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Beauveria brongniartii Sacc. (Petch) against *Melolontha* spp. white grubs in forest nurseries with different soil pH

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Abstract

In 2011, the General Directorate of State Forests in Poland managed 669 forest nurseries on the total area of 2411 ha that produced forest tree seedlings bare root systems, hence vulnerable to *Melolontha* spp. white grubs. Up to date, no chemical or biological plant protection product for control of cockchafer grubs in forests has been registered in Poland. The study was carried out with the aim to evaluate the efficacy of a biocontrol product Bovecol with BP strain of B*eauveria brongniartii* on sterilized wheat grain in control of *Melolontha* spp. white grubs in forest nurseries, established on acidic soils (with pH analogous to that of forest soils) as well as on those alkaline. The active substance of Bovecol was 10^8 fungal conidia per 1g of the product. Study plots were established in 3 bareroot nurseries, situated in the areas with different soil reaction values and abundant populations of *Melolontha* spp. white grubs (assessed before Bovecol treatments). The product was applied against L_1 larvae (rates in the Czerniawka and Bałtów nurseries: 120 kg/ha and 240 kg/ha) and against L_2 larvae (the Iłki nursery: 240 kg/ha). Grub population numbers were assessed 2, 3 and 4 months after the treatments. Application of Bovecol against L_1 into the soil with neutral reaction (pH 6.8) caused the reduction of white grub numbers in 2 months, down to the threshold recommended by the *Instruction of the protection of forests* (mandatory guidelines for the protection of Poland's State Forests), i.e. less than 1 grub/sampling pit. Bovecol treatment against L_1 cockchafer larvae into acidic soil (pH 4.8) had no statistically significant effect on the reduction of grub population numbers, even 4 months after product application. Bovecol treatment against L_2 larvae applied into the soil with medium pH value (5.3) gave poorer results when compared to the soil with pH 6.8, but considerably better – when compared to the soil with pH 4.8. The selection of a fungal strain with insecticidal properties against soil insect pests should include the evaluation of strain requirements with regard to soil pH range – optimal for germination, development and growth of a given strain.

Key words

Beauveria brongniartii, forest nurseries, *Melolontha* spp. white grubs, soil pH

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Intruduction

Beauveria brongniartii Sacc. (Petch) (Ascomycota: Cordycypitaceae) is a fungal species, well-known as a pathogen of larvae (grubs) of common cockchafer *Melolontha melolontha* L. and forest cockchafer *M. hippocastani* F. (Coleoptera: Scarabeidae) (Zimmerman 1992). In Poland, the earliest results on application of *B. tenella* (syn. *B. brongniartii*) against *Melolontha* spp. were published by Karpiński (1937) who studied the effect of large-scale sprays with fungal conidia against swarming adult cockchafers. The obtained results were unsatisfactory, as there was observed only 20% insect infection. In Europe, several studies on possibilities to reduce cockchafer grub numbers with the fungus *B. brongniartii* have been conducted among others in France, Switzerland, Germany and Italy. Mortality of *Melolontha* spp. white grubs observed in France was 50% after application of fungal conidia at the density 10^{11} per $1m^2$ soil (Ferron 1971). In Switzerland, Germany and Italy, treatments against cockchafer grubs with *B. brongniartii* were applied into the soil as fungal mycelium developed on grains. Otherwise, fungal blastospore suspension was used in sprays against swarming adults (Cravanzola et al. 1996; Fröschle 1996; Keller at al. 1997). In general, soil treatments with *B. brongniartii* mycelium growing on sterilized cereal grain were more successful. In Italy, a number of repeated treatments against low density grub population resulted in long-term effects (Zelger 1996). At the end of the 1900s, plant protection products with *B. brongniartii* were registered and used for soil treatments in several European countries – Switzerland, France and Austria (Strasser 2000). In Poland, the method of production of fungal material on sterilized wheat grains was initially elaborated during studies on utilization of entomopathogenic fungi against the Colorado beetle *Leptinotarsa decemlineata* Say (Bajan et al. 1975). In the years 2003–2008, the Company Rol-Eko Ltd., Poland, carried out large-scale research on the development of a biological plant protection product to register for soil treatments in fruit growing, agriculture and forestry. The tested product consisted of BP strain of *B. brongniartii* and was named Bovecol. *B. brongniartii* was also tested in studies concerning integrated pest control, with the aim to evaluate fungal efficacy when applied against cockchafer grubs jointly with entomopathogenic nematodes and chemical insecticides (Bednarek at al. 2004). Łabanowska and Bednarek (2011) reported high Bovecol efficacy against cockchafer grubs in orchards.

As said by the Central Statistical Office (Leśnictwo 2012), in 2011, the General Directorate of State Forests in Poland managed 669 forest nurseries which were established on the total area of 2411 ha. These produced bareroot tree seedlings, often damaged by *Melolontha* spp. larvae, especially within the areas where white grubs occurred in considerable numbers. There are a few reasons why in Poland, there has not yet been registered any chemical or biological plant protection product for control of cockchafer grub populations in forestry (Sierpińska and Grodzki 2012). Presently, the protection of forest nursery production against root pests, including cockchafer grubs, is based on agrotechnical practices that require considerable effort (Brodziak et al. 2013a, 2013b).

Kessler at al. (2003) studied the effects of various factors on the efficacy of *B. brongniartii* applied into the soil against cockchafer grubs. According to the results obtained, the value of soil pH ranging from 6.3 to 7.6 had no influence on *B. brongniartii* effectiveness against the grubs. On the other hand, the authors stated that in case of fungal treatments into acidic forest soils, soil reaction could in theory noticeably influence treatment efficacy. It is believed that weakly acidic or acidic soils are sufficient in forest nurseries (Biały and Biały 2009). Maintenance of appropriate soil pH is the good way of ensuring nutrient availability to seedlings growing under nursery conditions and forming symbiotic associations with mycorrhizae fungi. According to Biały and Biały (2009), in forest nurseries, there is frequently observed gradual increase of soil alkalinity as a result of specific soil cultivation techniques as well as mineral fertilization and spray irrigation with water with high contents of basic cations. The authors reported data indicating that soil reaction in nursery sites used for seedling growing for a short period of time was vaguely changed when compared to the typical pH of forest soils, whereas the soil in long-term utilized sites turned out to be alkaline (soil pH increased by more than 3 units when compared to the soil collected under tree canopy).

The aim of the present study was to evaluate the efficacy of the plant protection product Bovecol with BP strain of *B. brongniartii* in control of *Melolontha* spp. white grub numbers in forest nurseries established on soils with different soil reaction – similar to that of forest soils or else alkaline.

Material and methods

General description of experiments

The plant protection product Bovecol used to be produced in Poland in pilot plant scale, and consisted of mycelia and conidia of BP strain of *B. brongniartii* fungus on sterilized wheat grains. The active substance of the product was 10^8 fungal conidia/1g of the product.

In the present study, research plots were established within 3 bareroot forest nurseries, situated on the areas with abundant populations of *Melolontha* spp. white grubs (table 1). Starting in 2004, preliminary tests on Bovecol efficacy were conducted in the Iłki forest nursery. Further research was carried out in 2008 as a part of product registration procedure in the Bałtów and Czerniawka forest nurseries. In the Czerniawka nursery, the study plot was situated within a new nursery subdivision that was established after clearcutting a part of an adjacent stand and then grubbing up the area. Study plots in the Bałtów and Iłki forest nurseries were situated in nursery subdivisions used for seedling planting for more than 30 and 20 years, respectively.

In all the nurseries, the tests were carried to assure good Bovecol efficacy, taking into account local conditions. In preliminary and registration experiments, there were used various methods of product application. In the preliminary observations on Bovecol efficacy against *Melolontha* spp white grubs in the Iłki forest nursery, the product was dispersed manually into approx. 20 cm deep furrows between the rows of 1-year-old beech seedlings. Beech trees were planted in 6 strips with 4 rows/strip. During the observation period (June 2004-April 2006), standard management activities (fertilizing, weeding, etc.) were performed in beech seedling plantation, and no other plant protection product was applied. During Bovecol registra-

Table 1. Study areas in forest nurseries examined

	Iłki nursery	Bałtów nursery	Czerniawka nursery
Forest District	Smardzewice	Ostrowiec Świętokrzyski	Jarosław
Regional Directorate of State Forests (RDLP)	Łódź	Radom	Krosno
Latitude	51°28’34” N	51°0’22” N	50°2’56” N
Longitude	19°57’18” E	21°28’44 E	23°0’22 E
Bovecol dose applied	240 kg/ ha*	120 kg/ ha and 240 kg/ ha	120 kg/ ha and 240 kg/ ha
description of treated areas	3 plots treated with Bovecol, 500 m^2 each and 1 control plot (untreated) plot - 500 m^2	8 plots, each 50 m^2/ treatment and 8 control (untreated) plots, 50 m^2 each, randomized block experimental design	8 plots, each 50 m^2/ treatment and 8 control (untreated) plots, 50 m^2 each, randomized block experimental design
soil pH in nursery subdivisions**	5.3 ± 0.3	6.7 ± 0.2	4.8 ± 0.2
assessment of white grub population (larval instars, mean ± SD)	L_2 larvae (more than 90% of grub population); 9.0 ± 6.5grubs/ sampling pit	L_1 larvae (more 95% of grub population); 0.8 ± 0.7 grubs/sampling plot; after complementary transfer***: 5.0	L_1 larvae (more than 95% of grub population). 16.2 ± 9.2 grubs/ sampling pit

* One Bovecol rate was applied - 240 kg/ ha in the Iłki forest nursery; this was the maximum rate of the product tested in the study; lower rate (120 kg/ ha) was also applied - in another forest nursery, several dozens kilometers away); ** Information on soil pH value within the study area in the Iłki forest nursery (Bovecol preliminary testing) was provided by the Smardzewice Forest District (data based on biannual routine soil assessments performed in all Poland's forest nurseries). In the Czerniawka and Bałtów forest nurseries (Bovecol registration testing), soil reaction was assessed in 10 1 dm^3 soil samples using a soil pH-meter; *** A week before Bovecol treatment scheduled in the Bałtów forest nursery, white grubs were accidentally removed from the soil within the study area by nursery staff (following the agro-technical procedure recommended for cockchafer control – tillage and manual grub collection). Therefore, on the next day after Bovecol treatment, comparatively too low grub numbers observed in the study plots were complemented with 5 (L_1) grubs, collected in neighboring tree stand and then placed into the soil in the middle of each plot.

tion tests (in Bałtów and Czerniawka), the product was applied into the soil in a set-aside nursery subdivisin. Product doses were spread onto the soil and mixed with 10–15 soil layer with the use of field cultivators. All the study plots in the examined nurseries were spray irrigated when required.

In the preliminary tests, the assessment of initial (before the treatment) white grub population was carried out based on scrupulous examination of the soil collected from 2 sampling pits (1.0 m × 0.5 m × 0.5 m), dugout in each of the experimental plots. During registration experiments, overall initial grub population was assessed on the entire study area, based on scrupulous examination of the soil collected from 6 sampling pits (1.0 m × 0.5 m × 0.5 m) per 1200 m^2 (Tab. 1).

The evaluation of beech root system damage was conducted in the Iłki forest nursery, 22 months after Bovecol treatment (in April 2006). Beech seedlings just removed from the soil were examined. The seedlings with injured main roots and no lateral and hair roots were categorized as heavily damaged. On each experimental plot, there were evaluated 2500 beech seedlings.

Evaluation of white grub infection after the treatment

During preliminary testing, the number of white grubs in the soil after the treatment was assessed once – after 3 months. In registration tests, grub number assessments were conducted twice – 2 and 4 months after the treatment. Double assessment of grub population numbers after the treatment, allowed to better evaluate the speed of action of Bovecol tested under forest nursery conditions. During preliminary testing, white grub number assessment after the treatment was carried out following the sampling pattern described above. During registration experiments, white grub numbers were assessed based on scrupulous examination of the soil collected from 1 sampling pit/50 m^2 (8 pits/experimental treatment).

Statistical analyses

Statistical significance of the differences between the means obtained was assessed by means of the Kruskall-Wallis test with post-hoc comparisons ($\alpha = 0.05$). All calculations were performed using STATISTICA 10.0 software (StatSoft, Inc., USA 2010).

Results

Iłki forest nursery

Three months after Bovecol treatment, the numbers of white grubs observed in the treated plots were significantly reduced ($H_{(1, N=14)} = 4.41$, p = 0.036). The mean grub number was 1.7 ± 1.3 grubs/sampling pit. The mean number of white grubs in the untreated (control) plot was also reduced (3.5 ± 2.5 grubs/sampling pit), however, not significantly when compared to the initial white grub number found in the control plot before Bovecol treatment, ($H_{(1,N=10)} = 1.12$, p = 0.29).

In the treated plots, 22 months after the treatment, the number of 3-year-old beech seedlings with heavily damaged root systems was 3–5 times lower when compared to the control (tab. 2)

Table 2. Percentage of beech seedlings damaged due to white grub feeding, assessed 22 months after Bovecol treatment at a rate 240 kg/ha (Iłki forest nursery)

Plot	Damaged seedlings(%) ± SD
I	10.11 ± 4.58
II	6.68 ± 2.82
III	11.42 ± 9.82
Control	36.40 ± 21.81

Bałtów and Czerniawka forest nurseries

In the Czerniawka forest nursery, the assessment of white grub numbers was carried out 2 months after Bovecol treatment. Then, the numbers of white grubs ranged from 1 to 41/sampling pit. Regardless of product dose applied (120 kg/ha, 240 kg/ha or 0 kg/ ha), the results of K-W test ($H_{(3, N=30)} = 3.89$; p = 0.27) showed no significant differences between the number of white grubs before and after the treatment. Within the study are, 2 months after the treatment (June 2008), the mean number of white grubs found in all the sampling pits examined was 13.3 ± 9.1 grubs/sampling pit. The results of the second grub population assessment carried out in August 2008, also showed no statistically significant differences ($H_{(3, N=30)} = 5.85$; p = 0.12) between the initial number of grubs and that observed 4 months after the treatment (regardless of product dose applied). At that time, the mean number of white grubs observed within the study area was 9.5 ± 7.0 grubs/sampling pit.

Photo 1. *B. brongniartii* hyphae formed from fungal conidia on wheat grain used in Bovecol treatments, visible on a lump of soil

In the Bałtów forest nursery, the grub number assessed 2 months after Bovecol treatment (in June 2008) were significantly different when compared with those observed before the treatment; the difference was more distinctive for the higher Bovecol rate applied (240 kg/ha). *P*-values in post-hoc comparisons in W-K test ($H_{(5,N=48)} = 36.63$; p = 0.0000) for 120 kg/ha and 240 kg/ha were 0.027 and 0.004, respectively. The mean grub number for the higher dose was 0.1 ± 0.3 grubs/sampling pit, and for the lower – 0.6 ± 0.9 grubs/sampling pit. On the other hand, post-hoc comparisons showed no significance between grub numbers observed in the control before and 2 months after the treatment (p = 0.099). At that time, in 8 sampling pits, dugout in the control plot, there was found on average 1.00 ± 1.94 grubs/sampling pit. The assessment of grub numbers carried out 4 months after Bovecol application, showed no grubs in sampling pits (8) dugout in the plot treated with 240 kg/ha, 1 grub – in pits dugout in the plot treated with 120 kg/ha and 3 grubs – in pits dugout in the control plot.

In both observation terms (June and August, 2008), in the Bełtów forest nursery, there were observed white fungal hyphae growing on wheat grain (photo 1) applied as a part of Bovecol (then covered with *B. brongniartii* conidia). Similar observations were made in the Iłki nursery, during 2004 tests – 3 months after Bovecol treatment. No *B. brongniartii* vegetative growth on wheat grains was observed during the two white grub population assessments carried out in the Czerniawka nursery.

Discussion

The paper presents the results of the study on the efficacy of the plant protection product Bovecol containing *B. brongniartii* to control cockchafer grubs *Melolontha* spp., carried out in forest nurseries established on soils with different soil reaction. In Europe, there have been so far conducted no field studies on the comparison of efficacies of *B. brongniartii* products when applied to soils with acidic and neutral pH. In Romania, during the study on using *B. brongniartii* to control white grubs in forest nurseries conducted by Fatu et al. (2015), there was observed the reduction of grub population numbers to a level below the damage threshold after application of an experimental product BioMelCon (*B. brongniartii* on barley grain) at different rates – from 100 kg/ha to 200 kg/ha. Nonetheless, the authors gave no information about soil pH in the forest nursery tested. The highest rate of the plant protection product tested in the present study (240 kg/ha) was considerably high. A similar plant protection product with *B. brongniartii* – Melocont ® pilzgerste, consisting of BIPESCO 2 strain, was temporarily registered in Austria in 1998 – for 3-time application during the vegetation season application at a rate 30–50 kg/ha (label data, register no. 2582). Consequently, product rates applied in the present study were comparable to those in the aforementioned Romanian tests, also concerning forest nurseries. In our study, there were used different methods of treatment application, which was a result of characteristics of the study plots: in the Iłki nursery (preliminary tests), Bovecol was applied into the soil between already growing beech seedlings, and in the Bałtów and Czerniawka nurseries (registration tests) – onto set-aside nursery subdivisions. The experimental design was meant to help obtaining more complete information on product action at different application modes. It seems that the application mode had a minor effect on the obtained results when compared to the influence of soil pH on the efficacy of *B. brongniartii* against *Melolontha* spp. white grubs. The same method of product application was used in 2 forest nurseries (Bałtów i Czerniawka), most different in terms of soil reaction. As a result these two nurseries differed the most with regard to the reduction of white grub *Melolontha* spp. population numbers. A similar method of product application to that used in the Iłki ii nursery was described by Vestergaard at al. (2002) who tested another *B. brongniartii* product used against *Melolontha* spp. white grubs in Christmas tree plantations in Denmark.

In the present study, there was assumed the soil classification system referring to soil reaction Zawadzki (1999). The obtained results indicate that in the soil with acidic reaction, e.g. in the Czerniawka forest nursery with soil pH 4.8, *B. brongniartii* BP strain (Bovecol active substance) caused no mycosis in the white grubs observed, and consequently – no reduction in their population numbers. Regardless of a product rate applied, no population decrease was observed even 4 months after the treatment. Nevertheless, in case of slightly acidic soil – with pH 5.3 (Iłki forest nursery) treated with Bovecol at a rate 240 kg/ha, 3 months after the treatment, there was observed 5-fold decrease of grub population numbers. At the same time, in the soil with neutral pH (6.7, Bałtów nursery) treated with 120 kg of Bovecol/ha, 2 months after the treatment, grub numbers decreased 8 times, whereas at a rate 240 kg/ha – 41 times. The results of grub population assessment that was carried out 4 months after Bovecol treatment in the Bałtów forest nursery were not taken into consideration for the reason, that there also was observed grub population reduction in the control plot. The latter could be a result of transfer of complementary white grubs from older stand strips in the nursery area into the soil of the control treatment (description in tab. 1), which could weaken the grubs and increase their vulnerability to various environmental factors. When discussing the results obtained, there should be taken into consideration that Bovecol was applied against L_2 grubs in the Iłki nursery, whereas in Czerniawka and Bałtów – against L_1. It is commonly known that older insect larval instars are more resistant to the effects of various environmental factors. That is why, it can be assumed that the application of BP strain of *B. brongniartii* into slightly acidic soil (Iłki) could give better results if the product was used at the time when L_1 white grubs prevailed in the soil treated. Only in the Baltów nursery, where L_1 grubs in the soil with neutral reaction were treated with Bovecol at both rates tested (120 kg/ha and 240 kg/ha), when checked 2 months after the treatment, there were observed reductions of grub population numbers to the threshold recommended by the *Instruction of the protection of forests* (2012) – below 1 grub/sampling pit. The majority of Poland's soils are classified as acidic or strongly acidic. The report on the state of forests in Poland (Raport o stanie lasów 2004) states that at 0–5 cm soil depth, the average soil pH was 3.27 in Scots pine forests (coniferous and mixed coniferous forest sites), and – 3.58 in oak forests. In view of the results of the present study, this suggests that under Poland's conditions BP strain of *B. brongniartii* will not probably be a successful biocontrol agent against white grubs in strongly acidic (pH < 4.5) and acidic soils (pH below 4.8). In this context, it must be added that 5-fold reduction of white grub population in treated plots established in the Ilk nursery, observed 3 months after Bovecol treatment, resulted in 3–5 times lesser damages of beech seedling root systems when compared to those observed in the control (tab. 2). In the fall 2008, in the Bałtów and Czerniawka forest nurseries, there was planned oak seeding (after the assessment of grub numbers in the soil). In the spring 2009, the assessment of Scots pine and oak seedling survival was planned for both nurseries. However, oak seeding was not performed in the Czerniawka nursery due to too high grub numbers observed in the soil in August 2008. In the Baltów forest nursery, seedling survival was not evaluated due to minor numbers of cockchafer grubs observed after Bovecol treatments.

Up to date, there have been available no scientific publications with clear explanation of the reasons behind differences in the efficacy of *B. brongniartii* strains against *Melolontha* spp. white grubs when applied into soils with different pH values. Quazi (2008) pointed out that the differences in germination capability of *B. bassiana* conidia under differentiated conditions of substrate pH could be explained by specific optimal pH values needed for the expression of proteases produced by the fungus. Too acidic (or else – alkaline) reaction somewhat affects germination of conidia in *B. brongniartii*, and this could be the reason of hindered or discontinued development of mycosis in white grubs. According to Padmavathi et al. (2003), in the strains of

B. bassiana (closely related to *B. brongniartii*), there exists high variability with regard to optimal substrate pH values needed for proper development of this fungus. These authors investigated growth and development of 29 isolates of *B. bassiana* with reference to substrate pH value and described the isolates with a wide range of optimal pH (5–13) as well as those with a narrow range (pH 6–8). These results suggest that during the selection process on a fungal strain for application as an active substance in a given plant protection product against soil pests, there is a need to recognize strain requirements with regard to the optimum pH of its growing environment.

Conclusions

– Application of the plant protection product Bovecol with BP strain of *B. brongniartii* against *Melolontha* spp L_1 larvae into the soil with neutral reaction (pH 6.8) resulted in the reduction of white grub populations to the threshold recommended by the *Instruction of the protection of forest* in 2 months after the treatment.
– Application of Bovecol against L_1 larvae of *Melolontha* spp. into the soil with acidic reaction (pH 4.8) caused no significant reduction of white grub populations, even 4 months after the treatment.
– Bovecol treatment against L_2 cockchafer larvae applied into the soil with the medium pH value (5.3) gave poorer results when compared to the soil with pH 6.8, but noticeably better – when compared to the soil with pH 4.8.
– The selection of an entomopathogenic fungal strain for application against soil insect pests should also include the evaluation of strain requirements with regard to soil pH range – optimal for germination, development and growth of a given strain.

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