial medium) followed by one passage through test insect (*Plodia interpunctella*), for maintaining the strain virulence.

B. bassiana monosporal cultures

For obtaining monosporal cultures, one inoculating loop full of conidia was suspended in 1 ml of 0.01% water solution of Tween 80 and vortex for 10 sec. Then, 50 µl of the suspension was distributed on PDA and incubated for 24 hours at 24± 2°C. After that, one germinated conidia was transferred into a Petri dish containing PDA, formultiplication until the mycelium covered the entire plate (2-3 weeks). Multiplication of monosporal cultures in order to obtain the stock suspension was made by washing fresh, sporulated pure colonies with sterile distillated water amended with 0.01 % Tween 80. Using a hemacytometer, we determined the concentration for the spore-based liquid suspension (inoculum) at $6x10^9$ UFC / ml.

Plants. Young plants of *Capsicum anuum* L. (Solanaceae) were transferred in 25 pots with pre-sterilized soil, at 25 °C, 50% RH and 12 h photoperiod. The plants were watered every 2-3 days with sterile distilled water and fertilized 10 and 20 days after planting with a 6 g/L water solution of NPK 15-15-15 fertilizer. The seeds were not sterilized when planted.

Figure 1. Laboratory set up for soil drench method

The inoculation was done by watering plants with a spore-based liquid suspension (soil drench method, Figure 1) formulated at a concentration of $6x10^9$ UFC / ml, using a graduated cylinder to apply 10 ml of suspension to the surface of the soil at the base of the plant.

After 30 days of inoculation we uprooted the plants carefully and washed them thoroughly in running tap water (Figure 2). Then we sampled two leaflets, two pieces of root and two pieces of stem of 3 cm from each plant. They were sterilized by immersing for two

minutes in 0.5% sodium hypochlorite and two minutes in 70% ethanol, followed by washing the fragments (three times) in sterile water (Figure 3).

Figure 2. Uprooted plant for washing it with running tap water

At the end, outer edges of the fragments were cut, where endophytes might have been removed by disinfectants and subsequently placed on the PDA medium in Petri dishes and incubated at $24^0 \pm 2^0$ C for 20 days. PDA medium was not supplemented with antibiotics. The evaluation of results was done by analyzing the morphological characteristics of the fungi by checking the colonies once every 2-3 days.

Figure 3. Sampling and surface-sterilizing of plants

The assessment was made after one month from the application of the inoculum, by determining the colonization of the endophytic fungus in the roots, stem and leaves. Plant tissue colonization was assessed through re-isolation of *B. bassiana*. The morphologic characteristics defined by Barnett (1962) were used for identification.

RESULTS AND DISCUSSIONS

In our experiment, soil drench method resulted in endophytic colonization by *B. bassiana* in 68% of the treated plants, including foliage-level (Figure 4). The highest incidence of colonization was observed in absorbing roots, and the lowest was observed in the leaves (Figure 5).

Figure 4. Endophytic colonization by *B. bassiana*

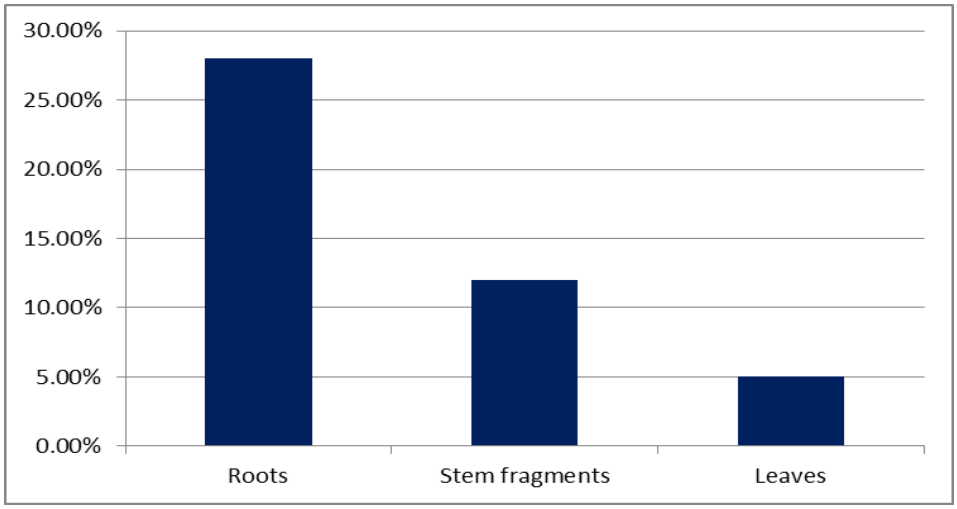

Figure 5. Endophytic colonization of vegetal samples

The inoculation of plants with entomopathogenic fungi was done using different methods: root and rhizome dipped in conidial suspension, injection, solid substrate inoculum, for banana *Musa* spp. (Akello et al., 2009) and onion *Allium cepa* (Muvea et al., 2014); seed treatment for beans *Vicia faba* and *Phaseolus vulgaris* (Akello & Sikora, 2012, Akutse et al., 2014), jute *Corchorus olitorius* (Biswas et al., 2012), pine *Pinus radiata* (Brownbridge et al., 2012), cotton *Gossypium* sp. (Castillo Lopez et al., 2014), tomato *Solanum lycopersicum* (Powell et al., 2009); dry conidia mixed with potting medium, root dip and also pot drench for strawberry *Fragaria* sp. (Dara et al., 2013); injection for tomato *S. lycopersicum* (El-Deeb et al., 2012) and for date palm *Phoenix dactylifera* (Gomez-Vidal et al., 2009); topical application to a wound site for date palm *P. dactylifera* (Gomez-Vidal et al., 2006); soil drench for cassava *Manihot esculenta* (Greenfield et al., 2016); topical spray for cocoa *Conopomorpha cramerella* (Amin et al., 2014), for artichoke *Cynara scolymus* (Guesmi-Jouini et al., 2014), for cotton *Gossypium* sp., wheat *Triticum aestivum*, bean *P. vulgaris*, corn *Zea mays*, tomato *L. esculentum*, pumpkin *Cucurbita maxima* (Gurulingappa et al., 2010), artichoke *Cynara scolymus* (Guesmi-Jouini et al.,2014); topical suspension in rice crops *Oryza* sp. (Jia et al., 2013), opium poppy *Papaver somniferum* (Landa et al., 2013), sorghum *Sorghum bicolor* (Mantzoukas et al., 2015), cauliflower *Brassica oleracea* (Razinger et al., 2014); sterile soil treated with hyphal plug for bean *P. vulgaris* (Behie & Bidochka 2015); topical spray and injection for corn *Zea mays* (Bing & Lewis, 1993).

It has been found that some plants can be inoculated by several methods as follows: bean *P. vulgaris* by topical foliar spray and soil drench (Parsa et al., 2013), coffee *Coffea arabica* topical foliar spray, stem injection and soil drench (Posada et al., 2007); topical suspension application, topical spray on cocoa *Theobroma cacao* and coffee *C. arabica*

(Posada & Vega, 2005, 2006; Posada et al., 2010); root and rhizome, dipped in conidial suspension, injection, use of a solid substrate inoculum on banana *Musa* sp. (Prabhavathi et al., 2013); topical foliar spray, seed treatment topical soil spray on Opium poppy *Papaver somniferum* (Quesada-Moraga et al., 2006); topical foliar spray, root dip on tobacco *Nicotiana tabacum*, corn *Z. mays*, wheat *T. aestivum*, soybeans *Glycine max* (Tefera & Vidal, 2009).

Inoculation method can influence the specific outcome of an experiment to establish a fungal entomopathogen as an endophyte (Parsa et al., 2013).

Other fungi have been identified in plants, predominantly *Fusarium* sp., *Alternaria* sp. and *Penicillium* sp. (Figure 6). The reason could be that *B. bassiana* was slower or a poor competitor.

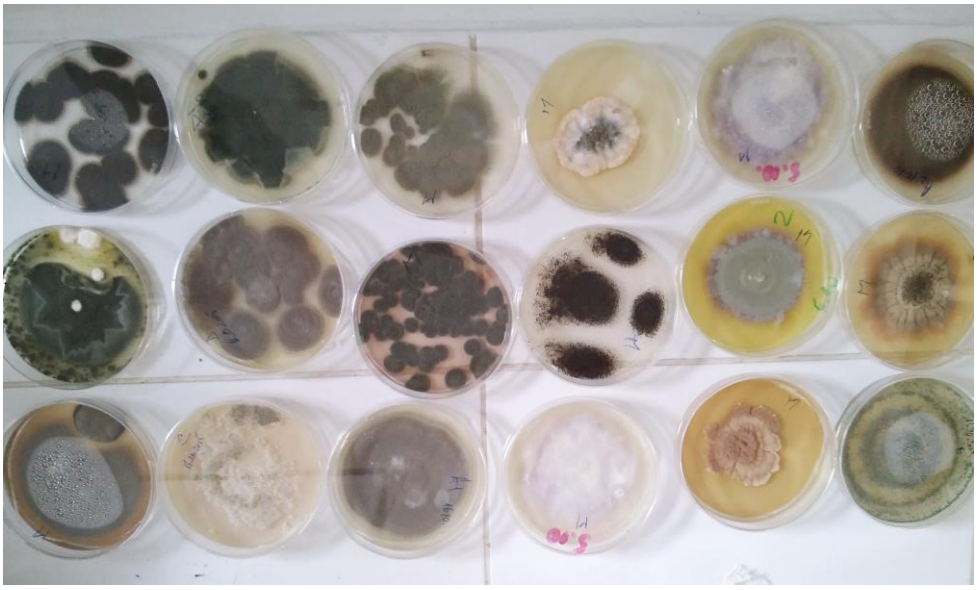

Figure 6. Fungal colonies of micromycetes belonging to various taxonomic groups identified in plants

We identified *Fusarium* sp. (Figure 7) and *Penicillium* sp. in roots and also bacteria in leaves (Figure 8). Therefore, for a faster and better reisolation of particular endophytes is preferable to be used selective PDA media, supplemented with antibiotics. The entomopathogenic fungus *B. bassiana* has competitors, so it is also preferable that the seeds from which the plants originated to be sterilized before planting, in order to obtain more conclusive results on the artificial inoculation success rate.

Figure 7. Roots infected by *Fusarium* sp.

Figure 8. Endophytic bacteria in leaves

In the experiment, a quick screening of the other endophytes besides *B. bassiana* was accomplished. Endophyte detection methods based on morphologic characteristics and media cultures are subject to false positives and false negatives. Methods based on polymerase chain reaction (PCR) are more trustworthy for detection and quantification. It is also necessary to evaluate the difference between two or more pepper cultivars or varieties and also to evaluate the difference between entomopathogenic fungal strains isolated from insects belonging to different orders.

Also, we consider that re-isolation of the inoculated endophyte should be repeated periodically for a correct assessment of method effectiveness during a crop season, at different temperatures and RHs.

CONCLUSIONS

B. bassiana strain isolated from *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) belonging to the RDIPP- Bucharest Collection of entomopathogenic microorganisms, was able to endophytically colonize *C. anuum* plants (68%), using soil-drench method.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian Ministry of Research and Innovation, CCCDI-UEFISCDI, project number PN-III-P1-1.2-PCCDI-2017-03-01/28PCCDI/2018.

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