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# Field evaluation of three biopesticides for control of the raspberry cane midge, *Resseliella theobaldi* (Barnes) in Bulgaria

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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**Abstract: The raspberry cane midge, *Resseliella theobaldi* is a key pest on red raspberry, *Rubus idaeus.* The larvae of the insect severely attack the raspberry canes, resulting in premature death of the plant canes. In the last decade, organic production of raspberry fruits has significantly increased in Bulgaria. At the same time there are few products of botanical or microbiological origin that might be used for control of this pest. In present study the effect of NeemAzal® T/C (azadirachtin A), Sineis 480 SC® (spinosad), and *Bacillus subtilis* on *R. theobaldi* was evaluated. The experiments were conducted in two raspberry fields at different altitude. In the field at lower altitude (196 m), the raspberry cane midge has developed four generation per year, while in the field at higher altitude (960 m) three generations of the pest have been completed. Lowest number of larvae in raspberry canes was observed after application of NeemAzal® T/C, and *B. subtilis* in both raspberry fields. Both products demonstrated highest efficacy at 7th day after treatment, when the number of larvae per splits was 67.1-82.5% for NeemAzal® T/C, and 75.1-81.2% for *B. subtilis* lower compared with the control at the two experimental sites.**

## 1. Introduction

Among the small fruit crops grown in Bulgaria, the red raspberry (*Rubus idaeus* L.) is the most valuable. During the last five years, the total area of raspberry plantation has increased by 43% and the yield has reached 3620 kg$^{-1}$ ha. At the same time approximately 54% of raspberry production is organic. In 2015 and 2016, about 75% of the total raspberry yield was exported mainly to western European countries, but also to several markets in Asia (Agrostatistics, 2015).

The most serious pest of raspberry is the raspberry cane midge, *Resseliella theobaldi* (Barnes) (Diptera: Cecidomyiidae), which causes premature death of the plant canes. In Bulgaria, the insect was first reported by Stoyanov in 1960. The author examined the life cycle of *R. theobaldi* under different conditions and reported the development of three-five overlapping generations a year. The larvae of the insect attack the plant primocanes, both of those fruiting in June-July as well as those fruiting in August-September (Stoyanov, 1963). The larvae feed under the bark of canes and clearly define dark brown spots appearing on the green surface of the canes. Pitcher (1952) stated that damage to raspberry plants caused by *R. theobaldi* was usually associated with fungal pathogens such as *Botrytis cinerea*, *Fusarium avenaceum* and *Didymella applanta*. The fungi cause necrosis of vascular cylinder through the larval feeding sites (Williamson, 1987). The complex of damage involved the raspberry cane midge and mycoses is known as "midge blight" (Pitcher and Webb, 1952). As a result of usually present of midge blight, there is no established relationship between population level of *R. theobaldi* and degree of plant damage caused by the insect (Williamson and Hargreaves, 1979). According to Shternshis *et al*. (2002), the effect of the control treatments against the pest should be assessed by estimating the severity of midge blight including fungal lesions.

To date, the biological control of raspberry cane midge is still poorly investigated. There are few reports concerning alternatives to chemical control for *R. theobaldi*. Sex-pheromone-based strategies are promising techniques to control of many economically important pests. Pheromone traps for raspberry cane midge were used for the first time in the UK in 2005 (Milenković *et al*., 2006). Cross and Hall (2006) and Hall *et al.* (2009) identified 2-acetoxy-5-undecanone as a major component of *R. theobaldi* female sex pheromone. Over the past ten years, the sex pheromones have been tested for monitoring the male emergence (Cross *et al.*, 2008, Tanasković and Milenković, 2010, Sipos *et al*., 2012). Therefore, little information concerning the application of biopesticides for control of *R. theobaldi* in raspberry organic production is currently available. For instance, use of products based on entomopathogenic bacteria, *Bacillus thuringiensis* (Bt) and *Streptomyces avermitilis* against raspberry midge blight have been reported (Shternshis *et al*., 2002). Further, there are no publications on the biological control of this raspberry pathogen complex.

The objective of this study was to evaluate the possibility to control raspberry cane midge using two commercial biopesticides and one noncommercial bacterial strain under field conditions. In particular, I attempted to assess the role of used products in reducing the number of *R. theobaldi* larvae in raspberry canes.

## 2. Materials and Methods

*Biopesticides and bacterium cultivation*

Commercial formulations NeemAzal® T/C (azadirachtin A, Trifolio-M, Germany), and Sineis 480 SC® (spinosad, DowAgroSciences, Bulgaria), and a bacterium *Bacillus subtilis* were used against midge in raspberry fields.

The strain of *B. subtilis* was grown in the dark for 48 h at 24°C on tryptic soy broth agar (TSBA). For inoculum production a loop of the bacteria was transferred into 100 ml of TSB and allowed to multiply for 48 h on a rotary shaker (160 rpm) at the same temperature. Bacterial suspensions were centrifuged at 4000 rpm for 20 min, and the bacterial pellet was resuspended in sterile¼ strength Ringer's solution (Merck). The bacterial suspension was adjusted to a final concentration of $10^8$ CFU $ml^{-1}$ by dilution with Ringer's solution. The bacterial strain identification was determined by FAME Analysis, following by BIOLOG Analysis.

*Experimental design*

The trials were conducted in 2016 in two commercial raspberry plantations in the regions of Bogdanovo (196 m) and Samokov (960 m). The first location (Bogdanovo) has a flat topography, with small hills. The soil type is Leptosols (Bulgarian Soil Taxonomy), and the landscape is dominated by agricultural land use. According to the climatic data (National Institute of Meteorology and Hydrology, BAS), the average air temperature from April through October 2016 was 22.4°C. The average rainfall for the investigation period was 284 mm. The geographical coordinates of the raspberry field in Bogdanovo are 42°36' N, 26°00' E. The second location (Samokov) is a valley between two mountains - Rila and Verila. The soil type is Fluvisols, and the landscape is dominated by arable land. The average air temperature from April through October 2016 was 15.4°C. For the same period, the average rainfall was 531 mm. The geographical coordinates of the raspberry field in Samokov are 42°21' N, 23°34' E.

The cultivar Heritage (USA) was grown in both three years old fields. Plants were located in spacing of 50 cm within rows and 2.0 m between rows. Experimental plots were 10 $m^2$, each plot containing approximately 100-120 raspberry canes. The size of the buffer zone between the plots was 4 m. The treatments were arranged in a completely randomized block design with four replications.

*Treatment and application methods*

The PheroNorm® standard large delta traps (Andermatt Biocontrol AG, Switzerland) were used to determine the population dynamic of the cane midge. The traps containing 10 µl cane midge sex pheromone lure per trap were mounted on bamboo sticks at height of 60 cm the 10th of April. Three traps were used in Bogdanovo (11 ha), and two in Samokov (7,5 ha). Two applications were made in Samokov, one against the first generation (on the 17th May), and one against the second generation (on the 14th July). Three applications were made in Bogdanovo on 9th May, 8th July and 13th August against the first, second and third generations of *R. theobaldi*, respectively. The timing of each treatment was chosen according to the number of males caught by the traps. The treatments were made during the period of midge oviposition and larvae hatching.

The test suspensions were: NeemAzal® T/C (0.2%), Sineis 480 SC® (0,025%), and *B. subtilis* (20 ml). The suspensions were applied at a volume application rate of 0.1 l $m^{-2}$, using a hand held sprayer. The plants in the control were treated with equal quantity (0.1 l $m^{-2}$) of water.

*Data collection and analysis*

The observations were made on the 3rd, 6th, and 12th day after treatments. Twenty canes per replicate, 20 canes were examined under stereomicroscope and the number of larvae in the natural splits was counted.

Obtained data were subjected to one-way analysis of variance (ANOVA) and the treatment means were compared with the control plants, according to the Duncan's test (P<0.05).

## 3. Results

In 2016, the raspberry midge cane flight pattern demonstrated four generations in Bogadonovo (196 m) and three generations in Samokov (960 m) (Fig. 1). In both commercial fields, the flight of midges started in the second half of April, a week later in Samokov (25.04) compared with Bogdanovo (18.04). At lower altitude, the first, the second, and the third generations of the pest showed three pronounced peaks of its flight dynamic. In the higher altitude, there were two peaks of midge cane flight - the first

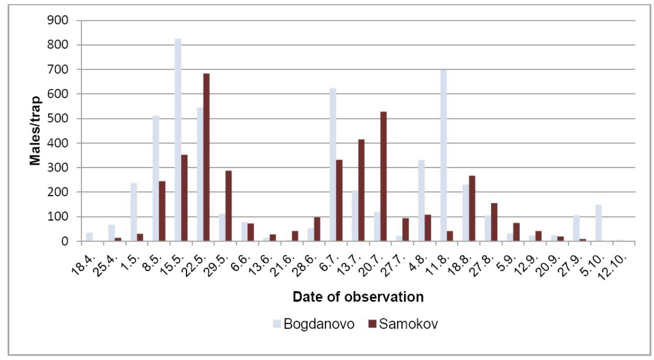


Fig. 1 - Trap catches of males of *Resseliella theobaldi* in the fields of Bogdanovo and Samokov during the spring, summer and autumn of 2016.

one between 19th and 25th of May, and the second one between 18th and 21th of July, for the first and second generations respectively (Fig. 1).

In general, the population density of the males was higher in Bogdanovo than at Samokov. The highest number of males was recorded during the intensive flight of the first generation of the midge in both Bogdanovo (824) and Samokov (683) sites (Fig. 1). Later in the season, the number of the males attracted by the traps in Bogdanovo was 623, 698 and 193 for the second, third and fourth generation, respectively. In Samokov, the number of the males was 528 and 267 for the second and third generation, respectively. The flight of the fourth generation of *R. theobaldi* continued until 12th of October in Bogdanovo, while the flight of the third generation of the midge in Samokov was completed on 27th of September (Fig. 1). The results obtained from this observation allowed finding the most appropriate date for treatments.

The evaluated bioinsecticides demonstrated biological activity against the raspberry midge cane applied during the period of pest oviposition and larvae hatching. Except for the treatment against the second generation of *R. theobaldi* in Samokov, in both raspberry fields, Bogdanovo and Samokov, the highest efficiency was found for the insecticide NeemAzal® T/C (Tables 1 and 2). This biopesticide also demonstrate the rapid initial effect against the penetration of larvae into raspberry canes. The number of midge larvae per split in raspberry canes treated with NeemAzal® T/C was significantly lower than the control at observations at 3rd, 7th and 12th day after applying the insecticide in Bogdanovo (Table 1, P<0.05). There was no significant difference between the efficacy demonstrated by NeemAzal® T/C and *B. subtilis*. After the treatment against the first generation the number of midge larvae per split varied between 1.08 and 1.54 for NeemAzal® T/C, and between 1.55 and 2.07 for *B. subtilis*. Similar results were observed after the second and third treatments. The insecticide Sineis 480 SC® demonstrated the lowest efficacy compared with NeemAzal® T/C and *B. subtilis* (Table 1, P<0.05). At the observation at 3rd day after treatments against the first and third generation of raspberry midge in Bogdanovo, the number of larvae in plots treated with Sineis 480 SC® was not significantly different from the number of larvae in control plots. In the second generation there was a statistical difference between the variant with the bioinsecticide and the control variant. After the treatments at 7th and 12th day, the insecticide showed better effect, and the number of larvae into the canes was significantly lower than the control but still higher compared with NeemAzal® T/C and *B. subtilis* (Table 1, P<0.05).

In the experiments conducted in Samokov Sineis 480 SC® demonstrated higher efficacy against raspberry cane midge and the number of larvae per split was statistically different than the control at all three observations (Table 2, P<0.05). In this field, *B. subtilis* was more effective than NeemAzal® T/C and the number of larvae per split varied between 2.86 and 2.91 after first treatment. After the same treatment, the number of larvae into the canes treated with NeemAzal® T/C varied between 3.15 and 2.94. After the treatment against the second generation of *R. theobaldi*, *B. subtilis* showed rapid initial effect than NeemAzal® T/C. There was significant difference in number of larvae at 3rd day - 2.63 and 4.86 for *B. subtilis* and NeemAzal® T/C, respectively (Table 2, P<0.05).

Table 1 - Efficacy of three biopesticides using against raspberry midge cane in Bogdanovo

| Treatments | Products/active ingredients | Number of midge larvae/split (day after treatment) | | |
|---|---|---|---|---|
| | | 3rd (±SD) | 7th (±SD) | 12th (±SD) |
| First generation | NeemAzal® T/C (azadirachtin A) | 1.54 (±0.18) a | 1.12 (±0.07) a | 1.08 (±0.37) a |
| | Sineis 480 SC® (spinosad) | 4.25 (±0.45) b | 2.93 (±1.04) b | 3.41 (±1.35) b |
| | *B. subtilis* | 2.07 (±0.67) a | 1.35 (±0.11) a | 1.55 (±0.64) a |
| | Control | 5.62 (±1.02) b | 6.28 (±1.22) c | 6.78 (±0.56) c |
| Second generation | NeemAzal® T/C (azadirachtin A) | 2.48 (±1.11) a | 1.74 (±0.64) a | 1.62 (±0.19) a |
| | Sineis 480 SC® (spinosad) | 5.77 (±1.32) b | 3.82 (±1.04) b | 3.87 (±0.94) b |
| | *B. subtilis* | 3.19 (±0.44) a | 2.17 (±0.05) a | 2.28 (±0.14) a |
| | Control | 8.44 (±1.12) c | 8.92 (±1.65) c | 9.41 (±0.27) c |
| Third generation | NeemAzal® T/C (azadirachtin A) | 1.24 (±0.72) a | 0.92 (±0.08) a | 0.98 (±0.34) a |
| | Sineis 480 SC® (spinosad) | 3.48 (±1.13) b | 2.14 (±0.22) a | 2.21 (±0.75) a |
| | *B. subtilis* | 1.37 (±0.18) a | 1.22 (±0.31) a | 1.43 (±0.86) a |
| | Control | 4.05 (±0.93) b | 4.89 (±1.16) b | 5.12 (±0.99) b |

Means within each column followed by the same letter are not significantly different, Duncan's test (p<0.05).

Table 2 - Efficacy of three biopesticides using against raspberry midge cane in Samokov

| Treatments | Products/active ingredients | Number of midge larvae/split (day after treatment) | | |
|---|---|---|---|---|
| | | 3rd (±SD) | 7th (±SD) | 12th (±SD) |
| First generation | NeemAzal® T/C (azadirachtin A) | 3.15 (±1.11) a | 2.13 (±0.82) a | 2.94 (±0.54) a |
| | Sineis 480 SC® (spinosad) | 6.05 (±1.32) b | 4.74 (±1.08) b | 5.38 (±0.67) b |
| | *B. subtilis* | 2.92 (±0.97) a | 2.39 (±0.17) a | 2.86 (±0.91) a |
| | Control | 10.21 (±1.24) c | 12.17 (±1.98) c | 14.71 (±1.15) c |
| Second generation | NeemAzal® T/C (azadirachtin A) | 4.86 (±0.46) b | 3.77 (±0.21) b | 3.58 (±1.06) a |
| | Sineis 480 SC® (spinosad) | 5.14 (±1.11) b | 3.98 (±0.87) b | 3.43 (±0.35) a |
| | *B. subtilis* | 2.63 (±1.07) a | 2.05 (±0.57) a | 2.73 (±0.97) a |
| | Control | 8.73 (±0.48) c | 10.84 (±1.54) c | 13.22 (±1.18) b |

Means within each column followed by the same letter are not significantly different, Duncan's test (p<0.05).

## 4. Discussion and Conclusions

*R. theobaldi* was described by Theobald in 1920 (Barnes, 1926). Since then, almost a century later, it has become a pest of economic importance of raspberry crop throughout Europe. The midge cane is widely distributed in Bulgaria, Greece, Rumania, Italy, France, Ireland, UK, Sweden, Czech Republic, Slovakia, Hungary and Poland. In these countries the insect has been introduced mainly with infested planting materials and somewhere with infested soil. When establishing a new raspberry plantation, it is critical to choose the cultivar that is well adapted to local soil and climatic conditions and it is less susceptible to infestation by the raspberry cane midge, as well. Normally, infested raspberry plants have demonstrated the symptoms of dark brown, clearly defined spots in the canes 3 to 5 weeks after laying the eggs (Stoyanov, 1963). For this reason, it is important to determine the most appropriate timing for control of *R. theobaldi*. The treatments have to be done before the larval feeding sites become visible.

Organic production of raspberry in many countries, including Bulgaria, is a challenge because of lack of products for plant protection, which have nonchemical origin. Moreover, in Bulgaria there are no officially registered biopesticides or even chemical insecticides, that can be specifically used for control of the midge cane. Meanwhile, NeemAzal® T/C has been registered for control of tomato borer, *Tuta absoluta*, Meyr. Considering this need, the present study met its objective - the results showing the possibility of biological control of the *R. theobaldi*. The tested biologically based preparations demonstrated their efficacy against the larvae of midge cane. The pest had four generations in the plantation at lower altitude (Bogdanovo) and three generation in the plantation at higher altitude (Samokov). Further, the data from the pheromone traps showed that the first generation had the highest population density in both raspberry plantations. The forth and the third generations in both sites showed the lowest population density. According to this information, the time and the number of treatments were determined.

Among the tested biopesticides, NeemAzal® T/C and *B. subtilis* demonstrated the highest efficacy, causing up to 82.5% (the former) and 81.2% (the later) reduction of number of midge larvae in the splits compared to the control. Sineis 480 SC® demonstrated slower initial effect than NeemAzal® T/C and *B. subtilis*, but comparatively high efficacy, causing up to 63.26% reduction in number of larvae in the splits compared with the control.

In fact, the present evaluation is the second attempt to apply only environmentally safe products for control of *R. theobaldi*. The first one was made by Shternshis *et al.* (2002). The authors tested the preparations based on *Bt* (BACTICIDAE®), and *S. avermitilis* (PHYTOVERM®) for control of the raspberry midge blight and reported significant reduction of disease complex severity compared with the control variants. *S. avermitilis* is the base of spinosad, the active ingredient of Sineis 480 SC®. Spinosad as an active ingredient of the product Audienz®, was tested by Barrofio *et al.* (2011) against the raspberry midge cane. The result from this experiment is ambivalent, showing comparatively high efficacy against the midge larvae, but not significantly differ compared to the control. In this study spinosad showed to be less effective to the raspberry cane midge compared with both, azadirachtin A and *B. subtilis*. The result is interesting, because the spinosad penetrates translaminarly in plant tissues and this suggests higher efficacy compared with the other tested products. Deleva and Harizanova (2014) stated the rapid initial effect of spinosad against the larvae of tomato borer, *T. absoluta*. The authors reported 73.33% larval mortality in tomato leaves at 3$^{rd}$ day after treatment.

Neem-based products have been evaluated for their efficacy against the different pest on berry plants. Kim (2014) reported insufficient activity of azadirachtin against the rednecked cane borer, *Agrilus ruficollis* F. on blackberries in USA. Contrary, Aguilera *et al.* (2009) commented the high efficacy of Neem against the raspberry weevil, *Aegorhinus superciliosus* G. in Chile. The authors reported significant embryogenesis inhibition after applying the Neem. The lowest number of larvae in raspberry canes observed in this evaluation is probably due to affect of azidarachtin on both larvae and adult of raspberry cane midge.

The results obtained after application of, *B. subtilis* indicate that the bacterium might be considered as an effective agent for control of *R. theobaldi*. *B. subtilis* was originally isolated from the soil and has been tested as a biocontrol agent of root-knot nematode, *Meloidogyne arenaria* on tomato (Mohamedova and Samaliev, 2011). The bacterium is able to colonize successfully the rhizosphere of the plants and affect different pathogens in this zone. This suggests that *B. subtilis* could influence the pupae of the raspberry cane midge in the soil.

I have not observed any phytotoxicity or negative influence of the three biopesticides on raspberry plants and beneficial insects. In several raspberry canes collected from the plantation in Samokov was observed parasitized 4$^{th}$ instar midge larvae of the third generation (Fig. 2). The midge larvae probably were infested by the parasitic larvae of *Aprostectus* genus.

Therefore the results of the present evaluation show the possibility of the tested biopesticides to control of the raspberry cane midge. These products are a good alternative to chemical insecticide and might be successfully integrated in the control strategies of *R. theobaldi* in both, conventional and organic raspberry production. Further research should focus on screening the pesticides and bioagents, which could be able to control the disease complex “midge blight”, causing very often the dead of raspberry plants.

Fig. 2 - Parasitized and nonparasitized 4th instar larvae of *Resseliella theobaldi* in splits of raspberry canes collected from Samokov raspberry plantation (September, 2016)

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