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Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251

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ABSTRACT

The plant parasitic root-knot nematodes *Meloidogyne* spp. are a devastating threat to agriculture. The urgent need to search for alternative root-knot nematode control methods that are less environmentally toxic is a demanding challenge to secure the increasing global food demand. Therefore, we combined chemical and biological control strategies to evaluate their management potential on *Meloidogyne incognita* infested tomatoes. To determine the combined effect of the nematicides Velum and *Purpureocillium lilacinum* strain 251 formulated as wettable granulate (BioAct WG), we evaluated tomato yield parameters, gall index, soil and root nematode populations. Velum is a chemical nematicide with fluopyram as its active ingredient and BioAct is a biological nematicide based on the egg pathogenic fungus *Purpureocillium lilacinum* (strain 251). As a single treatment, the nematicide Velum showed better *M. incognita* reduction at-planting, while BioAct controlled the nematode population throughout the growing season. Greenhouse experiments in two consecutive years, showed that by combining the two nematicides, the *M. incognita* controlling effect was enhanced and the tomato yield increased compared to single nematicide treatments. The controlling effect of *P. lilacinum* was lower when *M. incognita* population increased, presumably based on its limiting ability to parasitize the increasing numbers of nematode eggs. To conclude, we have shown that combining chemical and biological nematicides can successfully control the root-knot nematode *M. incognita* and increase yields. Velum downregulated the nematode population at-planting and reinforced the biological efficacy of *P. lilacinum* throughout the growing season.

1. Introduction

Root-knot nematodes are a major threat to commercial and private vegetable growers across the globe, causing severe root damage and yield losses (Jones et al., 2013). The obligate plant parasitic genus *Meloidogyne* has a broad host range and infects most economical important plants, such as tomatoes, cucumbers, soya and potatoes. Root galling is the primary symptom affecting the plant ability to uptake water and nutrients. The aboveground plant symptoms are not distinct from other root damage and therefore plant parasitic nematodes are often overlooked until the population has established and caused economical losses. To secure yield and profitable production, there is an urgent need to control these pathogens.

Commonly, *Meloidogyne* spp. are managed by chemical fumigants or nematicides. However, the ban of chemicals with a broad action on non-target organisms, or the implementation of new directives and regulations to reduce chemical applications, is increasing (Huang et al., 2018; Villaverde et al., 2016). Therefore, alternative strategies are needed to reduce *Meloidogyne* spp. populations with a durable solution and reduced pesticide usage. Resistant cultivars harbouring the *Mi*-gene, successfully introgressed from *Solanum peruvianum* L. to *S. lycopersicum* L. (tomato) conver high levels of resistance against *Meloidogyne arenaria, Meloidogyne javanica* and *Meloidogyne incognita* (Iberkleid et al., 2014). However, the *Mi*-gene does not confer resistance to *Meloidogyne hapla, Meloidogyne chitwoodi, Meloidogyne enterolobii* or *Meloidogyne exigua*. Beside this, temperatures above 28 °C are able to break the resistance, making the plant susceptible to all *Meloidogyne* spp. (Dropkin, 1969; Roberts et al., 1990). The usage of resistant cultivars helps to suppress

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Meloidogyne spp. populations, but the occurrence of virulent populations, such as *Mi*-gene resistant *M. incognita* are a threat to field and greenhouse growers (Gine and Sorribas, 2017; Jacquet et al., 2005; Schaff et al., 2007). Therefore, the use of new nematicides with preferable reduced ecotoxicological profiles such as fluopyram or fluensulfone, or biocontrol agents to target plant parasitic nematodes, are currently in high demand (Arthurs and Dara, 2018; Singh et al., 2017).

Nematophagous fungi are natural antagonists with the capacity to capture and/or parasitize nematodes (De Ulzurrun and Hsueh, 2018; Jansson and Nordbring-Hertz, 2017). One biological control agent used against *Meloidogyne* spp., is the nematode egg-pathogenic fungus *Purpureocillium lilacinum* strain 251 (formerly *Paecilomyces lilacinus* strain 251) with its commercial name, BioAct or MeloCon (Kiewnick and Sikora, 2006a, 2006b; Sikora et al., 2018). *P. lilacinum* is the only approved biological nematicide in Switzerland and one of the three listed biological nematicides in the European Union (EU) pesticides database. In the EU, they are listed together with the nematode parasitic bacteria *Bacillus firmus* I-1582 and *Pasteuria nishizawae* Pn1. *P. lilacinum* controls *Meloidogyne* spp. pre-plant, at-plant and during the vegetative plant growth, by colonizing nematode eggshells, the larvae cuticle, or through direct hyphal penetration (Gine and Sorribas, 2017; Holland et al., 1999; Khan et al., 2004, 2006a). The infestation is fostered through hydrolytic enzymes like proteases and chitinases (Khan et al., 2003, 2004; Wang et al., 2010). The effectiveness of these hyphomycete has been tested under controlled, greenhouses and field conditions (Anastasiadis et al., 2008; Gine and Sorribas, 2017; Kaskavalci et al., 2009; Kepenekci et al., 2018; Khan et al., 2006b; Kiewnick and Sikora, 2006b; Kiriga et al., 2018; Schenck, 2003), reducing gall formation on various crops. However, *P. lilacinum* effectiveness against *Meloidogyne* spp. populations were not always able to suppress nematode densities under greenhouse and field studies (Gine and Sorribas, 2017).

Generally, the nematode density at-plant is an important factor for the successful effect on keeping the nematode population below an economical threshold (De Leij et al., 1992; Kayani et al., 2017). Consequently, the use of new chemical nematicides in combination with a biocontrol agent to control plant parasitic nematodes during plant vegetation might be a more robust strategy then applying each treatment solely.

In this study, we investigated if *P. lilacinum* efficacy increases when nematode populations are downregulated with a nematicide treatment at-plant and whether the combination of a chemical and biological nematicides increase the yields.

2. Material and methods

2.1. Nematode inoculum and nematicides

The *Mi*-virulent *M. incognita* isolate (pathotype) population 2, described by Hallmann and Kiewnick (2018) were cultured on tomato plants cv. Oskar (*Solanum lycopersicum*) grown under greenhouse conditions at Agroscope Wädenswil. Eggs were extracted by cutting heavily galled roots into 1 cm pieces and then vigorously shaking in a 1% NaOCl water solution for 3 min. Eggs were collected in a 20 μm mesh sieve and thoroughly rinsed with water before storing in a fridge. Freshly hatched second stage juveniles (J2) were separated by applying rinsed eggs on an Oostenbrink dish and stored at room temperature for hatching (Walker and Wilson, 1960). The freshly hatched J2s were collected over a one-week period.

Velum Prime 400 SC (active ingredient fluopyram, Bayer) solubilised in water and *Purpureocillium lilacinum* strain 251 formulated as wettable granulate (WG) (PL251; BioAct WG, Andermatt Biocontrol AG, CH) were suspended in water and 0.2% wetting agent Trifolio S-forte (Trifolio-M, Germany) solution, used for all experiments. Each experimental setup were split into five treatments, including a negative control without nematodes, a positive control inoculated with nematodes, an inoculated PL251 treatment, an inoculated Velum treatment and a combined inoculated treatment with Velum and PL251.

2.2. Nematicidal effect on Meloidogyne incognita juveniles in soil

The nematicidal activity of Velum and PL251 was evaluated in steamed soil sand mix (1:2). 100 cc of soil were filed in a 5 cm plastic pot and inoculated with 250 *M. incognita* juveniles (J2s). 120 pots were prepared to test the treatments, Velum (1 ppm a.i./pot), Velum (1 ppm a.i./pot) + PL251 (0.2 g/pot), PL251 (0.2 g/pot), J2 inoculated control (positive control) and not inoculated control (negative control). Each treatment was replicated six times at four different time points (7, 14, 21 and 28 days). PL251 was applied straight after the J2 inoculation, followed by a second application one week post J2 inoculation, while Velum was only applied one week after J2 inoculation. Pots were stored at 23/20 °C at 60% humidity in the dark. One week post inoculation and only PL251 application, the first J2s from the PL251 treatment and controls were extracted for evaluation. Up to 4 weeks, J2s were extracted weekly with the Oostenbrink dish technique by using the entire 100 cc soil sample from each treatment. The number of J2s retrieved after two-days were counted under a light microscope.

2.3. Nematicidal effect on Meloidogyne incognita in small pot tomato rootstocks

Nematode population on tomato roots were evaluated in a 600 cc clay pot experiment. One week before planting, pots were filed with a steamed soil sand mix (1:2) and inoculated with 4000 eggs/juveniles per pot. The treatments were set as previously described and replicated five times. After J2 inoculation, 0.2 g PL251/pot was applied the first time. All pots were placed in the greenhouse with 60% humidity, day night cycle of 15/9 h and 25/19 °C. At planting, Velum (1 ppm a.i./pot) was applied around the planting holes. 4 weeks old tomato cv. Oskar were planted in all pots. Two weeks after planting, PL251 was applied at 0.2 g/pot. After six weeks the experiment was stopped, the soil was gently washed from the root systems and the root galling was determined after Zeck (1971); 0 = no gall and 10 = completely galled roots). *M. incognita* egg masses were stained with red food colour (Mapcol Ponceau 4R, E124) and counted (Thies et al., 2002). Root systems were placed in the mist chamber and extracted juveniles were collected weekly for 4 weeks and counted under a light microscope.

2.4. Large-scale greenhouse experiment

The greenhouse experiment took place in two consecutive years at Agroscope in Wädenswil. It consisted of 80 x 15L pots filled with heat-sterilized field soil. The pots were organized in the greenhouse as four rows and the treatments were arranged randomly as pairs of two pots. To minimize the edge effect, for each end of the row we planted two additional pots with the same tomato variety.

Two weeks prior to planting, a suspension with 5500 *M. incognita* egg/J2s was used for inoculation (2017; 99% eggs and 1% J2s, 2018; 90% eggs and 10% J2s).

One week prior to planting, 0.2 g of PL251 was applied as a 100 mL suspension per pot. One day before planting, the PL251 application was repeated as rootstock plantlet drench at 0.2 g/pot. PL251 application was repeated every five weeks for four months with the same concentration of 0.2 g/pot. Velum at the rate of 7.5 mg a.i. fluopyram per pot was solubilized in 50 mL water and applied at plant. Grafted tomato plants with the *Mi* resistant rootstock Maxifort F1 (De Ruiter) were used in both years. The top variety bearing the fruit was Climberly F1 (Syngenta) in 2017 and Tomaranto RZ F1 (72–722; Rijk Zwaan) in 2018. With a drip irrigation system, plants were watered and fertilised with water soluble NPK fertiliser (Kristalon Red Acid, Yara, UK) according to the plant growth stage. Red tomato fruits were harvested weekly, over a period of 14 weeks, quantifying the fruit weight (kg) and fruit numbers.

Root galling was indexed on a scale of 0 – 10 (Zeck, 1971). In 2017

root galling was determined at the end of the growth season. In 2018, 20 root systems, 4 replicates of each treatment, were rated at week 8 (early: 22.06.2018) and week 15 (mid: 10.08.2018) after planting. The last 40 plant root systems were rated at the end of the experiment (end: week 24, 05.10.2018). For each pot, soil nematode population was determined by extracting nematodes from 100 cc of well mixed soil using the Oostenbrink dish technique as described, followed by counting the *M. incognita* J2s under the light microscope.

2.5. Data analysis

The data were tested for homogeneity of variances (Levenes test) using the software SPSS 20. Root gall index data were log10 (x + 1) transformed before analysis. Treatments were separated by one-way ANOVA with post-hoc Tukey honestly significant difference test ($P \leq 0.05$).

3. Results

3.1. Nematicidal effect on Meloidogyne incognita juveniles in soil

The efficacy of *P. lilacinum* (PL251 formulated as BioAct WG) and Velum on *M. incognita* J2s were tested under laboratory soil conditions and recorded after 7, 14, 21 and 28 days (Table 1). Following the company request, PL251 was applied one-week pre plant and Velum was applied at plant giving a gap of 7 days. Table 1 shows that within the first week, the bio control agent PL251 significantly inhibited *M. incognita* J2s (92 extracted J2s) compared to the nematode control (143 extracted J2s). In general, the bare soil experiment showed that *M. incognita* population was significantly inhibited by all treatments. At time point 14, Velum had the strongest, but not significant, J2 reduction (28 J2s) compared to the other treatments. Over the time course of 28 days, PL251 decreased the J2 population slower, compared to Velum and PL251 + Velum treatments. Compared to the untreated soil at 28 days post treatment, the J2 reduction of the PL251 treated soil was 56% and Velum and PL251 + Velum showed the same J2 reduction, of about 68%. Nevertheless, no significant differences were seen between PL251, Velum and PL251 + Velum treatments, but the reduction of the J2 population relative to the untreated control was statistically significant higher.

3.2. Nematicidal effect on Meloidogyne incognita in small pot tomato rootstocks

To evaluate the efficacy of the nematicides on *M. incognita* under host plant conditions, we conducted a tomato pot experiment. *M. incognita* infested soil was treated as described for the bare soil experiment. The gall index, number of egg masses and juveniles were analysed after 6 weeks (Table 2). The tomato root galling of the inoculated control showed an average gall index of 3.8, which was significantly higher compared to plants treated with PL251 + Velum (1.8) and Velum alone (1.6). The PL251 treated plants had a lower gall index of 3.2, but not significant different compared to the control. With an average of 12.4 *M. incognita* egg masses per plant, PL251 + Velum treated soil had the least egg mass production per plant. An average of 20 egg masses were counted on the plants grown in Velum treated soil. 122.6 egg masses were counted on the inoculated control plants and PL251 treatment reduced the number of egg masses to 62.8 (Table 2).

With 94%, the J2 reduction was highest in the tomato roots grown in PL251 + Velum treated soil, closely followed by Velum alone with a J2 reduction of 92%. PL251 reduced the J2 population by 61%. Comparing the soil and the tomato pot experiment, the treatments showed similar performance in controlling *M. incognita*.

Table 2
Nematicidal effect on *Meloidogyne incognita* in small pot tomato rootstocks.

Treatment	Gall index	No. of egg masses per plant	No. of J2s per root system	Average reduction of J2
Inoculated control	3.8 ± 0.42^{a}	122.6 ± 44.31^{a}	43802^{a}	
PL251	3.2 ± 0.79^{a}	62.8 ± 36.17^{b}	16939^{b}	61%
Velum	1.6 ± 0.51^{b}	20.0 ± 17.33^{b}	3566^{b}	92%
PL251 + Velum	1.8 ± 0.79^{b}	12.4 ± 10.19^{b}	2571^{b}	94%

Nematicidal effect of PL251 (formulated as WG, 0.2g/pot), Velum (1 ppm a.i./pot) and the combination of PL251 + Velum on *Meloidogyne incognita* infestation rate on tomato plants grown for 6 weeks in greenhouse pots. Gall index, number of egg masses, juveniles (J2s) and the average J2 reduction were measured per root system. 4000 eggs/juveniles were inoculated at start. Values are means of 5 replicates, different letters in different columns indicate significant difference using one-way ANOVA with post-hoc Tukey HSD test ($P \leq 0.05$). Average reduction of J2 in percent (%) is in relation to the inoculated control plants.

3.3. Large-scale greenhouse trial

To evaluate the findings observed in soil (Tab 1.) and in small pot tomato rootstocks (Tab 2), we performed a large greenhouse experiment. The same treatments were repeated with PL251, Velum and PL251 + Velum under commercial standards in two consecutive years. During the research trial, the greenhouse temperature ranged between a minimum of 11.2 °C in 2017 and 15.7 °C in 2018 and a maximum of 33.0 °C in 2017 and 34.8 °C in 2018. The average greenhouse temperature was 19.6 °C in 2017 and 22.3 °C in 2018. The accumulated temperature were 3661 degree-days in 2017 and in 2018, 3562 degree-days.

In 2017, the root gall index was measured after the growth season (Tab 3). With a root gall index of 4.93, the combination PL251 + Velum treatment significantly reduced the galling compared to the untreated control (6.94). No significant relationships for Velum (5.20) and PL251 (5.77) treated samples were determined compared to the inoculated control. In general, the root gall index was lower in all treatments compared to the inoculated control.

In 2018, root galling was evaluated throughout the tomato growth season. Early root gall index evaluation showed significant reduction of root galling in the PL251 + Velum (1.25) and Velum (2.0) treatments compared to the inoculated control (3.25; Table 3). The PL251 + Velum combination did not differ statistically from the nematode free control.

Table 1
Nematicidal effect on *Meloidogyne incognita* juveniles in soil.

Treatment	*M. incognita* population in bare soil				Average reduction of J2
	7 days	14 days	21 days	28 days	
Inoculated control	143 ± 13.50^{a}	75 ± 12.74^{a}	70 ± 9.35^{a}	66 ± 12.94^{a}	
PL251	92 ± 28.63^{b}	39 ± 12.94^{b}	32 ± 7.58^{b}	29 ± 10.83^{b}	56%
Velum	–	28 ± 17.53^{b}	26 ± 11.93^{b}	21 ± 12.94^{b}	68%
PL251 + Velum	–	32 ± 15.24^{b}	23 ± 15.24^{b}	21 ± 8.21^{b}	68%

The nematicides PL251 (formulated as WG, 0.2g/100 cc soil), Velum (1 ppm a.i./100 cc soil) and the combination of PL251 + Velum were used. 250 *M. incognita* J2s were inoculated into 100 cc soil. Total nematodes were extracted from 100 cc of soil, using the Oostenbrink dish technique. Values are means of N = 6, followed by letters in the same column indicating significant differences if not the same, using one-way ANOVA with post-hoc Tukey HSD test ($P \leq 0.05$).

No significant differences were seen between the PL251 treatment and the inoculated control or between the PL251 and Velum treatments. At the midterm root galling evaluation, Velum with a gall index of 6.25 and PL251 with 6.75 did not show statistical significant differences compared to the inoculated control (7.25). On the other hand, PL251 + Velum significantly differed from the inoculated control, with a lower gall index of 5.75. For the final gall rating in 2018, the differences of the gall index were not significant, ranging between 7.15 for the PL251 treated samples and 8.0 for the positive control.

At the time point of the root gall rating, the *M. incognita* J2 soil population was analysed (Tab 4). After 8, 15 and 24 weeks, the *M. incognita* population increased over time and under all treatments. At the first (initial) time point, all treatments showed a significant reduction of the J2 population in the soil compared to the nematode control (88.3 J2s/100 cc of soil). With 17.1 J2s/100 cc of soil, Velum had the lowest *M. incognita* population followed by PL251 + Velum with 22.6 and PL251 with 47.3 J2s/100 cc of soil. At the second (mid) and third (end) time points, no significant correlation was found. Nevertheless, the treatment combining PL251 + Velum had the lowest nematode population at the second and third time points (Table 4).

Regardless of the treatments, in both years, the nematode free control plants had a higher yield, compared to the nematode infested plants (Table 5). In 2017, the yield potential of marketable tomatoes in nematode infested control plants were stronger affected than in 2018. In 2017, the infested plants reached a yield potential of 78.8% compared to 86.5% of the yield potential in 2018. However, all treatments reduced yield loss in both growth seasons (2017 and 2018). Overall, nematicide treated plants had a higher yield, more tomato fruits per plant, a higher average weight per harvest and consequently a higher total fruit weight per plant, compared to the untreated nematode infested control plants.

In 2017, PL251 + Velum treated plants had the highest total fruit weight per plant (12.0 kg), and differed significantly to the inoculated and the nematode free control plants. PL251 (11.2 kg) and Velum (11.5 kg) had a higher yield per plant, compared to the nematode infested control, but did not differ significantly between each other. In 2017, with 88.3%, PL251 + Velum showed the highest yield potential compared to the single treatments. Velum reaching 84.6% and PL251 reaching 82.5%. The inoculated control reached only 78.8% of the yield compared to the untreated plants.

In 2018, the nematicide treated tomato plants had a higher yield in terms of fruit weight per plant compared to the inoculated control, but no significant differences were observed between the treatments and the controls (Table 5). However, PL251 + Velum treatment had the highest yield potential with 96.7% of the nematode free plants. The same trend was observed following PL251 treatment with 94.1% and Velum treatment with 91.1% of the nematode free control plants, respectively. With 86.5% of yield potential, the inoculated control had the lowest yield of all nematode infested plants in 2018.

Table 3
Summary of the tomato root gall formation on the large-scale greenhouse trial.

Treatment	End 2017	Early 2018	Mid 2018	End
Inoculated control	6.94 ± 0.6^{a}	3.25 ± 0.4^{a}	7.25 ± 0.4^{a}	8.00 ± 0.3^{a}
BioAct	5.77 ± 1.1^{b}	2.75 ± 0.4^{ab}	6.75 ± 0.4^{a}	7.15 ± 0.4^{a}
Velum	5.20 ± 0.7^{bc}	2.00 ± 0^{b}	6.25 ± 0.4^{ab}	7.38 ± 0.9^{a}
Bioact + Velum	4.93 ± 0.9^{c}	1.25 ± 0.4^{c}	5.75 ± 0.4^{b}	7.63 ± 0.6^{a}

A suspension of 5500 *M. incognita* egg/J2s was used for inoculation of each pot. Pots were treated with PL251 (formulated as WG), Velum and PL251 + Velum pre plant. Gall index were measured according to Zeck (1971): 0 = no galls; 10 = dead plant. Averages and standard deviations of N = 16 in 2017. In 2018, N = 4 for early and mid-rating and N = 8 for the end. Mean values of gall index with the same letters denote no significant difference ($P \leq 0.05$) by one-way ANOVA with post-hoc Tukey HSD test.

Table 4
Soil population development of *Meloidogyne incognita* in the large-scale greenhouse trial.

Treatment	J2 population early	J2 population mid	J2 population end
Inoculated control	88.3 ± 41.3^{a}	763.3 ± 59.8^{a}	2026.9 ± 513.7^{a}
PL251	47.3 ± 18.1^{b}	512.0 ± 78.0^{a}	1766.8 ± 666.6^{a}
Velum	17.1 ± 9.7^{b}	495.7 ± 153.5^{a}	1687.1 ± 778.3^{a}
PL251 + Velum	22.6 ± 13.5^{b}	412.0 ± 90.3^{a}	1662.3 ± 321.2^{a}

Meloidogyne incognita juvenile (J2) population extracted from 100 cc soil, developed over 8 (early), 15 (mid) and 24 (end) weeks on tomato roots treated with the nematicides PL251 (formulated as WG), Velum and PL251 + Velum. A suspension of 5500 *M. incognita* egg/J2s per pot was used for inoculation. Values are means of N = 4 (early and mid) and N = 8 (end), different letters in different columns indicating significant difference, using one-way ANOVA with post-hoc Tukey HSD test ($P \leq 0.05$).

4. Discussion

This study demonstrated the potential of combining chemical and biological nematicides to control *M. incognita* in tomato cultivars.

In general, combining different nematode management practices are a good option to prevent disease outbreaks and secure yield. Grafted tomato plants using nematode resistant rootstocks are used successfully to control *Meloidogyne* spp. (Kaskavalci et al., 2009), but *Mi*-gene nematode resistant cultivars are not always effective to control *M. incognita* as shown in our experiment.

Furthermore, the nematicide Velum can only be applied pre-plant and up to 6 weeks post planting, whereas the nematode antagonist *P. lilacinum* (BioAct) can be applied before planting and during the entire crop season.

The two nematicides used in our experiment, BioAct (PL251) and Velum controlled *M. incognita* as shown by the different experiments, evaluation of the soil population, the tomato root gall formation and number of egg masses developed per plant (Tables 1–4).

The chemical nematicide Velum protected the plants from *M. incognita* by reducing the soil nematode population at planting (Tables 1–4) and protecting the plantlets against the initial penetration and significant root damage. *In vitro* experiments showed that a dose, as low as, 1.0 μg/mL fluopyram was able to paralyse *M. incognita* juveniles exposed to it for 2 h and protected tomato roots from *M. incognita* infestation (Faske and Hurd, 2015). Generally, the nematicidal effect of fluopyram has been described for multiple nematodes (Beeman and Tylka, 2018; Faske and Hurd, 2015; Kim et al., 2016; Mathew et al., 2016; Roper, 2017). However, over the tomato season, the controlling effect of a single Velum application was reduced, and at the end of the 2018 trial, the nematodes recovered to a level that no significant differences were seen when compared to the non treated inoculated control (evaluating the J2 soil population or the root gall index) (Tables 3 and 4). Based on the bare soil experiment, we assume that Velum has an effect within the first days. Seven days after the Velum application, the live nematode number decreased not stronger than the positive control (Table 1).

It is worthwhile mentioning that residual research on fluopyram, the active ingredient of Velum, showed moderate mobility in soil, and the residual level in cucumber fruits increased up to 20 days at an application dose of 0.056 mg/kg (Chawla et al., 2018). Subsequently, based on a daily intake, the dietary risk of the residual levels was assessed and a 15 days pre-harvest interval was suggested.

The fungal egg parasite, *P. lilacinum* controlled the *M. incognita* population in a less extend than the chemical treatment, Velum. Under the bare soil and small pot experiment, BioAct had a significant impact on *M. incognita* and reduced the J2 population by 56% or more (Tables 1 and 2). The J2 suppression in the soil is still remarkable, as *P. lilacinum* is primarily known as nematode egg parasite and only little research has

Table 5
Yield of tomatoes on *Meloidogyne incognita* infested plants after treatment with nematicie under the large-scale greenhouse trial.

Growth season	Treatments	Average weight g/ harvest	Average tomato fruit per harvest	Average fruit weight (g)	Total fruit weight per plant (kg)	Yield potential %
2017	Control	971.2 ± 546.2	13.9 ± 8.5	71.4 ± 13.8	13.6 ± 0.9[a]	100.0
	Inoculated control	765.5 ± 198.5	12.5 ± 4.0	63.7 ± 12.6	10.7 ± 0.6[c]	78.8
	PL251	801.2 ± 209.2	13.0 ± 5.8	67.8 ± 12.9	11.2 ± 0.7[bc]	82.5
	Velum	821.9 ± 230.1	12.8 ± 5.0	66.4 ± 11.6	11.5 ± 0.9[bc]	84.6
	PL251 + Velum	857.6 ± 270.7	12.6 ± 5.1	70.7 ± 12.5	12.0 ± 0.9[b]	88.3
2018	Control	1099.1 ± 605.2	13.1 ± 9.0	80.8 ± 16.3	14.2 ± 1.1[a]	100
	Inoculated control	993.4 ± 639.7	12.4 ± 9.8	76.1 ± 17.3	12.4 ± 1.1[b]	86.5
	PL251	1019.0 ± 649.2	12.6 ± 9.3	75.8 ± 13.0	13.3 ± 1.0[ab]	94.1
	Velum	1022.3 ± 667.5	12.5 ± 9.9	77.6 ± 16.5	13.0 ± 0.6[ab]	91.1
	PL251 + Velum	1057.3 ± 649.0	13.1 ± 9.7	76.8 ± 15.3	13.7 ± 0.5[ab]	96.7

Values are means of N = 16 (2017) N = 8 (2018), different letters in different columns indicating significant difference, using one-way ANOVA with post-hoc Tukey HSD test ($P \leq 0.05$). Yield potential in percent (%) is in relation to the nematode free tomato control plants.

been done on its ability to control *M. incognita* juveniles in the soil. During the 2017 greenhouse trial, the gall index of the PL251 treated plants were significantly lower than the inoculated control (Table 3). The reduced *M. incognita* control with PL251 compared to Velum might be due to its slower controlling "mechanism". Under *in vitro* conditions, *P. lilacinum* lipases, proteases and chitinases are used to colonize nematode eggs (Gine and Sorribas, 2017). We hypothesize that the effect of PL251 is limited by the increase of the *M. incognita* population, due to the fungal potential of parasitize the eggs. A similar finding showed that the fungus *Verticillium chlamydosporium* parasitizing *M. incognita* eggs was less effective, when the nematode population increased (De Leij et al., 1992). The reduced nematode control by *V. chlamydosporium* was attributed to the ability of reaching a limited number of nematode egg masses. A relationship study with *P. lilacinum* infecting *M. arenaria* on tomato showed that under optimal colonisation of egg masses, only 50% of the eggs were parasitized (Carneiro and Cayrol, 1991). These previous reports are supported by the pot experiment shown in Table 2, where the PL251 treatment had an average J2 reduction of 61%, compared to Velum that reached a higher reduction of 92%. Meaning that, not all the eggs were parasitized by *P. lilacinum*, leaving a high number of *M. incognita* inoculum for the following reproductive cycle. Therefore, a too high nematode pressure at planting might be an explanation if PL251 does not control *Meloidogyne* spp. as successful as previously described for *in vitro* and pot experiments, or in greenhouse and field trials (Gine and Sorribas, 2017; Kiewnick and Sikora, 2006a, 2006b). Besides egg parasitism, *P. lilacinum* could also reduce J2s in the soil as shown in Table 1. The effect on J2s was lower, since the *P. lilacinum* treatment needed 28 days to have the same effect on J2s as Velum showed after 7 days. However, that effect was substantial because *P. lilacinum* might have an effect as longer as the juveniles are exposed to the soil and suffer starvation.

The slower controlling effect of PL251 needs to be considered, since the nematode population has the possibility to infest and reproduce successfully on host plants if available. Therefore, by combining Velum and PL251, we demonstrated that *P. lilacinum* is more effective when nematodes are controlled pre-plant, starting the vegetable season with a lower nematode population. The combined results of the two nematicides were stronger at the large greenhouse trial, where multiple *M. incognita* populations developed compared to the small-scale experiments. Generally, root galling and nematode populations in the soil were reduced and the yield was increased in the consecutive growth seasons, 2017 and 2018.

In 2017, the root galling caused by *M. incognita* was significantly reduced by all treatments, with PL251 + Velum treated plants showing the lowest root gall index. During the greenhouse experiment in 2018, the combined treatment significantly controlled the *M. incognita* population, up to the second third of the trial (Table 3). At the end of 2018, no significant differences were seen between the root galling and the juvenile soil population of the infected plants.

To estimate the developed nematode populations for each consecutive year, the thermal requirement for *M. incognita* of 10.8 °C minimum and 400 degrees-days for the completion of one life cycle (Ploeg and Maris, 1999; Vrain et al., 1978), were reconciled with the accumulated temperature of 3661 degree-days in 2017 and 3562 degree-days in 2018.

According to the thermal requirement, we estimate that up to 9 *M. incognita* generations could have developed during each trial.

Comparing the gall index of 2017 with 2018, the intermediate gall rating of the 2018 roots reached a gall index of 7.25 for the positive control, comparable as the gall index of 6.94 observed at the end of the 2017 growth season. Generally, the gall index of 2018 indicates a better development of the nematode population, since the inoculation pressure of 5500 *M. incognita* eggs/J2s was the same in 2017 and 2018 growth seasons. One slight difference was that in 2018, 10% of J2s were present in the inoculum and only 1% of J2s were present in the 2017 inoculum. Therefore, more nematode eggs could have been parasitized by PL251 and therefore less J2s might have infested the plants at the start of the season 2017. This would further support the idea that PL251 is more effective against a lower *Meloidogyne* spp. population. However, no nematode measures were taken during 2017 growth season to compare the *M. incognita* development. For this reason, we cannot conclude whether the J2s in 2018 had a higher infestation rate than the inoculum in 2017 and therefore caused the differences in root galling.

The yield in both years was higher for the PL251 + Velum treated plants compared to the PL251 or Velum treated plants and the inoculated control. In 2017, the fruit weight per plant was significantly higher for the PL251 + Velum treated plants than the infested control, whereas in 2018, the PL251 + Velum treated plants had the highest yield, but did not differ significantly from the inoculated or nematode free plants. With a total fruit weight of 12.0 kg/plant in 2017, the yield was lower compared to 2018, reaching 13.7 kg of fruit weight/plant for the PL251 + Velum treated plants. The major differences in yield between the two consecutive years are due to the tomato cultivars used, Climberly F1 (Syngenta) in 2017 and Tomaranto RZ F1 (72–722; Rijk Zwaan) in 2018. Additionally, the climatic differences with an average temperature of 19.6 °C in 2017and 22.3 °C in 2018 might have had an effect on the tomato yield as well.

Therefore, the limiting controlling effect over time needs to be considered when growing tomatoes and other crops. The crop rotation and nematicide imput needs to be evaluated in a holistic management perspective. Since application cost and the suggested value of the marketable products will influence profit. We had a yield increase of 9.5% in 2017 and 10.2% in 2018 by using the combined treatment compared to the inoculated control. Comparing the combined nematicide treatment with the single nematicide treatments, the yield

difference of 3.7% in 2017 and 2.7% in 2018 is low. Whether this yield increase by a single or combined treatment is lucrative depends on the production system, the applied products and the marketable price of the tomato cultivars. A further aspect to consider is the growth period and the crop rotation. During the early and mid-season, the nematode population was significantly suppressed and the gall index was lower, using the combined treatments. Therefore, an earlier second crop could benefit from the combined treatment with PL251 + Velum.

5. Conclusion

This study revealed that the combination of a chemical treatment to downregulate the *M. incognita* population followed by the application of a fungal antagonist is more successful to control these nematodes compared to each treatment alone. The integrated chemical and biological strategies could become an important component to manage *Meloidogyne* spp. and other plant parasitic nematodes in the future. Velum and PL251 should be seen as a potential nematicide option, but other combinations to control plant-parasitic nematodes should be tested and used as integrated method rather than as a onetime solution. Besides the nematode controlling capacity, the profit of the additional application should be considered and essential to be evaluated for the particular market.

In regards to the chemicals and their residue effects as reported for fluopyram, pre-harvest losses could be overcome by alternative biological or chemical treatments to downregulate *Meloidogyne* spp. population at plant to give PL251 or a different organism the potential to suppress the nematode population during the entire growth period.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2019.104874.

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