ASSESSING THE NEMATICIDAL ACTIVITY OF *Bacillus firmus* STRAINS

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ABSTRACT

Root-knot nematodes (RKN) from the genus *Meloidogyne* are considered the most important group of the plant-parasitic nematodes, being able to parasitize a wide range of host plants. Field infestations lead to economic damage due to reduction or loss of crop yield. Different types of chemical nematicides are used for RKN control – most of them classified as fumigants, carbamates or organophosphates. Many chemical nematicides are no longer being used due to the high toxicity and are being replaced by the new generation of active ingredients such as fluopyram, bacterial secondary metabolites (avermectins), and biological agents such as fungi (*Pochonia chlamydosporium*, *Myrothecium verrucaria*, *Purpureocillium lilacinus, Trichoderma* spp., *Metarhizium* spp.) and bacteria (*Pasteuria* spp., *Bacillus* spp.). We evaluated the nematicidal activity of *Bacillus firmus* strains in *in vitro* and in pot experiments. Assessment of *in vitro* activity of three *B. firmus* strains against *Meloidogyne incognita* and *M. luci* showed comparable reduction in number of hatched and motile larvae for both species. Three different *B. firmus* strains exhibited different rate of nematicidal activity, with strain ZZV12-4809 isolated from garden soil being the most effective. *In vitro* egg-hatching in the presence of bacterial culture was up to 100 % lower in comparison to control treatment, while up to 77.9 % lower in the presence of washed bacterial cells. In pot experiments we evaluated the nematicidal activity of two most effective *B. firmus* strains - ZZV12-4809 and I-1582 isolated from bionematicide VOTiVO® (Bayer CropScience) and compared them to the untreated control, to the chemical nematicide Velum® Prime (Bayer) and to the bionematicide VOTiVO®. The reproduction factor (R_f) of *M. luci* determined at the end of experiment was up to 62 % lower in tomato plants where *B. firmus* was added compared to untreated control. All tested strains showed nematicidal activity; in *in vitro* experiments *B. firmus* ZZV12-4809 was the most effective, while in pot experiments strain I-1582 showed highest nematicidal activity.

Key words: *Bacillus firmus*, *Meloidogyne*, nematicidal activity, biological control agent

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IZVLEČEK

OVREDNOTENJE NEMATICIDNE AKTIVNOSTI SEVOV BAKTERIJE *Bacillus firmus*

Ogorčice koreninskih šišk (RKN) iz rodu *Meloidogyne* so najpomembnejša skupina rastlinsko-parazitskih ogorčic, saj zajedajo širok spekter gostiteljskih rastlin. Namnožitev RKN na njivi privede do gospodarske škode zaradi zmanjšanja ali izpada pridelka. RKN je mogoče zatirati z različnimi tipi kemičnih nematicidov, ki jih povečini uvrščamo med fumigante, karbamate ali organske fosforjeve estre. Mnogi kemični nematicidi niso več v uporabi zaradi visoke toksičnosti, nadomeščajo jih nove generacije učinkovin, kot so fluopiram, sekundarni metaboliti bakterij (avermektini) ter bionematicidi na podlagi gliv (*Pochonia chlamydosporium*, *Myrothecium verrucaria*, *Purpureocillium lilacinus, Trichoderma* spp., *Metarhizium* spp.) in bakterij (*Pasteuria* spp., *Bacillus* spp.). Ovrednotili smo nematicidno učinkovitost bakterijskih sevov *Bacillus firmus* v *in vitro* in lončnih poskusih. Vrednotenje *in vitro* aktivnosti treh sevov *B. firmus* proti RKN vrst *Meloidogyne incognita* in *M. luci* je pri obeh vrstah pokazalo primerljivo zmanjšanje števila izleženih in motilnih ličink. Opazili smo razlike v delovanju različnih sevov *B. firmus,* in sicer se je kot najbolj učinkovit izkazal sev *B. firmus* ZZV12-4809, izoliran iz vrtnih tal. Izleganje jajčec *in vitro* je bilo do 100 % nižje v primerjavi s kontrolo v obravnavanju, kjer smo jajčecem ogorčic dodali bakterijsko kulturo, do 77,9 % nižje pa ob dodatku spranih bakterijskih celic. V lončnih poskusih smo ovrednotili namaticidno učinkovitost dveh sevov *B. firmus* - ZZV12-4809 ter seva I-1582 izoliranega iz bionematicida VOTiVO® (Bayer CropScience), ter ju primerjali s kontrolami brez tretiranja ter z delovanjem kemičnega nematicidom Velum® Prime (Bayer) oz. bionematocidom VOTiVO®. V lončnih poskusih smo s štetjem jajčec ob koncu poskusa določali reprodukcijski faktor (Rf) RKN *M. luci*, ki je bil do 62 % nižji pri rastlinah paradižnika, tretiranih z *B. firmus*, v primerjavi z netretirano kontrolo. Vsi testirani sevi *B. firmus* so pokazali nematicidno aktivnost; v *in vitro* poskusih je bil najbolj učinkovit *B. firmus* ZZV12-4809, v lončnih poskusih pa sev I-1582.

Ključne besede: *Bacillus firmus*, *Meloidogyne*, nematicidna aktivnost, biotični agens

1 INTRODUCTION

Plant-parasitic nematodes (PPNs) are prolific pests of many economically important crop plants causing significant yield losses of over $100 billion per year. Considering the yield loss and because of their broad range of host plants, root-knot nematodes (RKNs) of the genus *Meloidogyne* are the most damaging of all plant-parasitic nematodes (Fuller et al. 2008). *Meloidogyne* spp. are soil-borne root pathogens that interfere with the normal uptake of nutrients and water into the plant by causing deformities of the root tissue – root galls. Chemical nematicides, many of which are toxic for the environment and human health are still the primary method used to control PPNs (Geng et al. 2016). Most of chemical nematicides are classified as fumigants, carbamates or organophosphates (Compendium of Pesticide Common Names, Nematicides, available at: http://www.alanwood.net/pesticides/class_nematicides.html). Out of these, many are

no longer approved for use due to the high toxicity and are being replaced by the new generation of active ingredients such as fluopyram, bacterial secondary metabolites (avermectins), and biological agents such as fungi (*Pochonia chlamydosporium*, *Myrothecium verrucaria*, *Purpureocillium lilacinus, Trichoderma* spp., *Metarhizium* spp.) and bacteria (*Pasteuria* spp., *Bacillus* spp.). Biological pesticides have become an important part of environmentally friendly pest management applications. Rhizobacterium *Bacillus firmus* is one of the most widely used agents in bionematicide preparations. Because of the scarcity of research having been done on *B. firmus* nematode-virulence factors, there is a clear need to further understand the ecology and mode of action of *B. firmus* when used as a bionematicide (Tian et al. 2007). Direct antagonism has been observed for different *Bacillus* species. Most rhizobacteria act against PPNs by means of various secondary metabolites, enzymes and toxins. The effects of these toxins can include suppression of nematode reproduction, eggs hatching and juvenile survival, as well as direct mortality of nematodes (Geng et al. 2016; Tian et al. 2007; Lian et al. 2007). Various *Bacillus* spp. proteases were shown to severely degrade nematode cuticle or internal body structures (Geng et al. 2016; Lian et al. 2007).
The aim of our investigation was to assess antagonistic *in vitro* and *in vivo* activity of three different *B. firmus* strains against the root-knot nematodes *Meloidogyne incognita* and *M. luci*.

2 MATERIALS AND METHODS

In *in vitro* experiments, three different *B. firmus* strains were tested for the nematicidal activity against *M. incognita* and *M. luci*. *B. firmus* strain I-1582 was isolated from the VOTiVO® (Bayer CropScience, Germany) formulation containing the bacteria, while *B. firmus* ZZV12-4809 and *B. firmus* ZZV12-4810 strains were isolated from pea (*Pisum sativum*) rhizosphere in the garden in Maribor, Slovenia. Nematode-eggs suspensions were prepared by harvesting egg-masses from the infested tomato (*Solanum lycopersicum* 'Val') roots as described by Hussey and Barker (1973). Eggs were then surface sterilised in 1% (v/v) sodium hypochlorite (NaClO; Kemika, Croatia) aqueous solution and placed on sterile 24-well microplates in concentration of 100 eggs per well. Bacteria were cultured in Luria-Bertani (LB) broth at 22°C with shaking at 130 rpm until reaching the culture density of approximately 1.5×10^8 CFU/ml (OD_{600} of 1 – 1.5). Bacterial culture was used to prepare three different treatments for testing the nematicidal activity on nematode eggs: unprocessed bacterial culture (B); bacterial culture filtrate (F); and washed bacterial cells - pelleted bacterial cells resuspended in phosphate buffer (W). Experiment was conducted four times using *M. incognita* and once using *M. luci*, with four replicates per treatment. The number of hatched second-stage (J2) juveniles was recorded under a stereomicroscope Nikon SMZ800 (Nikon, Japan) and compared to control treatments in sterile water, phosphate buffer and sterile fresh LB medium after 10 days at 23°C. The motility of hatched J2 larvae was inspected by shaking or probing with a fine nematological needle.
For *in vivo* pot experiments, tomato hybrid 'Horus F1' seeds (L'Ortolano, Italy) were surface sterilised in 3% NaClO and seeds were then either coated only with 2% (w/v) aqueous solution of carboxymethyl cellulose (CMC; Sigma-Aldrich, Germany) or with CMC and 2×10^6 bacterial spores. CMC was added for spore adhesion to the seed

surface as described by Razinger et al. (2018). Coated tomato seeds were potted in sterile 11 cm-diameter polypropylene pots (V = 1.3 L) with sterile substrate. Procedures for substrate mixture preparation, use of fertilizers during the experiment, final eggs counting and calculation of nematode reproduction factor (R_f) are described in details in Susič et al. (2018). After 35 days, tomato plants were inoculated with 4 × 10^3 *M. luci* eggs (equalling 3 eggs / cm^3 of substrate). Nematode-infested plants were subjected to 5 treatments with 4 biological replicates per treatment as follows: plants treated with VOTiVO® formulation (B1) according to manufacturers' instructions; *B. firmus* strain I-1582 previously isolated from VOTiVO® formulation (B2); *B. firmus* strain ZZV12-4809 (B3); chemical nematicide Velum® Prime (Bayer) according to manufacturer's instructions (B4, positive control); and untreated plants (B5, negative control). The experiment was conducted in duplicate in the controlled environment in the growth chamber (GC) DPC-420 (Kambič, Slovenia) at 23°C, 12 h/day illumination, 60% relative humidity (Rh); and in the glasshouse (GH) at the Agricultural Institute of Slovenia (Ljubljana, Slovenia) from January to April 2017. The pot experiments were terminated 50 days after nematode inoculation (DAI) when *M. luci* was expected to complete the first developmental cycle. At the end of the experiments, rhizospheric samples were collected and sporogenic bacteria were isolated (adapted from Földes et al. 2000; and Agrawal et al. 2013). Pure bacterial cultures were identified by 16S rRNA sequencing (Bandi et al. 1994). The following parameters were recorded at the end of the experiment: total number of nematode eggs, R_f, total root weight, and galling index (using the scale from 0 – no infestation, healthy plant and roots; to 10 – heavy infestation, plant and roots dead; Zeck et al. 1971). The data for parameters: galling index, total number of nematode eggs per plant, eggs per gram of roots, R_f, were normalised with Box-Cox transformation, while the data for total root weight from GC-replicated pot experiment were normalised with log-transformation (due to the zero lambda value (λ=0) obtained in Box-Cox). The data from *in vitro* experiments did not need normalisation, except for the *M. luci* treated with *B. firmus* ZZV12-4809 data subset. Normalised data were then statistically analysed with ANOVA and subsequently (where statistically significant) with Tukey's HSD (Honest Significant Difference) test at α=0.05 to separate means. Due to unequal variances between groups, the data for the total number of nematode eggs per plant, eggs per gram of roots, and R_f in GH-replicated pot experiment, were analysed with Welch's ANOVA and Games-Howell test. Statistical analyses were performed with R (R Core Team, 2018).

3 RESULTS AND DISCUSSION

In *in vitro* experiments, inhibitory effect on nematode-eggs hatching and larvae motility was observed with all tested bacterial strains and in all three tested treatments. The effect was the most significant in treatment B (Table 1) where unprocessed bacterial cultures were added to the nematode eggs. Different *B. firmus* strains displayed different levels of antagonistic activity; *B. firmus* ZZV12-4809 achieved the highest inhibition of J2 larvae hatching and strain I-1582 the lowest. Treatments F (bacterial culture filtrate) and W (bacterial cells washed in phosphate buffer) were included to discern the nature of inhibitory action in *B. firmus*. Both, treatments F and W, showed comparatively similar inhibition strength in hatching of

M. incognita and *M. luci,* which was lower in strength than in treatment B. As this nematicidal effect was shown for both treatments W and F, we presume that molecules secreted by bacterial cells play a role in nematicidal activity as well as the bacterial cells themselves. *B. firmus* I-1582 seemed to be more effective against *M. luci* than *M. incognita* (Table 1). As different bacterial strains exhibited different levels of antagonistic activity the same nematode-virulence factor(s) might be expressed at different rates in different strains; or a spectrum of nematode-virulence factors differ between strains of *B. firmus*.

Table 1: Results of *in vitro* experiments testing the potential of three different *Bacillus firmus* strains for inhibition of eggs hatching of two *Meloidogyne* species under treatments: B (bacterial cultures), W (washed bacterial cells) and F (bacterial culture filtrates). Data for inhibition of eggs hatching (%) presented as averages with the standard error of the mean (±SE), ANOVA statistic and Tukey's HSD results. Means sharing a letter are not significantly different at $p < 0.05$.

		Bacterial strains		
Nematodes	**Treatment**	***B. firmus* I-1582**	***B. firmus* ZZV12-4809**	***B. firmus* ZZV12-4810**
M. incognita	B	71.1 ± 9.1 a	97.9 ± 0.8	97.4 ± 1.2
	W	27.4 ± 7.0 b	78.9 ± 6.7	55.8 ± 13.7
	F	14.5 ± 13.7 b	77.9 ± 8.0	54.7 ± 14.9
	ANOVA statistics	$F_{2, 9}$=8.53, p=0.008	$F_{2, 9}$=3.51, p=0.075	$F_{2, 9}$=4.33, p=0.05
M. luci	B	86.4 ± 1.9 a	100.0 ± 0.0 a	96.4 ± 2.2 a
	W	39.5 ± 10.9 b	72.7 ± 5.9 b	52.3 ± 3.9 b
	F	54.5 ± 6.9 b	68.2 ± 0.0 b	38.6 ± 2.3 c
	ANOVA statistics	$F_{2, 9}$=11.66, p=0.003	$F_{2, 9}$=249.17, p<0.001	$F_{2, 9}$=125.47, p<0.001

Within *in vivo* pot experiments, the original VOTiVO® formulation was tested in parallel with the *B. firmus* I-1582 isolated from this bionematicide in order to account for any additional effects caused by various additives, rather than the *B. firmus* bacteria included in the formulation. Additionally, only *B. firmus* ZZV12-4809 was chosen out of the two rhizosphere strains isolated in Maribor since it exhibited higher inhibitory effect on nematode-eggs hatching in the *in vitro* experiments. Results of the in pot experiments showed that all *Bacillus*-based treatments (i.e. treatments B1, B2 and B3) failed to reduce the overall root galling caused by the *M. luci* infestation (Table 2). Differing results, however, were obtained when total number of nematode eggs per plant, eggs per gram of roots, and corresponding R_f in *B. firmus* treatments were calculated and compared to controls. The reproduction factor (R_f) of *M. luci* determined at the end of the experiment was up to 62% lower in tomato plants where *B. firmus* was added compared to untreated control (Table 2). Our results revealed that observing galling index only would not be sufficient for thorough evaluation of *B. firmus* virulence effects to RKN. Both *B. firmus* I-1582 and ZZV12-4809 strains

inhibited the formation of nematode eggs, with strain I-1582 showing somewhat higher inhibitory action against *M. luci* than ZZV12-4809. At the end of the experiment, sporogenic bacteria were isolated from all rhizosphere samples from *Bacillus*-based treatments (i.e. B1, B2 and B3). PCR amplification and subsequent 16S rRNA sequencing showed that the isolated bacteria belonged to the genus *Bacillus* thus indicating that the bacteria added in treatments B1, B2 and B3 were able to colonise the tomato rhizosphere during the course of the experiments. Differences in RKN reproduction potential and *B. firmus* performance were observed between the experimental duplicates in the growth chamber and in the glasshouse (Table 2). Nematode reproduction factors were comparatively lower in the growth chamber than in the glasshouse. In the glasshouse, more days with temperatures above 23°C positively influenced the reproduction of RKN. *B. firmus* ZZV12-4809 performed comparably in both experiments while the performance of the VOTiVO® and isolated strain I-1582 was inconsistent between the two experiments (Table 2).

Table 2: Results of pot experiments testing the efficacy of *Bacillus firmus* to control the *Meloidogyne luci* infestation in tomato in the growth chamber (GC) and the glasshouse (GH). Data presented as averages from 4 biological replicates with the standard error of the mean (±SE), ANOVA statistic and Tukey's HSD results. Means sharing a letter are not significantly different at $p < 0.05$. Treatments: B1 - VOTiVO®, B2 - *B. firmus* I-1582, B3 - *B. firmus* ZZV12-4809, B4 - Velum® Prime (positive control) and B5 - untreated plants (negative control).

Treatment	**Galling index**	**Number of eggs/plant (n × 10^5)**	**Number of eggs/g roots (n × 10^4)**	**Reproduction factor (R_f)**	**Root weight (g)**
B1 (GC)	3.5 ± 0.3 a	1.3 ± 0.3 ab	0.73 ± 0.14 ab	33.3 ± 6.7 ab	18.1 ± 0.4
B2 (GC)	2.5 ± 0.5 a	0.8 ± 0.1 b	0.39 ± 0.06 b	18.8 ± 3.6 b	19.1 ± 2.3
B3 (GC)	3 ± 0.0 a	1.4 ± 0.2 ab	0.63 ± 0.04 ab	35.5 ± 3.9 ab	22.4 ± 1.8
B4 (GC)	0 ± 0.3 b	0.01 ± 0.0003 c	0.006 ± 0.002 c	0.3 ± 0.1 c	20.5 ± 2.1
B5 (GC)	3.5 ± 0.3 a	2.0 ± 0.2 a	0.93 ± 0.17 a	49.6 ± 4.6 a	22.4 ± 2.7
ANOVA statistics	$F_{4, 15}$=19.98, p<0.001	$F_{4, 15}$=105.52, p<0.001	$F_{4, 15}$=81.64, p<0.001	$F_{4, 15}$=105.52, p<0.001	$F_{4, 15}$=0.911, p=0.48
B1 (GH)	3 ± 0.3 a	1.4 ± 0.5 ab	0.69 ± 0.15 ab	35.6 ± 12.0 ab	18.2 ± 4.5
B2 (GH)	3.5 ± 0.3 a	2.4 ± 0,2 a	1.04 ± 0.13 a	59.2 ± 5.4 a	23.3 ± 2.0
B3 (GH)	3 ± 0.0 a	2.8 ± 0.2 a	1.00 ± 0.12 a	68.7 ± 5.2 a	28.3 ± 2.1
B4 (GH)	0 ± 0.0 b	0.02 ± 0.02 b	0.008 ± 0.006 b	0.6 ± 0.4 b	27.5 ± 1.6
B5 (GH)	3 ± 0.3 a	3.4 ± 0.3 a	1.51 ± 0.20 a	85.5 ± 7.7 a	23.8 ± 3.3
ANOVA statistics	$F_{4, 15}$=48.75, p<0.001	$F_{4, 6.05}$=76.50, p<0.001	$F_{4, 6.02}$=38.86, p= 0.0002	$F_{4, 6.05}$=76.50, p<0.001	$F_{4, 15}$=1.96, p=0.15

Inconsistent performance of biological control agents in *in vitro* and *in vivo* systems has been observed previously. For example, *B. firmus* I-1582 was reported to give

inconsistent results in the management of the soybean cyst nematode (*Heterodera glycines*) infestations in soybean (*Glycine max*) (Beeman and Tylka, 2018). In case of *B. firmus*, effective nematode protection is achieved by colonisation of the plant rhizosphere. Rhizosphere is a very dynamic environment and many environmental factors and interactions between organisms may contribute to the overall performance of the biocontrol agent. Results from *in vitro* and pot experiment show that *B. firmus* strains studied here exhibit nematicidal properties, but nematicidal efficiency decreases when the system is more complex. The difficulty of "fine tuning" this biocontrol agent for maximum nematicidal effect is further exacerbated since the exact mechanism of nematode toxicity in *B. firmus* has still not been established. Bacterial proteases were shown to degrade nematode cuticle or internal body structures (Geng et al. 2016; Lian et al. 2007) and are thought to be the primary nematode-virulence factor. However, rhizobacteria such as *B. firmus* are known to produce an array of compounds to interact with their environment and are able to modulate plant defence systems and responses to stress (El-Esawi et al. 2018). Further studies into the genomics and secondary metabolite production should thus be conducted in order to enable the effective biotechnological exploitation of *B. firmus* bacteria as a biological nematocide.

4 CONCLUSIONS

All tested *B. firmus* strains showed nematicidal activity; in *in vitro* experiments *B. firmus* ZZV12-4809 was the most effective, while in pot experiments strain I-1582 showed the highest nematicidal activity. Nematode-eggs hatching *in vitro* was up to 100% lower compared to control with the addition of unprocessed bacterial culture, and up to 77.9% lower with the addition of washed bacteria. In pot experiments, the reproduction factor of *M. luci* was up to 62% lower in tomato plants where *B. firmus* I-1582 was added compared to the untreated control. Due to the lower overall effectiveness and inconsistent results of these bacteria against RKN in pot experiments compared to *in vitro*, further studies into the nematode-virulence factors are warranted to resolve the current performance issues.

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