ECOLOGIA BALKANICA
2020, Vol. 12, Issue 1 June 2020 pp. 31-40

Comparison of Endophytic Colonization of Bulgarian Variety of Tobacco by Enthomopathogenic Fungi - Beauveria bassiana and Beauveria brongniartii

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Abstract. In the modern breeding programs, the application and utilization of endophytic potential of microorganism is an opportunity to reduce damage from different pests and viruses on tobacco plants. In the present study, 48 plants of 56 day seedlings of oriental tobacco (Krumovgrad 58) plants treated with 2 strains 538 and 730 of the entomopathogenic fungi *Beauveria bassiana* and a strain 646 of *Beauveria brongniartii*. Two different inoculation techniques were applied by spraying the leaves and by directly placing the inoculant in the soil near the root of the plants. In order to compare the effectiveness of the colonization techniques of different tobacco tissues roots, stems and leaves, samples were taken for analysis on 7, 21 and 28 days after inoculation. Results have proven that all three strains of *Beauveria* endophyticaly colonize different tobacco tissues within 28 days after inoculation. The outcomes of the present study show the potential of *B. bassiana* and *B. brongniartii* to use for prevention and protection of tobacco plants.

Key words: *Beauveria bassiana*, *Beauveria brongniartii*, *Nicotiana tabaccum*, endophyte.

Introduction

Tobacco production is of great importance for the Bulgarian economy. Unlike other agricultural crops, tobacco occupies relatively small areas, and the products obtained from it have a great economic significance (Dimitrov et al., 2005). One of the main reasons for reducing tobacco crops is damage and attack from various diseases, pests, weeds and parasites. In addition to the application of various pesticides and herbicides today, a number of effective and more environmentally friendly methods for combating tobacco diseases are sought (Dimitrov, 2003). Trends in plant breeding require a more profound

understanding of the interactions between different components of the ecosystem and the use of this knowledge to applying new strategies in diseases and pests control (Vega et al., 2009). A key role in this relatively new approach to plant protection involves entomopathogenic fungi. Those microorganisms are usually endophytes, which mean that they exist inside plant tissue without causing any symptoms to the infected plant (Russo et al., 2015). The microorganisms can produce a series of chitinases, some of which act synergistically with proteases degrading the chitin shell of insects (Fan et al., 2013). One of the best studied and commonly used endophyte fungi are *B. bassiana* and *B. brongniartii*. Different techniques for introducing these endophytes in plants and soils have being conducted. As well as colonization rate of fungi has been evaluated. Considering the fact that endophytes have an antagonistic effect on specific insects and plant pathogens, the ultimate goal is to use them as a biological control agent against certain pests (Vega et al., 2009).

In addition, to pest and disease control a positive role of four *B. bassiana* strains has been published to decrease Zucchini yellow mosaic virus (ZYMV) infection in pumpkins (Jaber and Salem, 2014) has also been demonstrated. *B. bassiana* and *B. brongniartii* are effective against larvae of *Paraproba pendula* (Tajuddin et al., 2010), Stachys affinis (Goble et al., 2014; Konstantopoulou & Mazomenos, 2005) and the adults of *Tenebrio molitor* (L.), *Ceratitis capitate* and *Bactrocera oleae*. *B. brongniartii* produces secondary metabolites by which it kills the larvae of *Dendrolimus tabulaeformis* (Fan et al., 2013).

The aforementioned properties of *B. bassiana* and *B. brongniartii* are the reason for their artificial introduction into various economically important crop plants. The endophyte nature of insect pathogenic fungi in economically important plants such as *Vicia faba* (Jaber and Enkerli, 2016) plants has been proven. The study of C-sources utilization of isolates, collected from different regions of Bulgaria, has been studied and is compared, which included their phenotypic characterization and differentiation on their biochemical profiles. Each fungal isolate has been shown to exhibit a different specific profile, but sucrose, maltose and trehalose are assimilated to a higher degree than esculin, arabinose and dulcitol (Canfora et al., 2016).

In tobacco seedlings, foliar treatment results in 100% colonization of the leaves seven days after inoculation and decreases at the 28th day after inoculation. In maize, wheat and soybean, significant differences ($p < 0.001$) in endophyte colonization between different foliar, root and seed inoculation techniques (Russo et al., 2015) have been observed. Besides specifying the best inoculation technique for a given crop, another important aspect for maximally effective use of enthomopathogenic fungi is to determine the length of colonization of the fungus in the tissues. In a banana, *B. bassiana* was able to colonize the plant tissues for 4 months after the tissue-cultured plants were immersed in a spore suspension (Akello et al., 2009). The purpose of the study was to determine the ability of two *B. bassiana* strains (538 and 730) and a strain of *B. brongniartii* (646) to colonize different parts of the tobacco up to 28th day after inoculation and to compare the effectiveness of colonized leafy and soil inoculation of *Nicotiana tabacco* plants.

Materials and Methods

Plant material. The present study was conducted with 56 day seedlings from oriental tobacco (variety Krumovgrad 58, botanical classification: *N. tabacum*, *Basma*). The vegetative period, from planting to mass flowering, is 70-80 days. Each pot contains 400 g of peat mixture. All pot plants were watered with 50 mL of spring water in the pads to avoid inoculum loss in the soil-treated plants.

Fungal isolates. The fungal isolates were provided by prof. Slavimira Draganova Agricultural Academy – Bulgaria, Institute

of Soil Science, Agrotechnologies and Plant Protection (ISSAPP). The strains 538 and 730 of *B. bassiana* (Bals.) Vuill from Moniliaceae family, order *Moniliales*, class *Deuteromycetes*, were isolated from larvae of the *Coleoptera* family *Chrysomelidae* spp. Strain 646 strain of *B. brongniartii* was isolated from *Coleoptera* species (*Hylurgops palliatus* Gyll.) of the family *Curculionidae*. Fungal cultures, starting from dry conidia, were grown on Sabouraud's dextrose agar in dark at 22 °C.

Morphological evaluation of the isolates. The fungal isolates were cultured on Yeast extract agar (YEA) plate (0.5 g yeast extract, 10 g glucose, 20 g agar and 1000 ml distilled water) and were maintained in an incubation chamber at 25°C. After an 8-day period, the macroscopic characteristics of each colony were described through the observation of the following parameters: growth rate considering the colony diameter, aspect and color of conidial and reverse masses, and exudate production.

Molecular identification. DNA was isolated with HiPurA™ Fungal DNA Purification Kit (HiMedia, India). The control of purity and concentrations of genomic DNA was conducted by electrophoresis in an agarose gel. ITS1-5.8-ITS2 region of the nuclear ribosomal DNA was amplified with ITS1 and ITS4 universal primers (White et al., 1990). PCR analysis was performed in 20 µl reaction final volumes containing 1 µl (30-50 ng) of DNA and 2 µL 10 × reaction PCR buffer mixture, containing 200 nM solution of dNTPs, 5 µM $MgCl_2$, 1 µl of 10 µM primers and 0.25 µl of 5 U / µl of Red-Taq DNA polymerase (Canvax, Spain). The amplification reaction conditions consisted of 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C with a final extension of 5 min at 72°C. Expected amplicons of 500 bp are excised from the gel and purified with a Gel isolation kit (Exgene Cells SV, mini, Gene All, U.K.). PCR products were separated on 1% agarose gel stained with SafeView (NBS Biologicals, UK) at 100 V for 50 minutes using a VWR Mini Electrophoreis System for gel visualization. Gene Ruler 1 kb plus (Bioneer, South Korea) is used as a molecular marker. The resulting sequences were analyzed with BLAST software (Altschul et al., 1990) and compared with nucleotide sequences in the gene bank database ncbi.nlm.nih.gov).

Conidial suspension. Conidia were obtained from cultures grown on YEA after incubation for 10 days at 25°C in darkness. Conidia were harvested with glass cell scrapers and placed in test tubes containing 0.01% (v/v) Tween 80 (polyoxyethylene sorbitan monolaurate) (Merck® KGaA, USA). Suspensions were vortexed for 2 min, filtered through four layers of sterile muslin, and adjusted to 1 × 108 conidia ml–1 (Gurulingappa et al., 2010) after cell counting by camera. Conidial viability was assessed before every experiment (Goettel and Inglis, 1997). This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all cases, the average viability of the conidia was over 90% for isolate 538, 95% for isolate 646, and 98% for isolate 730.

Inoculation techniques. Soil inoculation was performed by using a total of 36 plants with a 10 mL a conidia suspension obtained from each of examined strains with a concentration of 1×10^8 and was placed in the soil in close proximity to the plant roots (Fig. 1). Control plants were free of inoculum treatment. The foliar treatment was performed with 10 mL conidia suspension with concentration of 1×10^8. Aluminum foil was also placed to prevent inoculation from the soil and the roots of the plants. Isolations of leaf-treated plants were removed 12 hours after inoculation (Fig. 1).

Endophytic activity evaluation. On the 7th, 21st and 28th days post tobacco inoculation, samples of treated plants were taken to detect the presence of *B. bassiana* and *B. brongniartii* by inoculation of leaf, stem, and root explants of YEA medium. Two whole plants (i.e., root, stem and leaf) treated with the 3 different strains and 3 control plants were taken from the soil drench tobacco. Plants were removed from

the soil and washed with dH_2O. Prior to introducing the explants into an in vitro medium, surface sterilization of the leaves, stems and roots was performed for 3 minutes in 0.008% Tween 80 w/v, 3 min in sodium hypochlorite NaOCl solution, 1 min in 70% ethanol and three times rinsed with sterile dH_2O for 50 s. To control the quality of antiseptic inoculation an antibiotic broth was made to the last washing sterile dH_2O (used in sterilization of the explants) to control the sterilization performed and to prove that the grown colonies of the fungus in the explants placed were not due to the surface layer of the plants. Six leaf disks of approximately 1 cm3 were incubated in culture medium with antibiotics added of concentration 0.02 g ampicillin, streptomycin and tetracycline. The presence of *Beauveria* fungi was recorded 10 day post incubation at 27 °C in the dark.

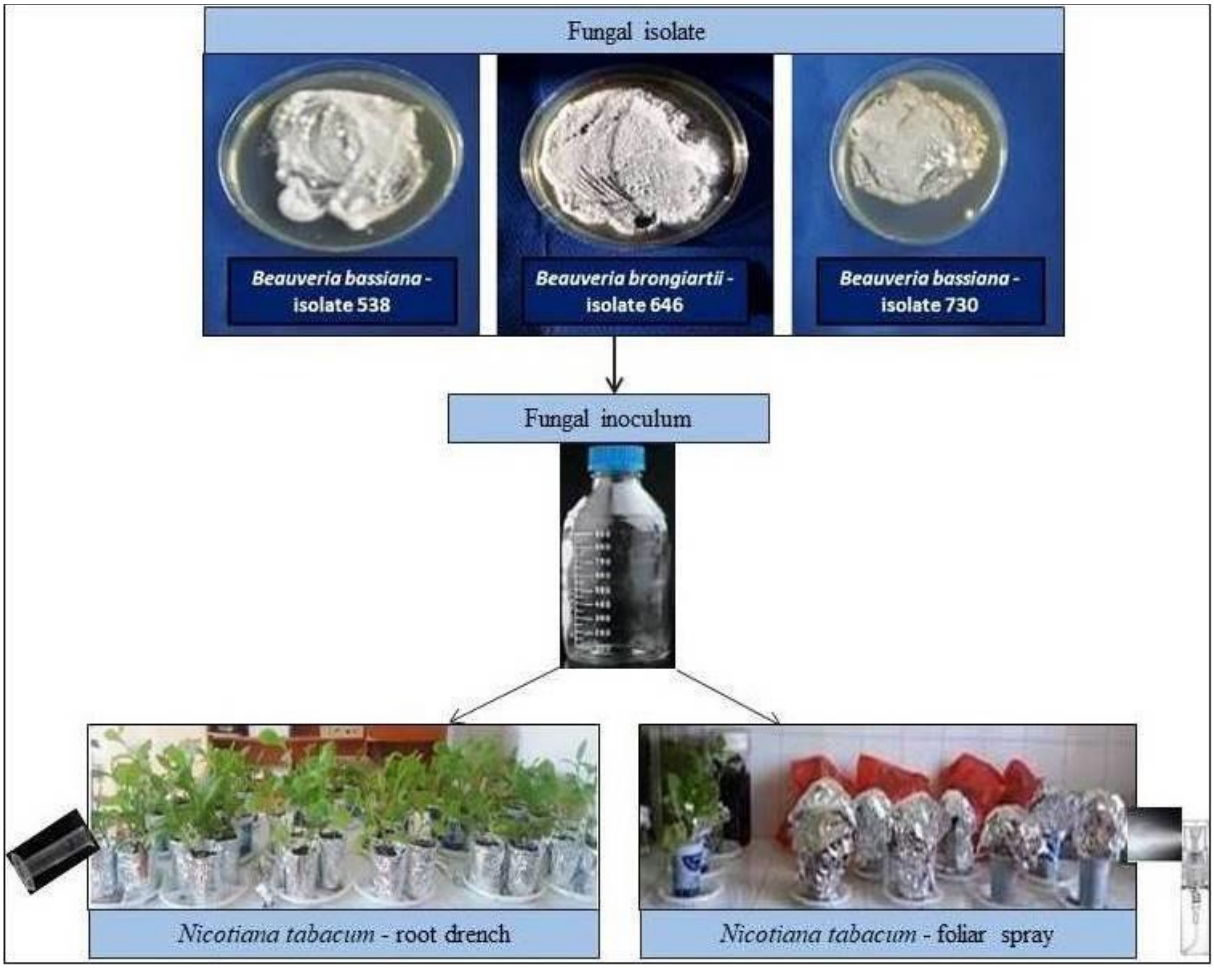

Fig. 1. Different inoculation techniques – soil drench and foliar spray with strains 730, 646 and 538.

Statistical processing of the results obtained. Isolation frequency (IF) and the degree of colonization (CR) of the *Beauveria* strains are calculated using the following formula: Isolation frequency (IF) = Ni/ Nt × 100 Colonization coefficient (CR) = Nc/ Nt × 100 Where Ni is the number of segments from which a fungi has been isolated; Nc is the total number of segments from which mushroom fungi were isolated from a sample and Nt is the total number of segments from which strains 538 and 730 of *B. bassiana* and strain 646 of *B. brongniartii* were isolated (Sun et al., 2011; Russo et al., 2015).

Results and Discussion

Morphological evaluation and molecular identification of the f isolates. The extensive overlap in conidia shape and dimensions among *Beauveria* species has limited their utility as key taxonomic structures (De Hoog, 1972; Parsa et al., 2013). Isolates could be divided into *B. bassiana* and *B. brongniartii* based on conidial dimensions; isolates with conidia longer than 3 µm were classified as *B. brongniartii*, isolates with shorter, spherical conidia were *B. bassiana*. On YEA *B. bassiana* grows slowly as a white mould with dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells, resulting in a long zig-zag extension. The fungi are characterized morphologically by globular to subglobular conidia. Although was strain 646 is determined morphologically as *Beauveria brongniartii* by its ellipsoidal conidia.

After processing the sequencing results and performing BLAST analysis with available data in GenBank the strain species identity was determined. Based on 18S gene sequences were compared with available in the database for the genera *Beauveria*. Nucletide sequence of 500 bp PCR fragments were used to define genetic similarity of the isolates with Mega 7.0 program by using neighbour-joining analysis (Kumar et al., 2016). After analysis, similarity between *B. bassiana* and *B. brongniartii* was very high (Fig. 2). Isolate 538 showed the high percent similarity with *B. bassiana* MG642849.1 (Vu et al., 2019; Mukawa et al., 2011). Strain 646 was established as *B. brongniartii*. Significant isolation-by-distance relationship was found (r = 0.33). Neighbor - joining analysis results showed that all the studied populations were divided into two discrete genetic groups with significant separation insignificant separation between two forms of *Beauveria* fungi. The sequencing of the ITS1-5.8 S - ITS2 rDNA regions also showed the insignificant separation of the two strains 730 and 538 of *B. bassiana*.

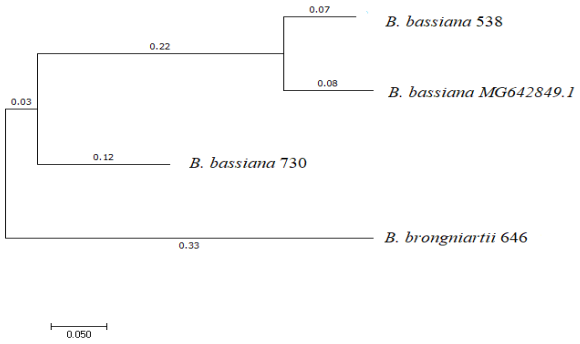

Fig. 2. Phylogenetic tree derived from neighbor-joining analysis depicting members of the *Beauveria* clades and a reference species. Branch lengths are proportional to the number of nucleotide differences. The marker bar denotes relative branch lengths.

Investigation of the frequency of fungal colonization. The frequency of colonization of the three isolates of insect pathogenic fungi (538, 646 and 730) was studied in a total of 36 soil treated plants and 12 leaf spraying plants. *B. bassiana* and *B. brongniartii* have not been applied to control plants.

On the seventh day after treatment of tobacco plants with 1×10^8 spore suspensions of *B. bassiana* strain 538, 730 and strain 646 of *B. brongniartii*, using the direct inoculation technique in the soil, the highest colonization rate was recorded in *B. bassiana* strain 538 - 16.6%, followed by strain 730 - 15.3%. *B. brongniartii* 646 has the lower colonization rate of tobacco roots around 7.14% (Fig. 3). The endophytic colonization of leaf explants on YEA media were relatively low - from 6.6% to 16.6%. Significantly high colonization of steam was recorded with root drench with strain 538 – 41.66%. Compared to this result when leaves were plated on YEA medium the amount of the inoculum was low - 6.6%.

In the first week after foliar spraying of the aerial parts of the plants, high colonization values of the leaves were found 27,20 - 49,65% and less of the stems 12,48 % - 33,33%. As a result of soil isolation during leaf inoculation, there is no development of the fungus in the roots of the test plants.

On day 7 after soil drench, the highest colonization rate shows *B. bassiana* 538, and *B. bassiana* 730 strains (Fig. 3).

On 21 days after the spore suspension was introduced into the soil, was observed an increase in the colonization rate of the fungal isolates of the three strains in the tobacco stems and leaves (Fig. 4). Similar results are also found with foliar treatment. There was a slight increase in stem and leaf colonization percentages in all strains tested. In order to better elucidate the vertical movement of mushroom endophytes, samples were taken from the lower and young upper leaves. It is particularly interesting that the old leaves have a higher colonization than the upper leaves. Results determined three times higher allocation of spores of isolate 538 and isolate 730 to the root in leaf-spaying treated plants on 21 DAT. In contract, when *B. brongniartii* isolate 646 was applied by leaf spraying, the root colonization was the two times lower compare to soil-inoculated plants.

On the 28th day after treatment, colonization was reduced in both inoculum delivery techniques as compared to the 21-day colonization rate (Fig. 5). In soil treatment, *B. brongniartii* strain 646 has the highest value. Foliar spraying of the plants showed he highest activity with *B. bassiana* strain 538. In leaf treatment, the colonization factor is higher on the lower leaf and decreases in the study of the upper young leaves.

Control plants are pure from colonization by *Beauveria bassiana* and *Beauveria brongniartii*.

Calculation of a colonization factor (CR). Fig. 6 presents the results of calculating a colonization factor (CR). On day 7 after soil introduction of inoculum from *B. bassiana* and *B. brongniartii* strain 538 was the most effective, followed by strain 646, and lowest value was determined at strain 730. In foliar treatment, highest rates of colonization were recorded when *B. bassiana* strain 730 was applied and lowest when strain 538 was used. *B. brongniartii* 646 is equally effective in soil drench application and foliar spraying inoculation till 28 day after treatment.

At the second and fourth weeks after treatment, the three *Beauveria* strains showed similar endophytic activity in both inoculum modes. On day 21, *B. brongniartii* 646 has with the highest colonization coefficient of 92.01% for soil treatment and 89.16% for leaf spraying. Analogous to the data in Fig. 6, at 28 days after the application of the fungal isolates, a significant reduction in colonization of tobacco plants was observed, the lowest being in soil treatment with strain 538 was 14.32%.

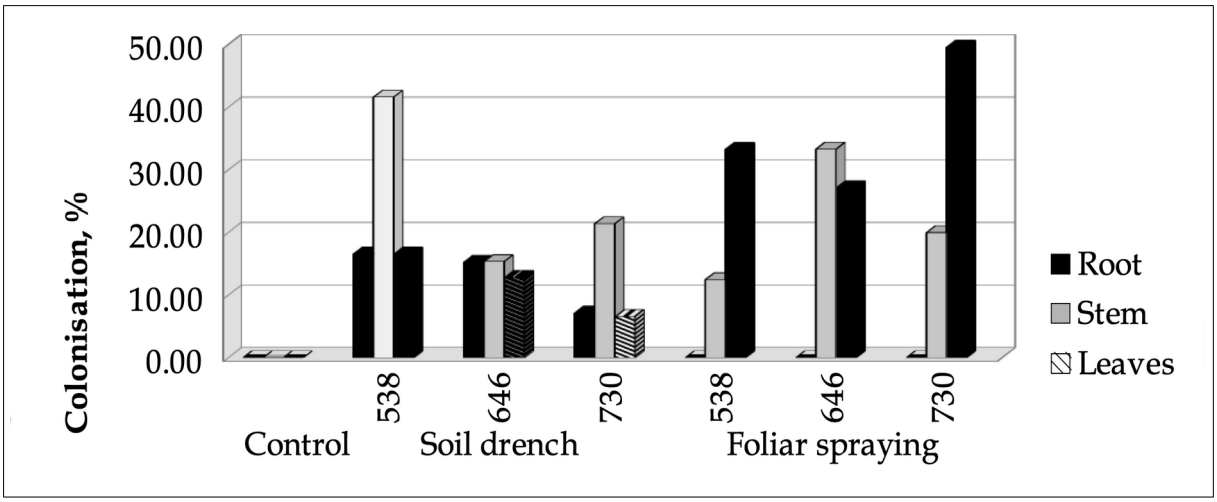

Fig. 3. Recovery percentage of *Beauveria* strains colonization of tobacco plants by soil drench and foliar spraying on 7th day post inoculation.

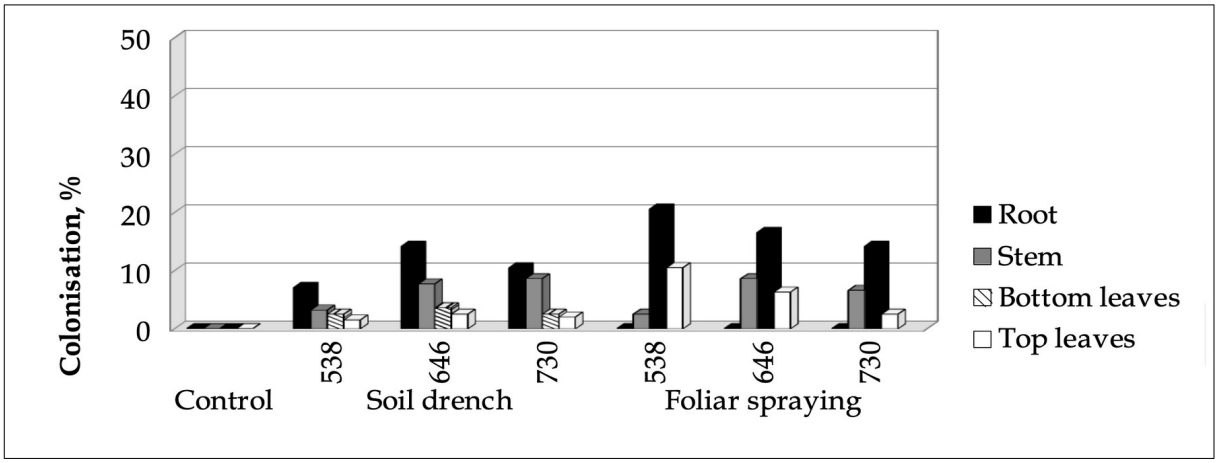

Fig. 4. Recovery percentage of *Beauveria* strains colonization of tobacco plants by soil drench and foliar spraying method on 21st day post inoculation

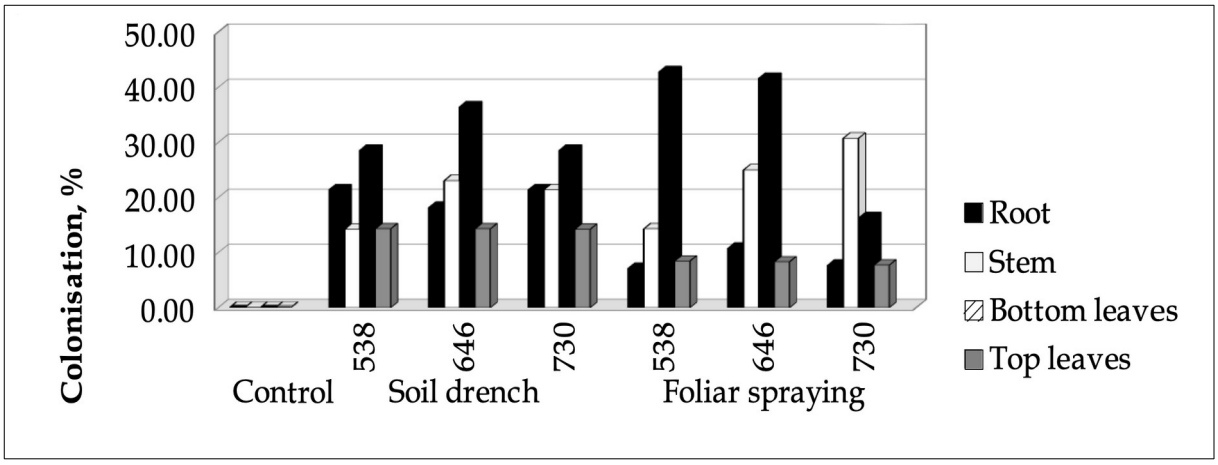

Fig. 5. Recovery percentage of *Beauveria* strains colonization of tobacco plants by soil drench and foliar spraying on 28th day post inoculation.

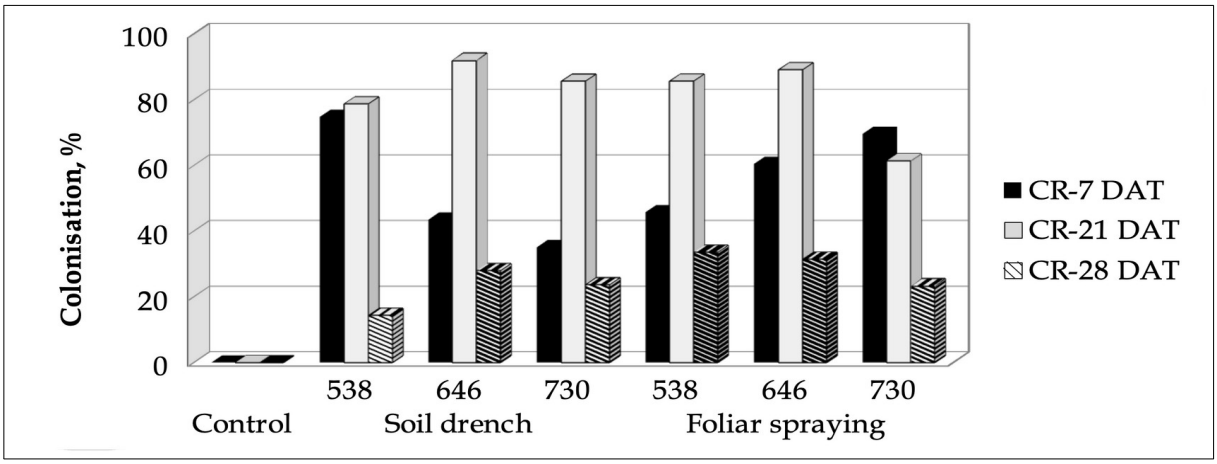

Fig. 6. Recovery percentage of CR of *Beauveria* fungi by different inoculation technique at 7, 21 and 28 days after soil drench or foliar spraying.

The present study found that *B. bassiana* and *B. brongniartii* successfully colonized tobacco plants. This result is similar to previous studies conducted with tobacco, corn soybean (Russo et al., 2015), opium (Quesada-Moraga et al., 2006) and tomatoes (Ownley et al., 2004). When different inoculation techniques were applied to several plants, alterations were observed and the date of the highest rate of colonization was recorded. In wheat, the highest percentage of colonization was achieved by leaf treatment on 14 day, and in root immersion and seed inoculation, the highest colonization was obtained on the seven day. In maize, the highest rate of colonization was achieved by foliar treatment on days 7 and 14 days (Russo et al., 2015).

According to Russo et al. (2015) data from the method of inoculation of tobacco plants with *B. bassiana*, the highest percentage of colonization was achieved by foliar treatment and the highest rate of colonization was recorded on 7 day. In contrast to the data obtained by Russo et al. (2015), current experiment shows relatively uniform rates of colonization with both applied techniques, with the exception that the leaf-treated plants with *Beauveria* was established root infection only on 21 day. The highest activity was recorded on the 7th day after inoculation, and in the present study, the highest colonization rates were 21 days after inoculation. Possible explanation is the activity of those fungi isolates, the type of inoculation and climatic conditions in the country. In support of this explanation is the result observed in two of the applied strains of *B. bassiana*. As a result of soil drench application of strain 538 on tobacco Krumovgrad 58 the percentage of the colonization rates were with similar values on 7th and 21st day. In leaf treated plants with strain 730 again the highest colonization rate was recorded on 7 days. Only in strain 646, distinguished by sequencing analysis as *B. brongniartii*, the highest colonization rate was found at 21st day for both inoculation methods. The results obtained by Russo et al. (2015) and current results demonstrated the tendency to reduce the colonization rate after the 21st day of treatment. In current experiment the endophyte activity of the fungus on day 28 was greatly reduced.

Most studies in other crops tend to reduce the fungal colonization of the various tissues of the plant over time (Greenfield et al., 2016), although vertical transmission of *B. bassiana* to the generations of endophyte colonized mother plants (Quesada-Moraga et al., 2014).

Conclusions

All three tested strains of *Beauveria* (646, 730 and 538) exhibit endophyte nature in the tobacco. Present study observed differences in efficacy among the two inoculation techniques. Both applied techniques with the three strains under examination have been found to colonize the different parts of the plants. When root immersion was used, the highest percentage of colonization of tobacco was detected at 21st day for the all the *Beauveria* strains applied. Leaf treatment with fungal strains 538 confirmed the highest percentage of colonization of tobacco on 7 day till 21 day after colonization. For strain 730 the highest percentage was recorded on 7 day post inoculation. There was no difference in colonization efficiency when applying the two different inoculation techniques. The strains of *B. bassiana* and *B. brongniartii* have been shown to be preserved in the different parts of the tobacco until 28 day, but the percentage of the inoculum decreases on the 28 day. Plant colonization does not affect the normal physiological development of tobacco. However, there is an obligation for better understanding of the biology of the entomopathogenic fungi in order to use them as biocontrol agents.

Acknowledgements

We are grateful to prof. Slavimira Draganova from Agricultural Academy – Bulgaria, Institute of Soil Science, Agrotechnologies and Plant Protection (ISSAPP) for providing fungal strains. We

thank to prof. Vili Harizanova from the Department of Entomology, Agricultural University of Plovdiv, for valuable revision of the manuscript.

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Received: 02.03.2020
Accepted: 15.04.2020