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Fluopyram: Optimal Application Time Point and Planting Hole Treatment to Control *Meloidogyne incognita*

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Abstract: Research into new nematicides that provide adequate control against root-knot nematodes in a more environment-friendly way is of great interest to vegetable growers. Thus, the effect of fluopyram, a relatively new chemical nematicide, was evaluated against a Swiss population of *Meloidogyne incognita* in vitro, in soil and as a planting hole treatment for tomato, lettuce and cucumber plants. Fluopyram treatment in vitro revealed LC50 (lethal concentration, 50%) ranging from 2.15–0.04 µmol of fluopyram/L after 1–14 days of exposure. However, some nematodes (visually categorized as dead) were able to recover and infect cucumber plants. Fluopyram's optimal application time appeared to be up to 1 day after planting, with a significant control effect on *M. incognita* up to 14 days after planting. A root penetration assay showed that only nematodes that remained in the rhizosphere were controlled by fluopyram. Furthermore, fluopyram planting hole treatments on lettuce, tomato and cucumber plants, successfully controlled *M. incognita* in the root zone under greenhouse conditions. Overall, this study contributes to an optimized application of fluopyram for the control of *M. incognita* in vegetable crops, highlighting its effectiveness in soil and showing its limitation to control juveniles that have already invaded the root systems of plants.

Keywords: fluopyram; application time; planting hole treatment; *Meloidogyne incognita*

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1. Introduction

The obligate plant parasitic root-knot nematodes (RKN) of the genus *Meloidogyne* are devastating sedentary endoparasites, causing significant crop losses globally [1,2]. In their life cycle, second-stage juveniles (J2s) enter the root tip near the elongation zone and migrate intercellularly to establish a permanent feeding site by inducing the formation of giant cells in the vascular cylinder. During the development into the adult female that lays eggs in a gelatinous matrix outside the root system, they drain the nutrients from the plant and cause root galling. RKN infection can cause reduced plant growth, stunting and leaf discoloration, to total crop loss [1,2]. Within RKN, *Meloidogyne incognita* is one of the most common RKN in agriculture [3,4], and as the common name "southern root-nematode" indicates, *M. incognita* is distributed in warmer climates. However, this nematode can cause significant damage in greenhouses in Switzerland, and it has been found in several locations across the Swiss territory. In tropical and subtropical areas, *M. incognita* is assumed to be the most widely distributed and economically important plant parasitic nematode (PPN), infecting over 200 genera of plants [3,5].

The control of RKN can be diverse and depends on the species present. Nematode species and race-specific resistant cultivars, physical methods and cropping-based management, as well as biological and chemical control, are commonly used. Soil fumigation and other synthetic chemical nematicides have often been used as fast "reliable" means to control RKN [6]. Whilst, due to human health safety and environmental concerns, most chemical nematicides are no longer authorized, or they are strictly regulated in Europe and most of other countries worldwide [6–8], chemically synthesized nematicides still account

for 48% of the means used for RKN management globally [9]. Therefore, much attention has been drawn to new non-fumigant nematicides developed in recent years that have a lower risk of causing environmental and health hazards than older nematicides [6,8,10]. One of the more recent synthetic nematicides is fluopyram, initially discovered and registered by Bayer as a fungicide [11] and later discovered as a powerful nematicide by Nihon Nohyaku [12]. This broad-spectrum nematicide belongs to the pyridinyl-ethylbenzamide class, produced by Bayer Crop Science and marketed in Europe under the Velum label. Its mode of action (MOA) belongs to the class of succinate dehydrogenase inhibitors (SDHI), sub-grouped under a new subclass of complex II respiration inhibitors in fungi [13]. The MOA is likely to be the same in nematodes, as *Caenorhabditis elegans* succinate dehydrogenase knock-down lineages had a 2.6-fold reduced sensitivity to fluopyram compared to the wild-type lineage [14]. Although fluopyram is an effective and fast-acting nematicide, it has low water solubility, and its half-life in soil is up to 746 days, which is reported to be relatively long compared to other new nematicides [15,16].

Therefore, in this study, the impact of fluopyram on *M. incognita* second-stage juveniles (J2s) from a Swiss population was investigated in vitro, in the soil and in the plant root systems at different application time points, in addition to its use as a planting hole treatment to protect young root systems from *M. incognita*-infected soil.

2. Materials and Methods

2.1. Preparation of Nematode Inoculum and Chemical Nematicide

Meloidogyne incognita (isolate Reichenau 2 [17]) were reared on tomato (*Solanum lycopersicum*) cv. Oskar under greenhouse conditions (25 °C/19 °C, 60% humidity, 15/9 h day/night cycle). Freshly hatched second-stage juveniles (J2s) were extracted from heavily galled root systems, which were washed free of soil and placed over filter paper on glass funnels with a collection tube under a mist chamber at 22 °C [18]. Hatched J2s were collected daily from the collection tubes and stored in the fridge at 6 °C until use.

Velum Prime 400 SC (supplied by Bayer CropScience) containing the active ingredient (a.i.) fluopyram at 400 g a.i./L was solubilized in water according to the manufacturer's instructions.

2.2. In Vitro Effect of Fluopyram on Meloidogyne incognita

The nematicidal effect of fluopyram on *M. incognita* J2s was evaluated in an in vitro aqueous assay. The experiment was conducted in 250 mL Erlenmeyer flasks filled with 120 mL of a nematode suspension (130 J2s/mL) at 20 °C in the dark. Fluopyram was added to each nematode suspension to reach final concentrations of 0.25, 0.5, 1.0, 2.0, 4.0 or 8.0 µmol/L of fluopyram, and an aqueous suspension of nematodes was used as a control. The activity of 100 J2s was monitored under an optical light microscope (40× magnification) after 1, 2, 3 and 14 days for each treatment. Aliquots of the nematode suspension (1 mL; n = 3) were evaluated according to J2s motility, normal J2 sinusoidal motion (normal), strongly affected (affected showing twisted or coiled but still with slight motility) and immotile elongated J2 (immotile or dead). In addition to the visual J2s categorization, a biotest was done in order to allow the assessment of the nematode infectivity capacity. From each treatment (fluopyram concentration and time point), 250 J2s were applied to a small pot (30 cc), containing a pre-germinated cucumber (*Cucumis sativus* cv. Landgurken, Bigler Samen) seedling (n = 6). The inoculated cucumber seedlings were grown at 23 °C ± 2 °C, 16:8 day:night photoperiod and 60% relative humidity. After 28 days, cucumber roots were washed free of soil, and the root galling index (GI) was graded according to Zeck's scale of root-knot infection [19], where 0 refers to no root gall and 10 to a 100% galled root.

2.3. Optimal Time Point of Fluopyram Application

Different application time points of fluopyram were tested to control *M. incognita* using the tomato cultivar Moneymaker. The greenhouse trial was conducted in pots (ϕ = 13 cm) filled with 650 g (500 cc) of soil:silver sand mix (1:3, *v*/*v*) and inoculated with

5000 J2s/pot (12 days before planting). Fluopyram was applied at a rate of 1.12 µg/pot, 5 days or 1 day pre-planting (dpp) or 1, 2, 3, 6, 10, 14, 21 or 28 days after planting (dap) two-week-old tomato seedlings (Figure 1). Each treatment, including the untreated control, was replicated six times. The gall index was determined according to Zeck's scale of root-knot infection [19].

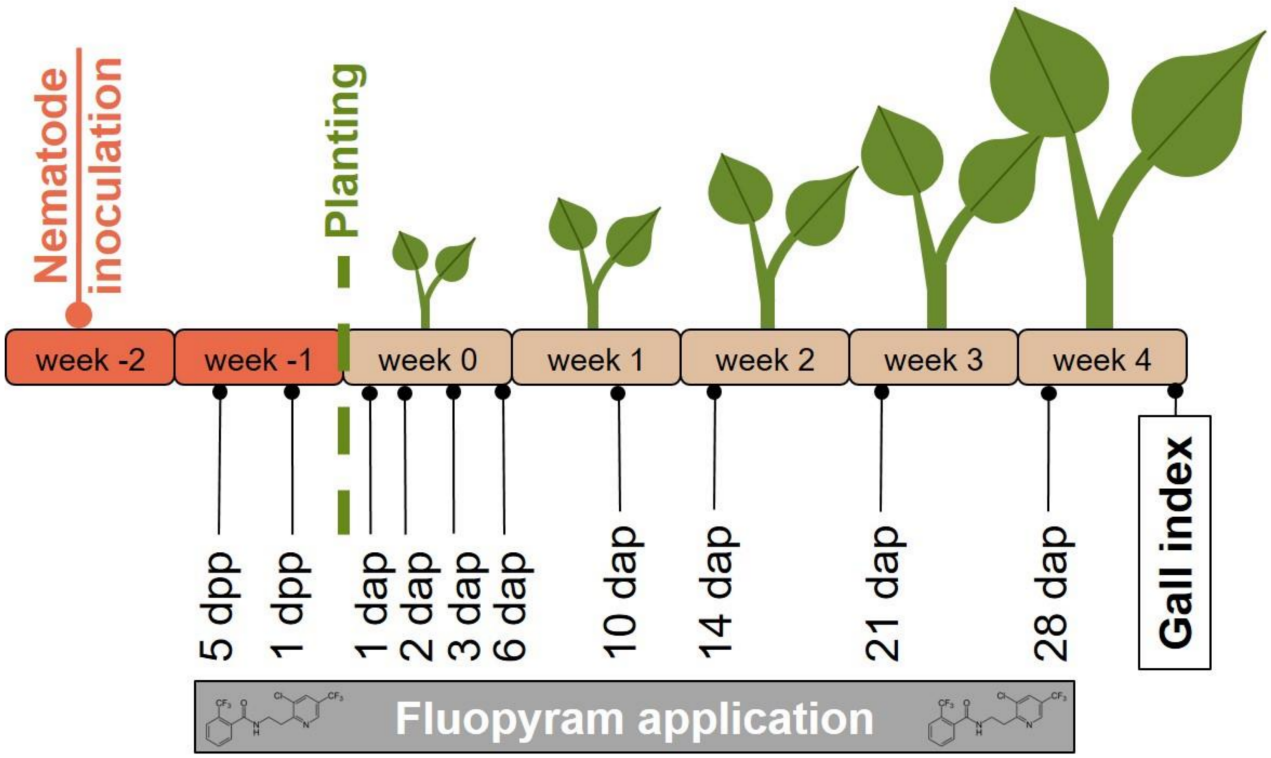

Figure 1. Schematic representation of fluopyram application at different time points to assess the control of *Meloidogyne incognita* in soil and plant roots.

Penetration Assay

Germinated seven-day-old seedlings (12 days from sowing) in 50 cc pots filed with 65 g of soil:silver sand mix (1:3, *v*/*v*) were inoculated with 150 J2s per pot, grown in a climate chamber at 24 °C and 60% relative humidity with a 16/8 h light/dark cycle. Velum was applied at a concentration of 0.15 µg per pot (n = 14), 0, 1, 2, 3, 6 and 10 days after J2s inoculation. One day after each treatment, the number of J2s that penetrated the root system was evaluated by staining nematodes in the root systems using an acid fuchsin stain solution [20]. Stained nematodes in the root systems were counted under the light microscope (40×) and compared to the untreated nematode control (n = 7). Seven plants from each treatment remained for 28 days in the growth chamber for GI determination according to Zeck's scale of root-knot infection [19].

2.4. Application of Fluopyram as Planting Hole Treatment

Fluopyram application was tested in four independent large-scale greenhouse trials, using 17 cm × 56 cm × 36 cm (h × l × w) trays filled with 40 kg of soil naturally infected with *M. incognita*. A tomato (cv. Moneymaker) trial, where soil was infected with 250 J2s/100 cc of soil (n = 8 plants); a trial with lettuce (*Lactuca sativa* cv. Crispa; n = 12 plants) and two trials with cucumber (cv. Landgurken; n = 12 plants, for each of the trials), where soil was infected with 500 J2s/100 cc of soil, were conducted. For the cucumber trials, the assessment of the impact of nematode-infected roots from the previous culture were included. The roots were manually removed and cut into 4–5 cm pieces; 266 g of roots/tray were reapplied to the soil and compared with the infected soil, the untreated and soil treated with fluopyram.

Planting holes were made by manually removing a sufficient amount of soil to fit the root ball, followed by the application of 7.5 mg of fluopyram diluted in 50 mL of water. Control planting holes were treated with 50 mL of water. Six weeks after planting, cucumber height and lettuce weight were measured, and root galls were rated for all cultivars as described above. Nematodes from the tomato trial were extracted from three aliquots of soil (100 cc) using the Oostenbrink dish technique [21], and the J2s were counted under the light microscope (40×).

2.5. Data Analysis

LC50 (Lethal concentration, 50%) was determined using the probit analysis with log-transformed data according to Finney [22].

Statistical differences between multiple treatments were determined by one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) post hoc test ($p \leq 0.05$) of log-transformed data. The mean, standard errors and standard errors of mean of root-gall index, abundance of nematodes, and weight and height of indicator plants were visualized using the software R (version 4.1.2; 2021) with the package ggplot2 [23].

3. Results and Discussion

3.1. In Vitro Effect of Fluopyram on Meloidogyne incognita

Increasing fluopyram concentration showed a clear toxic effect on *M. incognita* J2s in an aqueous solution, which is in line with previous reports by Faske and Hurd [24], which resulted in a LC50 of 5.18 µg/mL after 2 h of exposure. In our experiment, the LC50 calculation further revealed a time-dependent effect of fluopyram on J2s (Table 1) with LC50s of 2.15 µmol/L (0.85 µg/mL) and 0.04 µmol/L (0.016 µg/mL) after one day and 14 days of exposure time, respectively. However, the LC50 already decreased within the first 3 days to 0.05 µmol/L (0.02 µg/mL). Interestingly, despite the visual observation that 100% of J2s were affected or immotile (elongated) at 8 µmol/L of fluopyram after 14 days of exposure, some nematodes were still able to successfully infect cucumber roots, causing root galling (Table 1). In addition, the LC50 calculation of the visual determination did not correspond with a significant reduction in root galling, as seen for the statistical analysis of the gall index in Table 1. Faske and Hurd reported a similar recovery [24] after 2 h of exposure to fluopyram at concentrations ranging from 1.3 to 5.2 µg/mL. However, even though some J2s could recover from exposure to fluopyram, root galling was significantly reduced compared to the water control. This could be due to an effect on the nematodes chemotaxis, affecting their capability to localize the root tips, and/or to the fact that the nematodes were too weak to establish a suitable feeding site in the root system.

Table 1. In vitro effect of different concentrations of fluopyram on *Meloidogyne incognita* second-stage juveniles (J2s) after 1, 2, 3 and 14 days of exposure and recovery assay using *Cucumis sativus* root systems as bio indicator.

Exposure Time	Concentration [µmol/L]	N [%]	A [%]	I [%]	LC50 [µmol/L]	Gall Index
Day 1	Control	95.1	1.9	3.0	2.15 (1.281–3.610)	7.17 ± 0.69^{a}
	0.25	81.1	13.2	5.7		7.33 ± 0.75^{a}
	0.5	83.2	11.9	5.0		7.17 ± 0.69^{a}
	1.0	74.5	18.1	7.4		6.33 ± 0.47^{abc}
	2.0	59.7	28.0	12.3		5.83 ± 0.37^{bcd}
	4.0	28.2	39.2	32.6		5.83 ± 0.37^{cd}
	8.0	6.1	35.3	58.6		4.83 ± 0.69^{d}

Table 1. *Cont.*

Exposure Time	Concentration [µmol/L]	N [%]	A [%]	I [%]	LC50 [µmol/L]	Gall Index
Day 2	Control	95.0	2.0	3.0	0.45 (0.242–0.836)	7.00 ± 0.58 ^{a}
	0.25	58.5	36.2	5.3		6.83 ± 0.69 ^{a}
	0.5	53.1	42.4	4.5		7.17 ± 0.69 ^{a}
	1.0	18.3	74.7	6.9		7.17 ± 0.69 ^{a}
	2.0	9.2	80.1	10.7		6.25 ± 0.43 ^{ab}
	4.0	4.9	70.4	24.6		6.33 ± 0.75 ^{a}
	8.0	0.8	27.5	71.7		4.83 ± 0.90 ^{b}
Day 3	Control	94.9	2.5	2.7	0.05 (0.016–0.159)	7.00 ± 0.58 ^{a}
	0.25	27.0	66.7	6.3		7.00 ± 0.58 ^{a}
	0.5	18.9	75.4	5.7		7.00 ± 0.82 ^{a}
	1.0	4.5	85.2	10.3		6.83 ± 0.69 ^{a}
	2.0	1.7	87.0	11.3		6.60 ± 0.49 ^{a}
	4.0	0.4	70.7	28.9		6.67 ± 0.47 ^{a}
	8.0	0.0	31.8	68.2		4.83 ± 0.69 ^{b}
Day 14	Control	83.5	7.9	8.6	0.04 (0.024–0.080)	5.83 ± 1.07 ^{a}
	0.25	6.0	66.9	27.1		5.80 ± 1.47 ^{a}
	0.5	1.5	76.9	21.6		5.50 ± 1.38 ^{a}
	1.0	0.7	79.1	20.2		5.17 ± 1.07 ^{ab}
	2.0	0.0	80.5	19.5		6.50 ± 0.50 ^{a}
	4.0	0.0	48.3	51.7		5.33 ± 0.75 ^{ac}
	8.0	0.0	23.6	76.4		3.33 ± 0.75 ^{bc}

Normal (N) sinusoidal motion juveniles, affected (A) and immotile (I) elongated second-stage juveniles (J2s) are displayed in percentages (%) (n = 3). Log-transformed data of A and I were pooled for LC50 (Lethal concentration, 50%) calculation. Fluopyram-exposed J2s were inoculated on pregerminated *Cucumis sativus* cv. Landgurken seedlings, and 28 days later, the root-gall index was determined according to Zeck's scale of root-knot infection [19] (n = 6). Significant differences within the same column are indicated by different superscript letters, calculated using a one-way ANOVA with post-hoc Tukey HSD test ($p < 0.05$).

3.2. Optimal Time Point for Fluopyram Application

As usually, fluopyram is applied before planting. The goal was to verify the optimal application time point before planting and evaluate how long after planting fluopyram could be applied with sufficient protection against *M. incognita*. In the experimental setup, the strongest control effect was before planting up to one day after planting. Roots treated with fluopyram two days after planting already had root galls caused by surviving *M. incognita*. With increasing delay of fluopyram application, galling gradually increased until there were no significant differences from the control on day 14 (Figure 2).

Penetration Assay

As fluopyram is reported to be systemically active and moves through the plant acropetally (Bayer CropScience,), and as already described that a minimum concentration of 16 mg/L of fluopyram is required for a significant reduction of sedentary *Meloidogyne javanica* in tomato roots, there may be a systemic effect [25]. However, a similar experiment was done using a smaller soil volume, and it studied nematode infection in the root system by staining the nematodes in infected roots over time (Figure 3 A,B).

Based on the results of root penetration and root-gall index, it was concluded that in the experimental setup, nematodes were only affected when in the soil by fluopyram, ruling out a potential systemic effect, when 1.12 mg/L of fluopyram was applied. In the root penetration test, we noted a difference based on the time delay of nematode staining in the roots one day after fluopyram application. During this day, untreated nematodes still infected the control plants, supporting our conclusion that only nematodes in the soil were affected by fluopyram (Figure 3B).

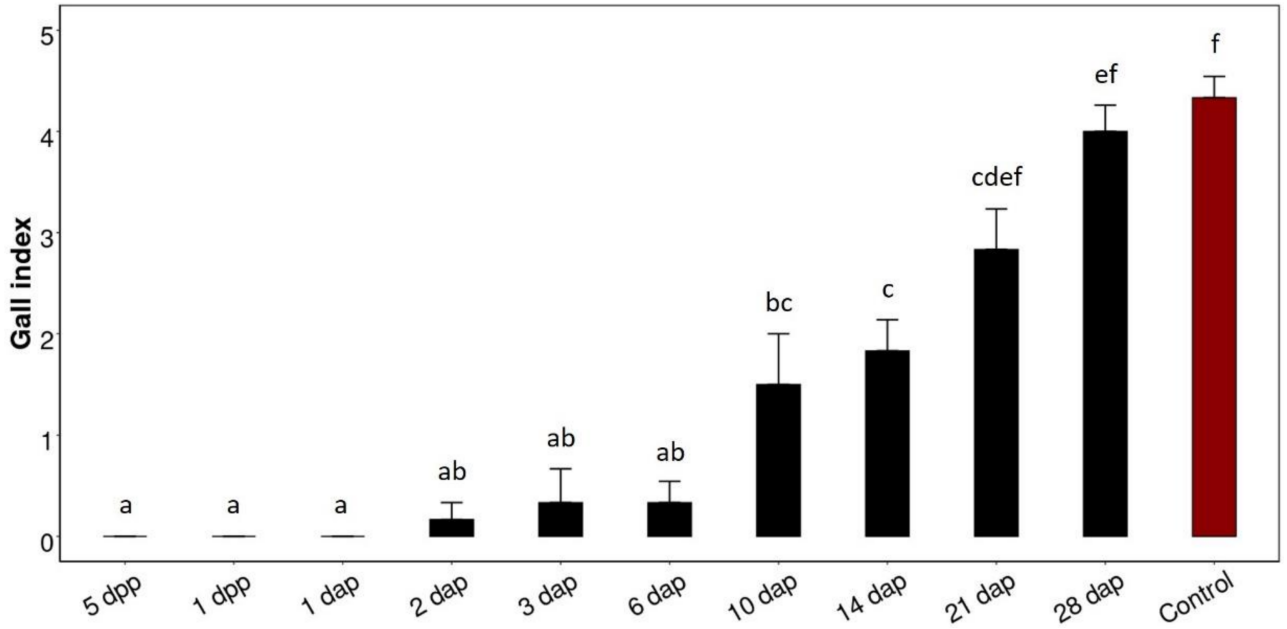

Figure 2. Root-gall rating of tomato roots grown in *Meloidogyne incognita* second-stage juvenile infected soil, treated with fluopyram at different time points. Pots were inoculated with 5000 J2s/pot and treated with fluopyram 5 days or 1 day pre-planting (dpp) or 1, 2, 3, 6, 10, 14, 21 or 28 days after planting (dap). The gall index was recorded according to Zeck's scale of root-knot infection [19] (n = 6). Different letters indicate significant differences compared to the control according to a one-way ANOVA with post-hoc Tukey HSD test ($p \leq 0.05$).

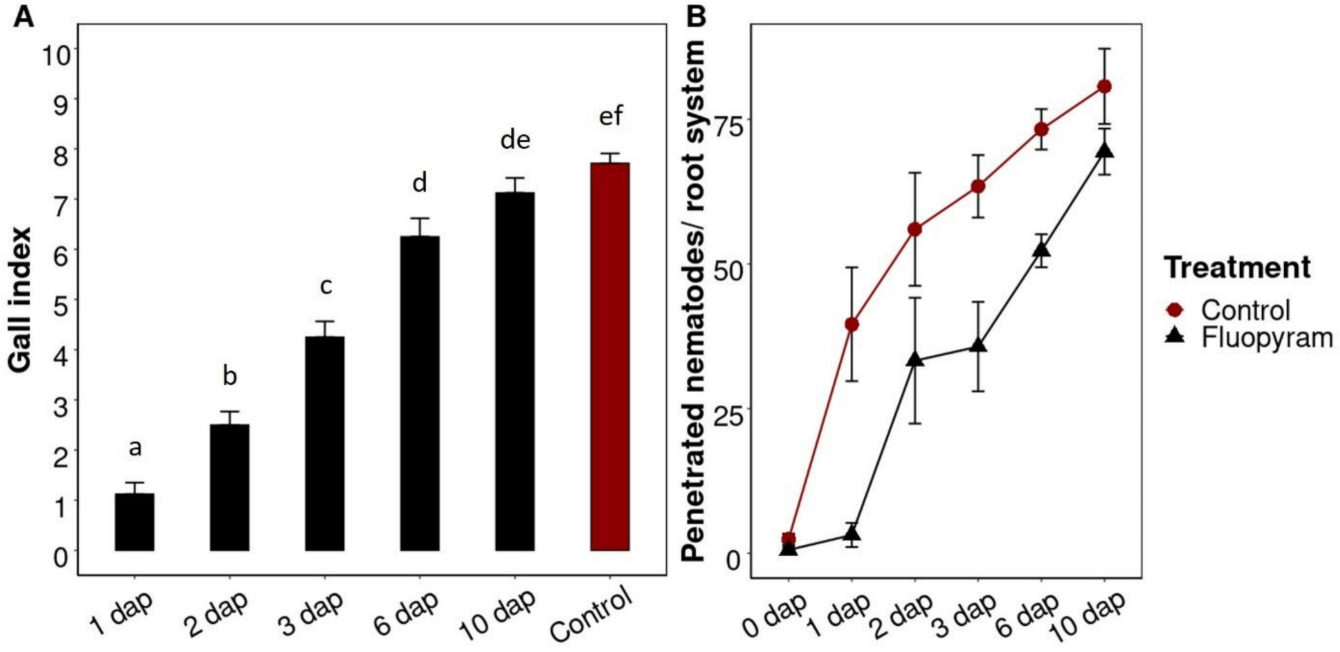

Figure 3. *Meloidogyne incognita* second-stage juveniles controlling effect of fluopyram at different application time points, 0, 1, 2, 3, 6 or 10 days after planting (dpa). (**A**) The root gall-index was rated according to Zeck's scale of root-knot infection [19]. (**B**) The number of *M. incognita* J2s that successfully penetrated tomato roots one day after each fluopyram treatment (n = 7). Initial *M. incognita* suspension of 150 J2s/plant. Different letters over the bars indicate significant differences compared to the control according to a one-way ANOVA with post-hoc Tukey HSD test ($p \leq 0.05$).

However, as only limited studies have investigated whether RKN development can be inhibited by fluopyram application after planting and whether soil type and its absorption/adsorption plays a critical role in availability [15], additional research is needed to study potential minor effects on population changes due to the systemic effect of fluopyram on the plant.

3.3. Application of Fluopyram as Planting Hole Treatment

As fluopyram is reported to be retained mainly in the top layers of different soils (0–10 cm) based on its low water solubility and high adsorption to soil particles [13,16], on-site applications of fluopyram in planting holes were further investigated.

The planting hole study showed promising results for all three crops tested: tomato, lettuce and cucumbers (Figures 4 and 5 and Table 2). Tomato grown in planting holes treated with fluopyram had a significantly reduced root-gall index of 3.00 compared to roots grown in untreated planting holes (GI of 5.40; Figure 4A). In addition, the reduction in root galling resulted in an overall decrease of J2s in the soil (Figure 4B). The reduction of J2s in the soil due to the application of fluopyram might support the establishment of the following crops, as the nematode population would not be as high as in the untreated soil.

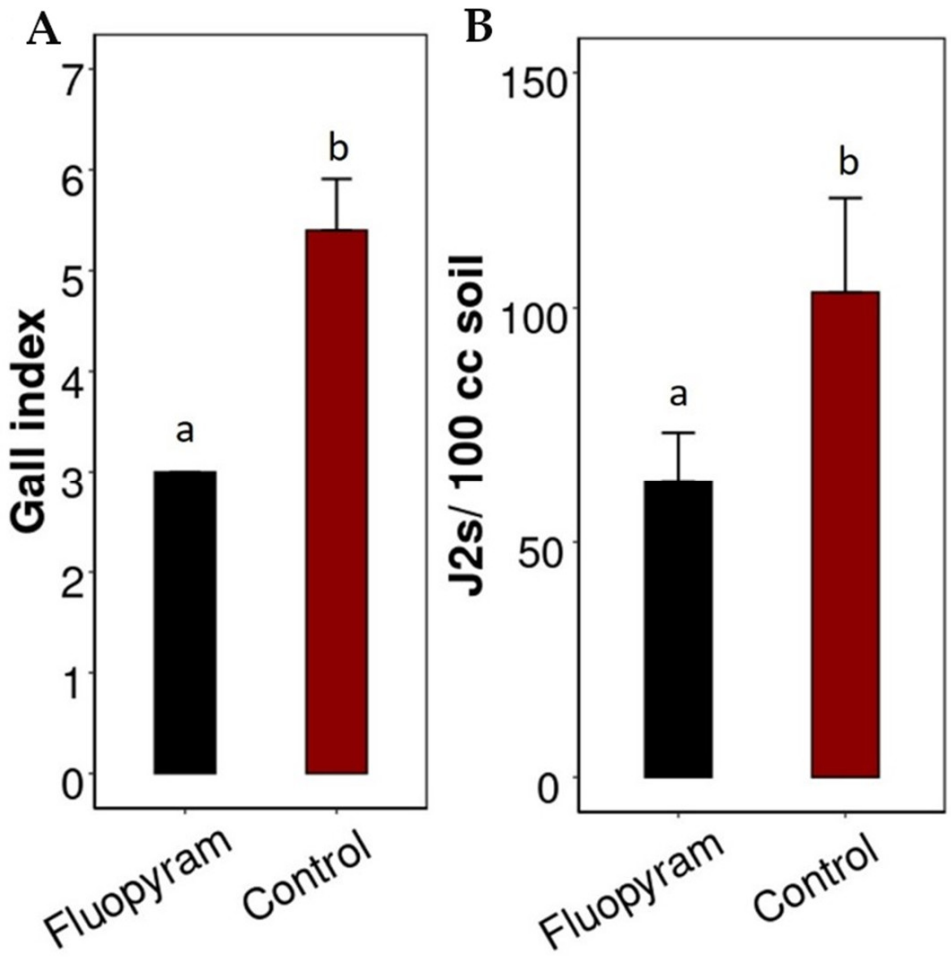

Figure 4. The effect of planting hole treatment with fluopyram on tomato root-gall rating (**A**) and *Meloidogyne incognita* second-stage juveniles in the soil (soil population) (**B**). Tomato plants were planted in greenhouse soil infected with *M. incognita* (250 J2s/100 cc of soil). Root galling was indexed according to Zeck's scale of root-knot infection [19], with 0 = no root galls and 10 = severe galled-up roots (n = 8). Different letters indicate significant differences compared to the control, according to a one-way ANOVA with post-hoc Tukey HSD test ($p \leq 0.05$).

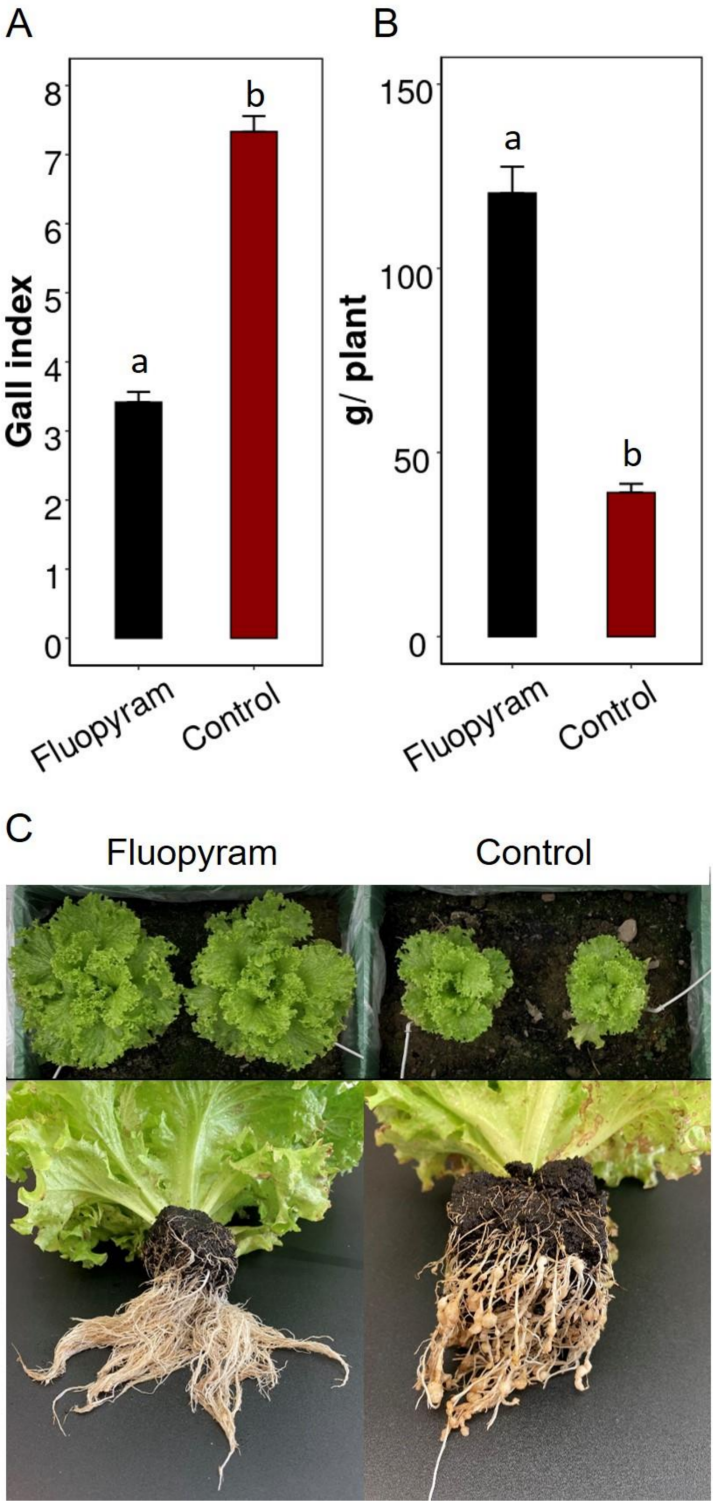

Figure 5. Effect of planting hole treatment with fluopyram on lettuce root-gall rating (**A**) and lettuce weight (**B**) grown in soil infected with *Meloidogyne incognita* (500 J2s/100 cc of soil). Representative photos (**C**) show the differences between lettuce treated with fluopyram and untreated (top) and lettuce roots (bottom). Root galling was indexed according to Zeck's scale of root-knot infection [19], with 0 = no root galls and 10 = severe galled-up root (n = 12). Different letters indicate significant differences compared to the control according to a one-way ANOVA with post-hoc Tukey HSD test ($p \leq 0.05$).

Table 2. Effect of planting hole treatment with fluopyram on cucumber root galling and plant growth in *Meloidogyne incognita*-infected greenhouse soil, where *M. incognita*-infected root systems of previous crops were kept or removed.

Treatment	Cucumber Height [cm]		Gall Index	
	Trial 1	Trial 2	Trial 1	Trial 2
Soil	138.08 ± 51.32 [a]	93.93 ± 20.95 [a]	8.08 ± 0.75 [a]	7.14 ± 0.64 [a]
Soil + fluopyram		124.71 ± 15.34 [b]		4.79 ± 0.56 [b]
Soil + roots	95.92 ± 73.87 [a]	75.50 ± 23.98 [ac]	8.75 ± 1.01 [a]	7.14 ± 0.52 [ac]
Soil + roots + fluopyram	224.75 ± 20.20 [b]	118.07±34.04 [abd]	6.33 ± 0.47 [b]	5.64 ± 0.89 [d]

M. incognita-infected greenhouse soil contained 500 J2s/100 cc of soil. Root galling was indexed according to Zeck's scale of root-knot infection [19], with 0 = no root galls and 10 = severe galled-up root (n = 12). Different letters indicate significant differences compared to the control according to a one-way ANOVA with post-hoc Tukey HSD test ($p \leq 0.05$).

The treatment of planting holes with fluopyram for cucumber and lettuce plants confirmed the successful practice (Figure 5A,B, Table 2). Lettuce plants showed a significant reduction in root galling. Roots treated with fluopyram had a root-gall index of 3.42, while the untreated nematode control plants had a root-gall index of 7.33 (Figure 5). The reduction in root galling resulted in a significantly higher lettuce weight of 120.44 g compared to plants grown in the untreated soil (39.11 g) (Figure 5B,C).

For the treatment of the cucumber planting hole, the impact of removing the infected roots of the previous crops was additionally evaluated, compared to leaving the root systems, and their incorporation into the soil was simulated with a rotary tiller, cutting the roots into small pieces. Comparing the results of treated and untreated soil with and without nematode-infected roots, we see a better control effect and improved growth of cucumber shoots when the infected roots were removed and the soil was treated with fluopyram (Table 2).

Because the low water solubility and strong affinity of fluopyram to soil particles typically reduces movement into the upper 10 cm soil layers [16], we hypothesize that a targeted application of fluopyram in the planting hole will have reduced side effects on beneficial organisms and the soil microbial community [25,26]. Furthermore, a wide field application of fluopyram may not have sufficient RKN control, since RKN populations are found in similar amounts at both 0–30 cm and 30–60 cm soil depths [27], generally where plant roots and moist soil are present [28].

Despite the fact that fluopyram has been reported to have a longer half-life in the soil, recent publications could not show a "residual" effect and/or longer nematode suppression during a six-month trial with tomatoes [29]. The main effect of fluopyram was observed at early application time points.

As the planting hole treatment was successful for all three vegetable crops tested, and, as the experiments revealed, only nematodes in the soil were affected when the recommended concentration of fluopyram was used, fluopyram can therefore be recommended as a planting hole treatment to support early rooting of the seedlings. However, additional studies with different cultivars/crops should be conducted to further investigate the systemic effect of fluopyram and its metabolites, not only to control PPN, but also whether fluopyram accumulates in plant organs, as described previously for cucumbers [30], and whether this accumulation may have a negative effect on consumers.

4. Conclusions

The in vitro effect of fluopyram on *M. incognita* was shown to be in line with previous investigations, suggesting a specific effect of fluopyram on *M. incognita*. Furthermore, it can be assumed that the control effect is mainly based on direct contact of the nematodes with fluopyram in the soil and not a systemic effect in the plant. Although fluopyram has been reported to have a systemic effect on *M. incognita*, the results showed that the maximum recommended application of fluopyram only controlled the nematodes in the soil but neglected those nematodes that had already penetrated the root systems. Therefore,

it was concluded that an effective method with reduced environmental side effects is to apply fluopyram as a planting hole treatment to target nematodes in the soil layers around the root systems (Figure 6). Based on our results, we can recommend that growers use fluopyram to manage RKN to treat planting holes before planting, instead of applying fluopyram over the entire field.

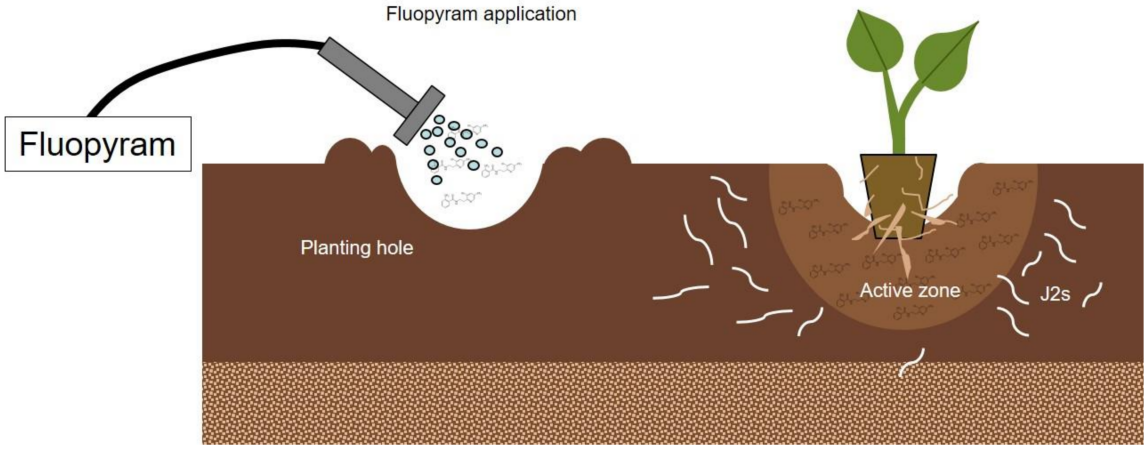

Figure 6. Schematic diagram of planting hole treatment for the control of *Meloidogyne incognita* in the root zone of the plant. The active zone represents the area in which *M. incognita* can be controlled by the application of fluopyram into the prepared planting hole.

Author Contributions: T.S. performed the experiments, analyzed the data and wrote the material and methods draft for the manuscript. P.D. conceptualized, planed and designed the study, wrote the draft and revised to the subsequent manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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