APPLICATION OF *BEAUVERIA BRONGNIARTII* AGAINST WHITE GRUB LARVAE IN FOREST NURSERIES IN THE N-E PART OF ROMANIA

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Abstract

In Romanian forest nurseries, May beetle larvae cause significant damage by gnawing on the roots of seedlings in their early planting years. The use of bioinsecticides represents an alternative, especially in nurseries certified in accordance with FSC criteria (which prohibits certain chemicals and active ingredients). Beauveria brongniartii is an entomopathogenic fungus most commonly used in the biological control of May beetle larvae. During 2017-2023, the efficacy of an native strain of B. brongniartii applied as a sporulated barley kernels was evaluated in some forest nurseries from the N and N-E part of Romania against different white grub species (Melolontha melolontha, Amphimallon sp. and Anomala sp.), in doses varied from 150- 200 kg/ha (1x10^{15} spores/ha), soil treatments were applied annually for seven years.The requirements of certain pedoclimatic conditions, larval age and mechanical treatments for causing a successful epizootic with this fungus will be discussed.

Keywords: *Beauveria brongniartii, forest nurseries, white grubs*

1. INTRODUCTION

M. melolontha is one of the most harmful pests in the agricultural and forestry economy. In the forest nurseries located in the Northeast of Romania, *M. melolontha* (Coleoptera: Scarabeidae) is the most common root pest species. *M. melolontha* is a polyphagous species that attacks both as an adult and as a larva. Their larvae are also called white grubs due to their whitish appearance. In the climatic conditions of our country, the larvae live in the soil for 2-3 years, going through several development stages (instars) during which they cause significant damage by gnawing on the underground parts of plants. In forest nurseries, the larvae prefer all species of conifers, and among the deciduous trees, they prefer oak, maple, cherry, and less so walnut and ash. Plants whose roots are attacked dry out and die. The affected areas in the crop appear as patches with poorly developed or even dried vegetation, known as attack spots.

In the 1950s and 1960s, organochlorine products were exclusively used in forestry protection practices, especially lindane (HCH) under the names Nitroxan or Heclotox. This product was recommended for preventive application to deter egg laying and thus prevent the soil from being populated with beetle larvae. Applied in powder form, it was spread on the soil surface and then raked in (Georgescu et al., 1957). Subsequently, its use was restricted and banned in many countries due to the associated risks of toxicity and environmental persistence. In Romania, lindane was withdrawn from the market in 2007. After the 1980s, organophosphorus and carbamate insecticides, which are more environmentally friendly, were introduced in forestry protection practices in Romania. The following plant protection products were used to combat soil larvae in nurseries: Basudin 10 G, Counter 5 G, Dursban 10 G, Sinolintox 10 G, Sinoratox 5 G (granular formulations), Onevos 35 EC, and Lorsban L16EC (liquid). The product containing carbamates as active substances was Vydate 10 G (granular formulation). The application dose depended on the method of application and the age of the larvae. During the same period, synthetic pyrethroids such as Karate 0.8 ULV, Polytrin 200 EC, and Supersect 10 EC were recommended for combating adults during flight (Simionescu et al., 2000). Starting in the 1990s, forest certification in accordance with Forest Stewardship Council (FSC) standards became a global practice, promoting the avoidance of pesticide use and the adoption of alternative methods for pest and disease control. These standards were adopted by Romania in the 2000s (Iorgu and Turtica, 2008), and currently, the country has approximately 2.5 million hectares certified (80% by the state Forest Department) (RNP-Romsilva, 2023). The withdrawal of soil treatment insecticides following the adoption of the new European regulation on plant protection products (e.g., Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009) along with the limitations imposed by FSC certification have resulted in no authorized products for controlling root pests in forest nurseries in Romania. Adapting to new challenges and requirements has meant focusing protection measures on preventive and repressive actions of a biological, agrotechnical, and physical-mechanical nature.

In the case of biological control of Melolonthinae, the use of entomopathogenic microorganisms, especially the fungus *Beauveria brongniartii*, is paramount (Rombach et al., 1994; Vestergaard et al., 2002; Keller et al., 2003; Easwaramoorthy et al., 2004; Srikanth et al., 2011; Ciornei, 2006; Fatu et al., 2015, Fatu et al., 2017). It is known that the entomopathogenic fungus acts through contact, adhering to the insect's cuticle, penetrating it, colonizing its interior, causing death, and then sporulating on the cadaver under optimal humidity and temperature conditions.

The identification of natural infections with *B. brongniartii*, recorded in areas of the Neamț Forestry Directorate (Northeast Romania), provided the conditions for using infected biological material (L_3 instar *Melolontha* larvae) to produce a bioinsecticide that was experimented with for several years in the Neamț Forest Districts.

This paper presents the results obtained during seven years of investigations on the effects of consecutive applications of *B. brongniartii* as sporulated barley kernels on *M. melolontha* population density in some Romanian forest nurseries.

2. MATERIALS AND METHODS

The experiments were conducted in five forest nurseries situated in N and N-E of Romania: Voivodeni (NFA Branch Mures), Basta- Roman and Pipirig-Preluca (NFA Branch Neamt) and Ghedeon-Moinesti and Vermesti-Reghin (NFA Branch Bacau) respectively (Table 1). The soil Ph values in nurseries belonging to NFA Branches Bacau and Neamt was slightly acidic (above 5.1) (Geambasu et al., 2004) while in the forest nursery belonging to NFA Branch Mures was neutral (6.9-7.2) (Dumitru et al., 2011). The experiments commenced in 2017, with experimental sites selected due to the feeding damage inflicted by *M. melolontha* larvae in forest nurseries. A density of more than one *M. melolontha* larva per square meter ($1L_3/m^2$) signifies a high threshold, as just one L_3 larva can entirely destroy a one-year-old forest crop (Georgescu, 1957). The assessment of the *M. melolontha* density was carried out before treatments (in spring) and four – five months after the treatment by surveys that consisted in digging 10 survey pits per hectare in order to identify the presence of grubs, pupae or adult beetles. The holes had 1 x 1 x 1 m dimensions. When larvae of different species and ages were found, the average number of larvae per square meter was established as the average number of third instar *M. melolontha* larvae using the 5-3-1 scale as follows:

Average number of larvae = $L_1 \times \frac{1}{5} + L_2 \times \frac{1}{3} + L_3$, where L_1, L_2, and L_3 represent the number of first, second, and third instar larvae, respectively.

Biological treatment effect was annually evaluated during four to seven years (2017-2023).

The bioinsecticide obtained at pilot scale in RDIPP consisted in sterilized barley kernels colonized by autochthonous *B. brongniartii* fungus isolated from *M. melolontha* larva was used in experiments. The bioinsecticide was applied manually followed by incorporation in soil with a disc harrow attached to a tractor. Treatments were performed with doses of bioinsecticide between 150 and 200 kg / ha (2-5 x 10^{15} spores/ha) in April-May to bare soil (without growing plants) or between rows in planted areas, by incorporation into the first 30 cm of soil. Treatments were applied three-seven years consecutively.

National Forest Administration Branch	Forest district	Forest nursery	Established crop	GPS Coordinates	Altitude	Treated area (ar)	Soil type
Neamt	Roman	Basta	*Abies nordmanniana* (two years old crop)	46°52'42'' 26°56'31''	179	30	Eutric Cambisol (typical)
Neamt	Pipirig	Preluca	none	47°23'32'' 26°41'51''	660	50	Molic-Gleyic Aluviosol
Bacau	Moinesti	Ghedeon	none	46° 46′ 59 26° 48′ 0''	465	100	Eutric Cambisol (typical)
Bacau	Darmanesti	Vermesti	none	46°25′18″ 26°28′35″	411	23	Eutric Cambisol (typical)
Mures	Reghin	Voivodeni	none	46°43'27'' 24°36'12''	377	100	Typical Preluvosoil

Table 1. Location of nurseries treated against white grubs, treated surface and soil type

3. RESULTS

In the Basta nursery (Fig.1), comparing the dynamics of *M. melolontha* population density before application (spring 2017), when the larval density was approximately 1.6 larvae/m², a rapid decrease to almost 0 larvae/m² was observed in the fall of 2017 after the bioinsecticide application, followed by moderate increases and fluctuations, peaking at nearly 1.4 larvae/m² in spring 2022 and subsequently decreasing to 0 in fall 2023. Despite significant fluctuations, the overall trend of decreasing larval density of *M. melolontha* suggests an influence of the bioinsecticide in reducing the larval population. Additionally, in this nursery, in the years 2021 and 2022, larvae infected with *B. brongniartii* were found through soil excavation before the bioinsecticide application, indicating that this fungus persists in the soil throughout the year (Fig 2-3).

In the Preluca nursery (Fig.1), comparing the dynamics of *M. melolontha* population density before application (spring 2017), when the larval density was approximately 1.2 larvae/m², significant fluctuations were observed with an overall decreasing trend, reaching 0 larvae/m² in fall 2021. The general trend of decline and disappearance of larvae after the bioinsecticide application suggests a positive influence in reducing the larval population.

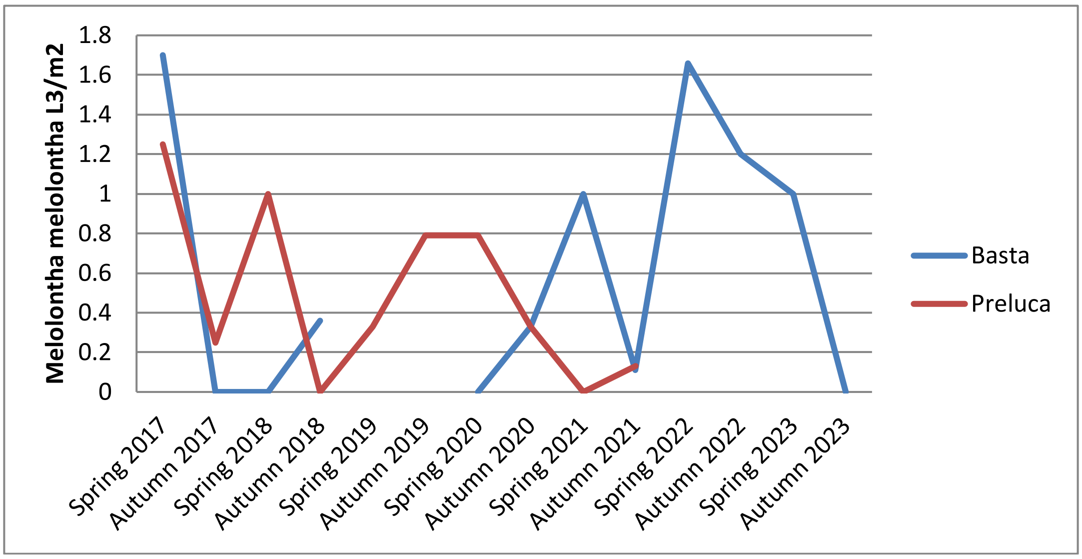

Fig. 1. Density of Melolontha melolontha larvae (L_3/m^2) in two different locations in Neamt County: Basta and Preluca nurseries, over multiple time periods (spring and fall) between 2017 and 2023.

Fig. 2. *Melolontha melolontha* L_3 instar larvae in different stage of disease (from left to right): the first- a healthy larva; the second larva showing signs of mycosis, observable through the change in color and texture of the body; the third larva shows a more advanced stage of the infection, where the structure and color of the larva are significantly altered, indicating more pronounced degradation; the last larva is covered with *Beauveria brongniartii* mycelium and soil

Fig. 3. Soil fragments containing *Beauveria brongniartii* mycelium, indicating the active presence of the fungus in the soil

In the Ghedeon nursery (Fig. 4), comparing the dynamics of *M. melolontha* population density before application, when the density was approximately 1.7 larvae/m², a decrease to 0 larvae/m² was observed in fall 2017, followed by a peak of nearly 3.7 larvae/m² in spring 2019, and then a continuous decline to 0 in fall 2020. Although there was a significant increase in spring 2019, the long-term general trend shows a decrease in population density after the bioinsecticide application.

In the Vermesti nursery (Fig. 4), a constant decrease in density was observed, from approximately 0.25 larvae/m² in fall 2018 to 0 larvae/m² in spring 2019, remaining at 0 until fall 2021. The constant decrease and maintenance of density at 0 larvae/m² after the bioinsecticide application suggest a positive influence in reducing the larval population.

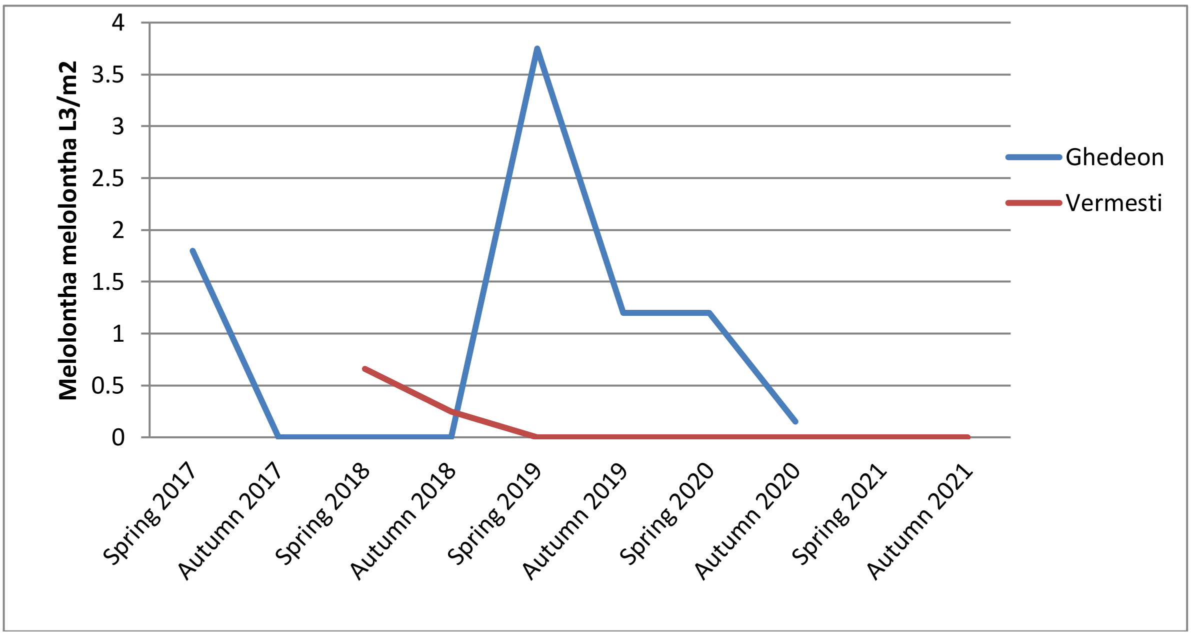

Fig. 4. Density of Melolontha melolontha larvae (L_3/m²) in two different locations: Ghedeon and Vermesti, over multiple time periods (spring and fall) between 2017 and 2021

In the Voivodeni nursery (Fig. 5), comparing the dynamics of *M. melolontha* population density before application, when the density was approximately 3 larvae/m², a decrease to 0.3 larvae/m² was observed in fall 2018, with subsequent increases and decreases, but an overall decreasing trend to 0 larvae/m² in fall 2021. The overall decreasing trend in population density after the bioinsecticide application indicates a positive influence of the bioinsecticide in reducing the *M. melolontha* larval population.

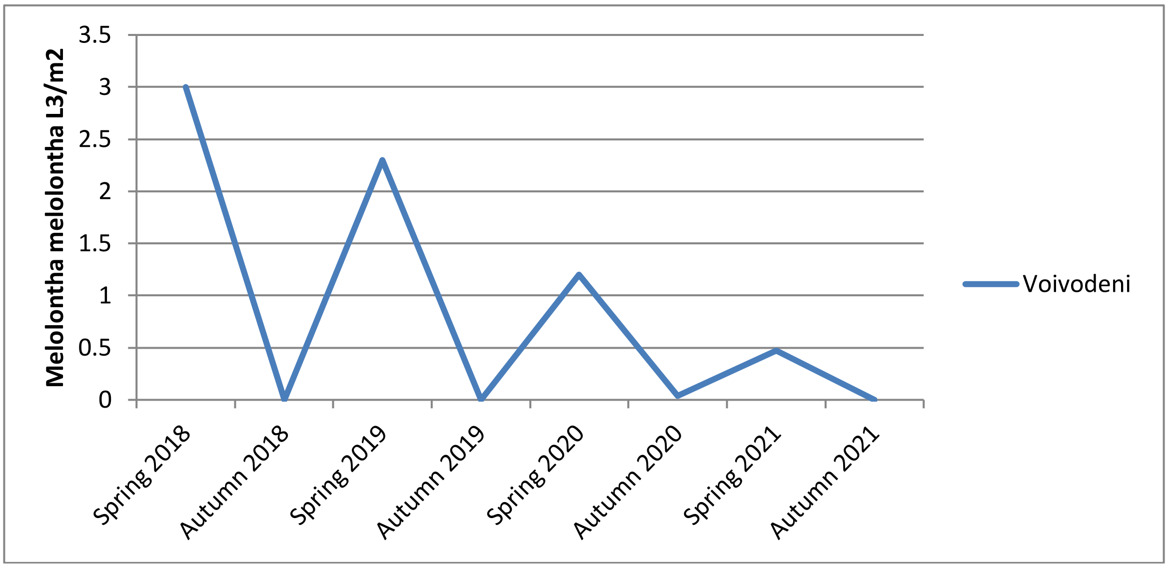

Fig. 5. Density of *Melolontha melolontha* larvae (L_3/m²) in the Voivodeni location over multiple time periods (spring and fall) between 2018 and 2021

4. DISCUSSION

Our observations have shown that the density of *M. melolontha* larvae exhibits significant annual fluctuations in all studied locations. Since the policy of applying the fungus required treating all targeted areas without a control area for comparison, we cannot conclude that the decrease in larval density is due to the fungal treatment. There are periods with very high densities, followed by sharp declines to very low values or even to 0. For example, in the Ghedeon nursery, the density peaks at nearly 3.7 larvae/m² in the spring of 2019, followed by a decrease to 0 in the subsequent years. Additionally, the density and dynamics of larval populations vary considerably between different locations. For instance, in Voivodeni, the maximum density was 3.1 larvae/m² in the spring of 2018, while in Ghedeon, it was 3.7 larvae/m² in the spring of 2019. In the Vermesti nursery, the density gradually decreased and remained constant at 0 after the spring of 2019.

There is a general trend of decreasing larval density after peak periods. For example, in Basta, the density peaked in the spring of 2022, but gradually decreased until the fall of 2023. Similarly, in Voivodeni, after a peak in the spring of 2019, the density decreased and varied between low values in the following years.

At certain times, no larvae were detected in the soil. For example, in Ghedeon, the density was zero in the fall of 2017 and dropped to zero again in the fall of 2020. In Preluca, the density was zero starting in the fall of 2021 and remained so until the fall of 2023.

Seasonal fluctuations are evident in all locations. Typically, spring presents higher densities compared to fall. The spring reinfestation with *M. melolontha* could be due to the application of treatments on second instar larvae, which are known to be less susceptible to fungal infections than those in the third instar (Ferron, 1967). Another possible explanation for the increased larval densities in spring could be the presence of continuous reinfestation sources from adjacent fields.

Laznik and Trdan (2015) found that using a mixture of biological control agents (fungi and nematodes) can provide a more effective solution for managing these larval populations. They demonstrated that first and second instar larvae populations of certain Scarabeidae species can be effectively controlled using a combination of entomopathogenic fungi, such as *B. brongniartii* and *B. bassiana*, along with entomopathogenic nematodes.

Annual fluctuations and differences between locations regarding the density of *M. melolontha* larvae can also be attributed to the fact that the fungus's efficacy is influenced by a series of abiotic and biotic factors (Kessler et al., 2003). The presence of other microorganisms in the soil, such as bacteria and other fungi, can influence competition for resources, thereby reducing efficacy by consuming the nutrients necessary for the fungus (Meyling & Eilenberg, 2007). Additionally, certain microorganisms can produce antagonistic substances that inhibit the growth and sporulation of the fungus. Predators and parasites, such as nematodes and various species of mites or small insects, can affect populations of entomopathogenic fungi in the soil by preying on spores and mycelium (Meyling & Eilenberg, 2007). Furthermore, the presence of mycorrhizae and symbiotic bacteria in the soil can indirectly influence the fungus by modifying the soil's structure and chemistry, which can affect the available microhabitat for the fungus (Jaronski, 2007).

The density of the host population plays an important role, as higher larval densities increase the probability of contact between fungal spores and larvae, which can lead to a higher infection rate (Keller et al., 2013). Additionally, the method of applying the bioinsecticide influences the persistence of the fungus in the soil. The barley kernels colonized with the fungus must be properly incorporated into the soil to prevent fissures through which moisture could be lost.

5. CONCLUSIONS

The analysis of *M. melolontha* population densities before and after the application of the bioinsecticide based on *B. brongniartii* generally indicates a decreasing trend in population densities across all studied locations. This suggests that the bioinsecticide had a positive effect on controlling *M. melolontha* populations, reducing larval densities in the long term. However, the observed fluctuations may also be influenced by other environmental factors, so further studies might be necessary to confirm these conclusions and eliminate other possible variables.

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